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RESEARCH ARTICLE

The Establishment of Tandem Mass Spectrometric Fingerprints of Phytosterols and Tocopherols and the Development of Targeted Profiling Strategies in Vegetable Oils

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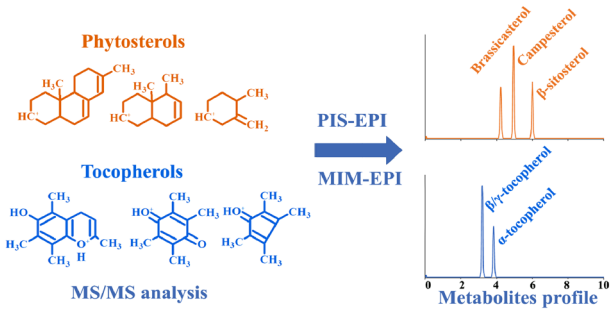

Abstract. Phytosterols and tocopherols are essential for plant biochemistry, and they possess beneficial health effects for humans. Evaluating the tandem mass spectrometric (MS/MS) behavior of phytosterols and tocopherols is needed for the development of a qualitative and quantitative method for these 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 tocopherols (i.e., alpha (α), beta (β), gamma (γ), and delta (δ)). As such, a universal MS/MS fragmentation pathway for each group was 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. A precursor ion scan-triggered-enhanced product ion scan (PIS-EPI) method was established. Due to enhanced chromatographic peaks, 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. A similar approach was attempted for tocopherols and was only hindered by the low concentration of these bioactive metabolites present in the oil samples.

Keywords: MS/MS fingerprints, Phytosterols, Tocopherols, Profiling, Precursor ion scan, Multiple ion monitoring, PIS-EPI, MIM-EPI, Vegetable oil

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Introduction

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 [1, 2], and they also possess beneficial health effects [3–5]. Phytosterols are responsible for

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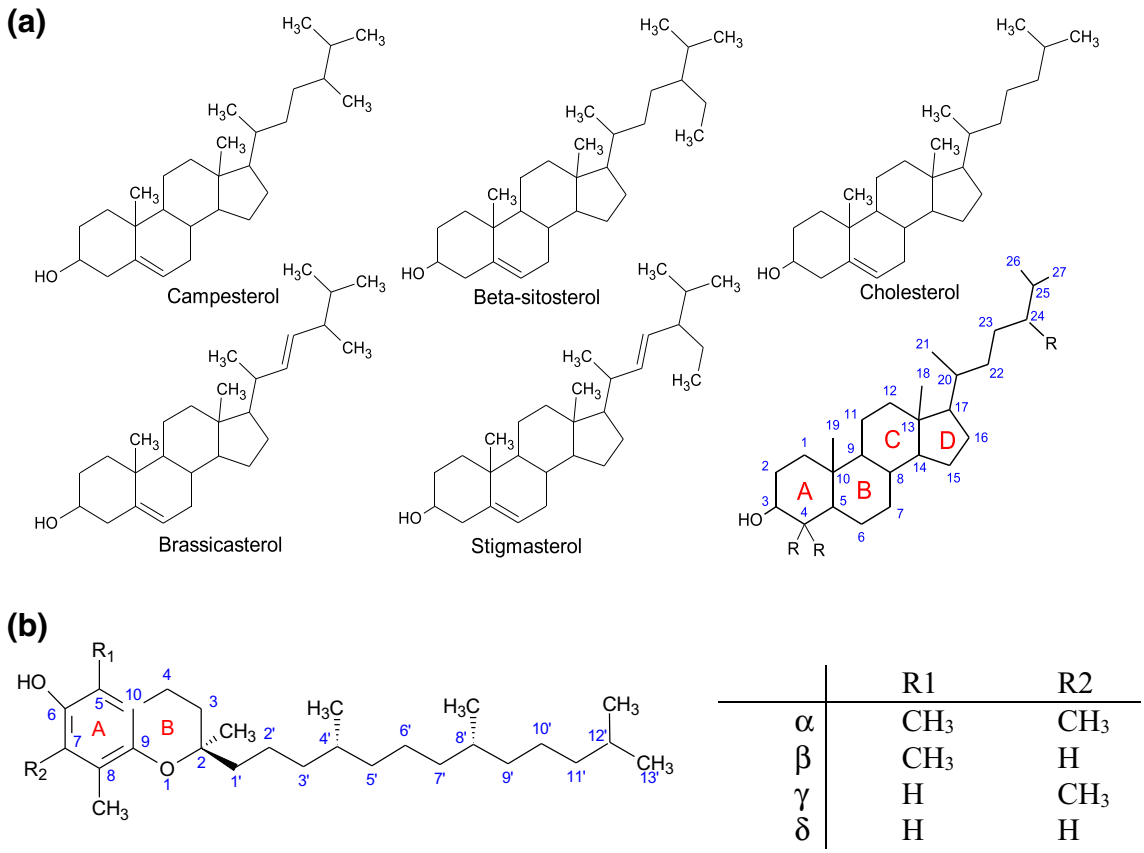
the stabilization of the phospholipid bilayer of the cell membrane by regulating the fluidity and permeability properties of the membrane [3]. They also control other membrane-associated metabolic processes, such as the activity of membrane-bound enzymes [1]. Phytosterols are structurally and functionally similar to cholesterol; however, they possess an extra methyl or ethyl group on the side chain and sometimes with an extra double bond. As cholesterol analogs, they compete with the absorption of cholesterol [6] if taken orally, and they are currently incorporated in food due to their well-established blood cholesterol-lowering abilities [7–9]. In addition, they have been reported to show anti-inflammatory [10], antibacterial [11], and antitumor [12] activities. There are many structural forms of phytosterols, but the most abundant are β-sitosterol, campesterol, brassicasterol, and stigmasterol [13] (Figure 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 (Figure 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 [14–16], and they have shown promising effects as a preventative and therapeutic agent against cancer [4, 17, 18]. Additionally, it has been suggested that tocopherols may enhance the immune response [19] and inhibit the progression of cardiovascular diseases [5]. In sum, tocopherols and phytosterols have a wide range of health applications and are used as additives in food, pharmaceutical, and cosmetic products [20–22].

Phytosterols and tocopherols are found in plants, such as seeds, grains, and legumes [23], with high concentrations in unrefined vegetable oils [23, 24]. 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 [25]. It contains a substantial amount of bioactive metabolites including tocopherols (i.e., vitamin E) and phytosterols. However, the amount or compositional distribution of phytosterols and tocopherols will depend on the seed oil [23, 24, 26]. Several strategies have been developed to recover and purify these compounds from plant sources as well as from DD of different vegetable oils [27–29].

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 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 [30–32]. For example, Tan and coworkers [30] 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



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

Figure 1. Schematic representation of the structure and nomenclature of (**a**) phytosterols, cholesterol, and (**b**) tocopherols

(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 [28]; which is the case in most published methods [30–32]. However, Mo et al. [33] 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 et al. [34] 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 [35–37]. The data was subsequently utilized to develop targeted MS methods to selectively quantify these compounds in cellular matrix [38–40]. Similarly, MS/MS can be used to develop screening methods, based on the precursor ion (PI) and neutral loss (NL) scans [41, 42].

To the best of our knowledge, no detailed MS/MS analysis of either phytosterols or tocopherols was reported for the establishment of 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.

Materials and Methods

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

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) analyses, each stock solution was further diluted to 5 μg/mL with acetonitrile (0.01% acetic acid).

For olive and sesame oil, the unsaponifiable matter was analyzed. The extraction was done as reported with some modifications [43–45]. Briefly, 5 g of each oil was saponified with 1 M 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 rotavap, 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 being dried under vacuum. For MS analysis, approximately 5 mg of each sample (extracts from olive, sesame, and CODD) was dissolved in chloroform and further diluted to the required concentration with acetonitrile (0.01% acetic acid).

MS Analysis

Full Scan MS Using Qq-LIT MS analysis of four standard phytosterols (β-sitosterol, stigmasterol, campesterol, and brassicasterol) was 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). 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 40 V and vaporization temperature of 400 °C. The various optimized MS parameters are shown in supplementary materials, Table S1. Among the various source parameters, temperature was the most critical to optimize; for example, ion counts were reduced to 30% and 70% if the temperature was changed from the optimum value of 400 to 350 °C or 450 °C, respectively.

High-Resolution MS and MS/MS For high-resolution MS and MS/MS analysis, a Thermo Scientific Q Exactive™ Quadrupole-Orbitrap (Thermo Fischer Scientific, Waltham, MA, USA) equipped with APCI source 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 and with isocratic elution (99% acetonitrile and 1% methanol). The injection volume was 10 μL, and the overall run time was 0.5 min. To provide backpressure for the pump, a CSH C18 pre-column (130 Å, 1.7 μm, 2.1 mm ×

5 mm, waters) was used. Full scan data were acquired from *m/z* 50 to 500 with a resolution of 98,995 (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. It should be noted that the process remains CID-MS, despite adopting the term HCD by the manufacturer.

Software

Structures of all the phytosterols, tocopherols, and product ions generated during MS/MS analysis were drawn using the open source ACD/chemsketch (freeware) software, 2018, 1.1 (File Version C50E41), Advanced Chemistry Laboratory Inc. The software was also used for calculating the theoretical monoisotopic masses.

Precursor Ion Scan (PIS)- and Multiple Ion Monitoring (MIM)-Triggered Enhanced Product Ion Scan (EPI)

Information-dependent acquisition (IDA) methods were conducted on the Qq-LIT platform. 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 (Schematic representation of PIS- and MIM-EPI can be found in supplementary material, Scheme S1).

PIS- and MIM-EPI were carried out on an Agilent 1290 UHPLC (Agilent, Santa Clara, CA, USA) connected to AB SCIEX 6500 QTRAP® Qq-LIT-MS (AB Sciex, Concord, ON, CA). The analytical column was an Agilent Poroshell C18 column (2.1 mm × 150 mm, 5 μm) protected by a guard column (2.1 mm × 4.7 mm, 2.7 μm) of the same packing material. 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 °C, and the injection volume was 3 μL.

The parameters used for PIS were similar to those already applied in MS/MS analysis and listed below. Product ions observed at *m/z* 109, 147, 161, 215, 297, and 301 were selected as the product ions for PIS, and 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. The threshold for 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 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 minimal CE (5) in the collision cell [46]. 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 5. For tocopherols, the threshold for IDA trigger for the EPI acquisition was set at 50,000 ion counts and the CE was 5.

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.

Results and Discussion

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; a representative APCI-MS is shown in supplementary materials, Fig. S1 using Quadrupole-Orbitrap system. APCI showed better ionization than ESI particularly for phytosterols during direct infusion. Ionization efficiency was substantially better using APCI-MS in comparison to ESI-MS. In fact, ion counts increased many orders of magnitudes; for example, APCI-MS for stigmasterol demonstrated ion counts of 2.01×10^7 in comparison to 4.40×10^5 using ESI-MS, using the same instrument Qq-LIT (data not shown). This was consistent findings among all tested phytosterols.

Tocopherols, however, showed similar ionization performance for both APCI and ESI. APCI was therefore chosen for its compatibility in the detection of both groups of metabolites, simultaneously. 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, stigmasterol mass spectrometric analysis showed a base peak at *m/z* 395.3662 corresponding to $[M + H\text{-}H_2O]^+$ (Figure 2). However, $[M + H\text{-}2H_2]^+$ was also observed as a lower abundant ion during phytosterol ionization (Fig. S1, supplementary materials); such ion was reported in the literature [47].

On the other hand, tocopherols ionized primarily as the molecular ion, $M^{\cdot+}$. Exact mass measurements using high-resolution mass spectrometer (HRMS), Quadrupole-Orbitrap, were conducted for both phytosterols and tocopherols. Observed mass accuracies were better than 3 ppm (Table 1). Exact mass measurements confirmed the molecular structures of the tested compounds, as well as the proposed ionization mechanism (Figure 2).

Multi-Stage MS Analysis

MS/MS Analysis of Phytosterols The $[M + H\text{-}H_2O]^+$ was selected for MS/MS and MS^3 analysis of phytosterols, using Quadrupole-Orbitrap and Qq-LIT instruments, respectively. It should be noted that MS/MS analysis using Qq-LIT produced similar product ions to those observed by the Quadrupole-Orbitrap (data not shown); however, we used the MS/MS with the high resolution data as it helps in structural assignment.. During MS/MS analysis, most product ions were formed by cleavage of the C-ring and/or the penta cycle of the

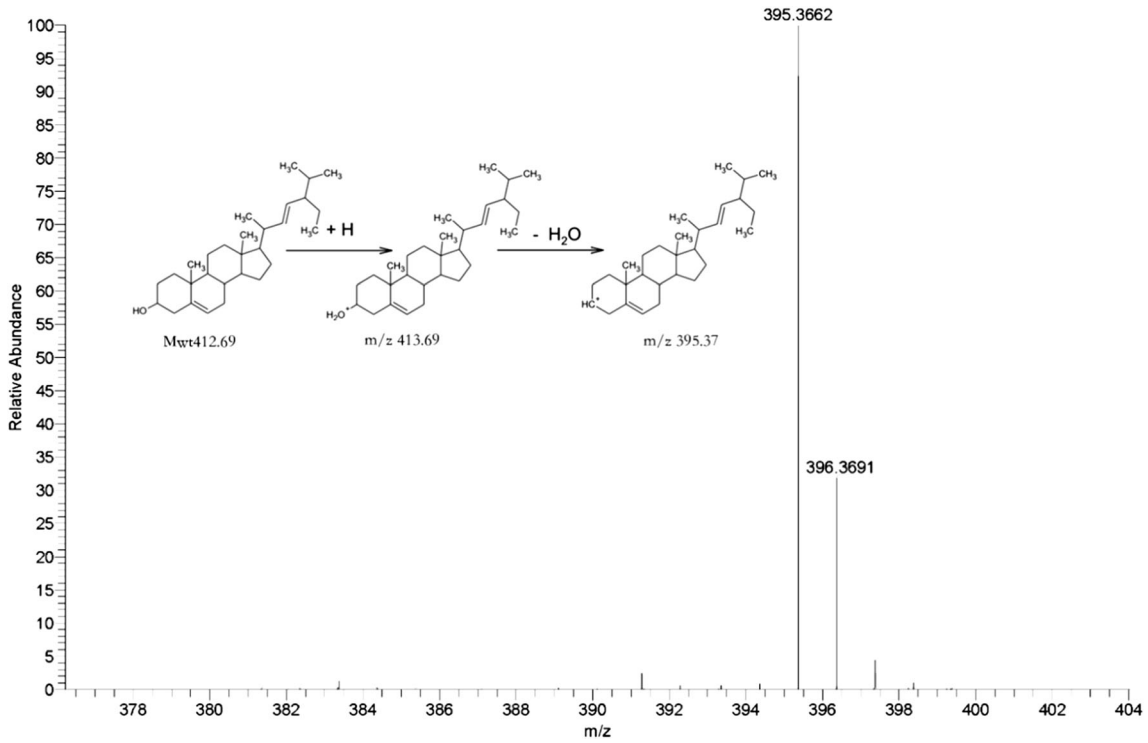


Figure 2. Full scan MS analysis of stigmasterol showing the precursor ion as $[M + H\text{-}H_2O]^+$ at *m/z* 395.3662 from Thermo Scientific Q Exactive™ Quadrupole-Orbitrap and the predominant ionization pathway of stigmasterol via the loss of a water molecule

phytosterols. Figures. 2 and 3a show the full scan MS and MS/MS spectra of 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.

The MS/MS analysis of stigmasterol showed a complex spectrum, and the structure of major product ions was rationalized and confirmed via MS^3 analysis. The fragmentation process starts with three unique pathways that result in the formation of three singly charged product ions observed at *m/z* 311.27, 297.26, and 285.26 (Figure 3b), 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 pathways are identified. It was observed that the initial dissociation of the side chain will affect the dissociation of the core part of the molecule, each resulting in unique product ions. However, subsequent to S1, S2, and S3 formation, there are still some identical product ions that are shared among the three main fragmentation pathways.

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) (Figure 3b). 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 (Figure 3b). 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, as well as at ring C yielding C1 at *m/z* 201.16.

The product ion S4 at *m/z* 269.22 can undergo two main fragmentation processes. The first one involves the 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

Table 1. Full scan MS analysis of phytosterols (a) and tocopherols (b) using Quadrupole-Orbitrap instrument

Compound	Monoisotopic mass	*m/z* (theoretical) $[M + H\text{-}H_2O]^+$	*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
Campesterol	400.7	383.3672	383.3664	2.16
Compound	**Monoisotopic mass**	***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

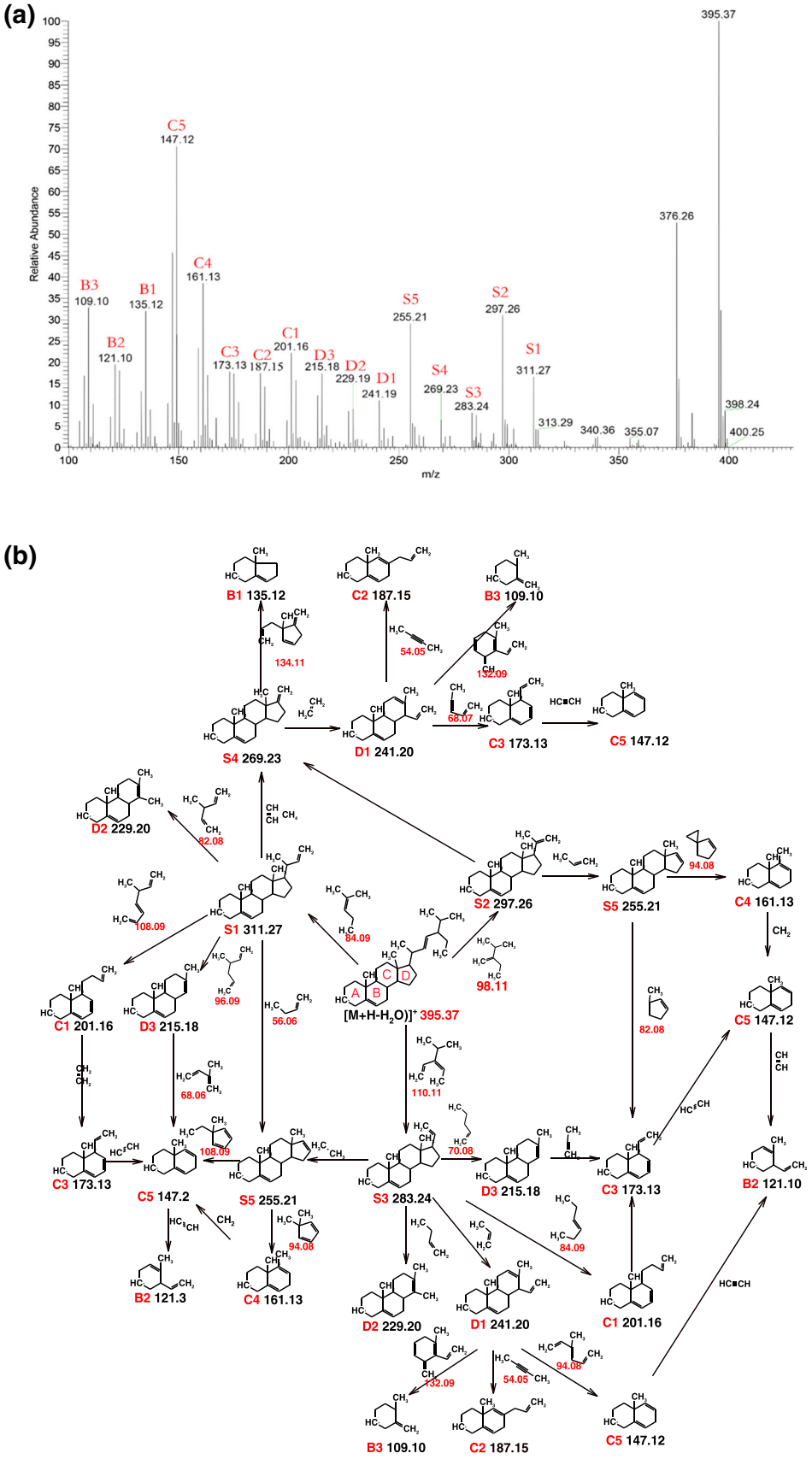


Figure 3. (**a**) MS/MS spectra of stigmasterol from Quadrupole-Orbitrap instrument. (**b**) The proposed fragmentation pathway showing the genesis of the various product ions, as confirmed by MS^3 analysis

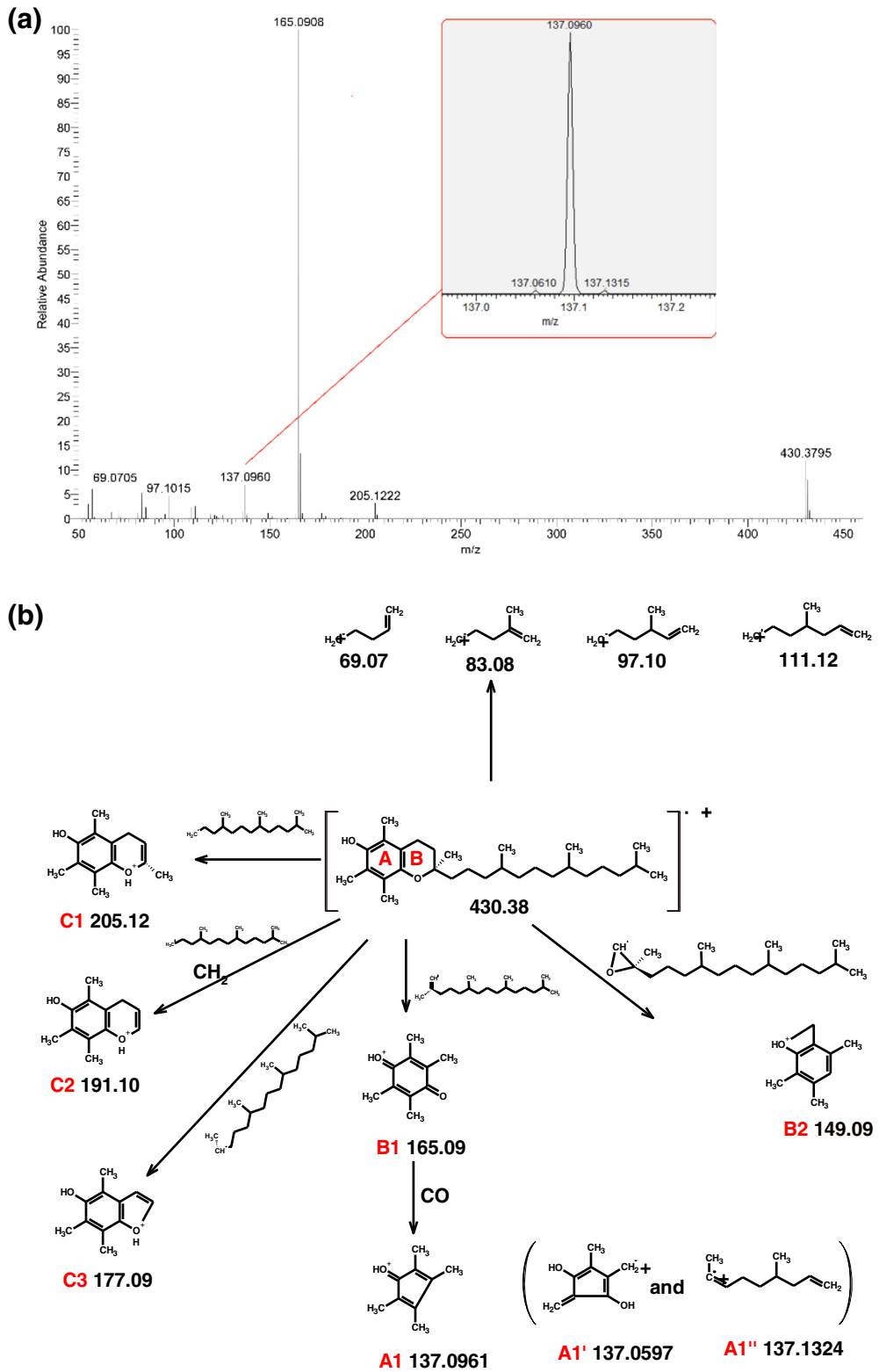


Figure 4. (**a**) MS/MS of α-tocopherol from Quadrupole-Orbitrap instrument. (**b**) The proposed dissociation behavior, showing the genesis of the various product ions as confirmed by MS^3 analysis

product ion D1 at *m/z* 241.20. D1 is further dissociated to form three product ions from the cleavage of ring B or ring C, forming ions B3 at *m/z* 109.10, C2 at *m/z* 187.15, and C3 at *m/z* 173.13; the latter through the neutral loss of C_5H_8. Additional product ion, C5, is further formed through a loss of ethyne moiety as shown in Figure 3b.

The second major pathway during CID-MS/MS dissociation involves a C=C double bond cleavage at the side chain. Cleavage

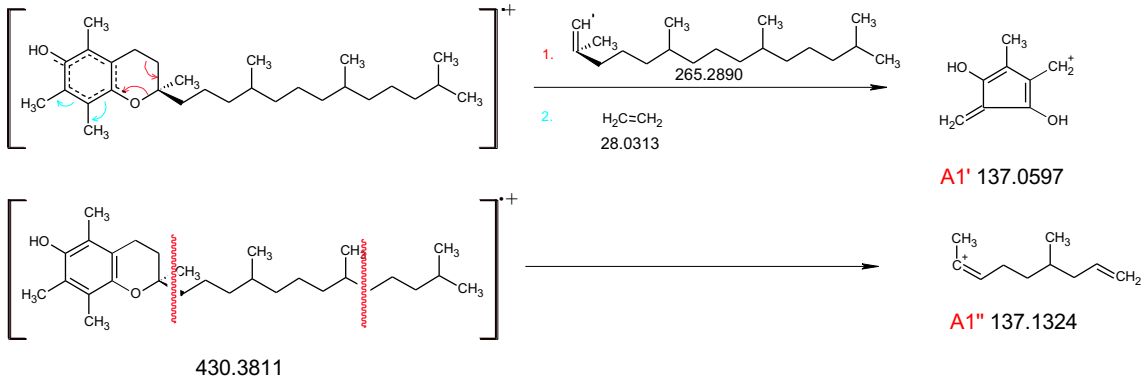


Figure 5. The proposed fragmentation mechanism of the ions designated as A1' and A1"

at the c22-c23 bond yields the product ions S2 at *m/z* 297.26 (Figure 3a) 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 (Figure 1a). The subsequent partial loss of the remaining part of the side chain at position 17 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 (Figure 1a). Subsequently, cleavages within ring C of S5 produces two product ions, designated as C3, and C4 as shown in Figure 3b. Ions C3, C4, and C5 are common ions observed in all tested phytosterols, due to common structural features (Figure 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 the formation of B2. The genesis of all observed ions was confirmed by MS^3 analysis (Supplementary materials, Table S2).

The third major pathway 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, Figure 3b), which is supported by MS^3 analysis (Supplementary materials, Table S2). D1 at *m/z* 241.20 is produced by inner-ring cleavage of ring D at the 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 (supplementary material, Scheme S2) 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 Figure 3b were supported by MS^3 analysis (Supplementary materials, Table S2) and exact mass MS analysis (Supplementary materials, Table S3). It was observed that ion clusters were present at most major ions, such as ions C1, B1, C5, C4, C3, C2, C1, D2, D1, S5, S4, S3, S2, and S1. This cluster ions are most likely linked to the dominant species; however, the analysis was focused on rationalizing the structure of the most abundant species within each cluster since the abundant ions are the most stable and can be used for qualitative and quantitative analysis.

MS/MS Analysis of Tocopherols α-Tocopherol has been used as a representative example to illustrate the CID-MS/MS behavior of tocopherols (Figure 4a). The fragmentation pathway for the tocopherol molecular ion $[M]^{\cdot+}$ at *m/z* 430.38 is shown in Figure 4b. 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 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 values below and above this flow rate favored the formation of the protonated and the molecular ion, respectively. Both the protonated and molecular ions were reported for MS/MS analysis in different studies [48, 49]. However, both the 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, but it was reported as observed.

In the present work, the molecular ion was chosen for MS/MS analysis since the PIS/MIM profiling (discussed below) 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 [50] 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 Figure 1b). The molecular ion of tocopherol dissociates via inner ring fragmentation at the sites 1–2 and 3–4 (Figure 1) with rearrangements to form the highly conjugated stable ion B1 observed at *m/z* 165 bond. 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*

Table 2. Summary of product ions of tested phytosterols. Checkmark indicates the observation of the ion during MS/MS analysis

	Stigmasterol	Brassicasterol	β-Sitosterol	Campesterol
5A	√	√	×	×
5B	√	√	×	×
5C	√	×	×	×
5D	√	×	×	×
5E	√	√	×	×
4A	√	√	√	×
4B	√	√	√	√
4C	√	√	√	√
3A	√	√	×	×
3B	√	√	√	√
3C	√	√	×	×
3D	√	√	√	√
3E	√	√	√	√
2A	√	√	√	√
2B	√	×	√	√
2C	√	√	√	√
2D	√	√	√	√
1A	√	√	×	√
1B	√	√	√	√
1C	√	√	√	√

137.0960 confirming the proposed structure as shown in Figure 5b. 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", $C_{10}H_{17}^+$) (Figure 4). These product ions are different from the abundant ion at *m/z* 137.0961 (A1, $C_9H_{13}O^+$) and indicates that additional product ions (Figure 5) were also formed but in a low abundance; A1 formation is probably favored due to its highly conjugated structure in which the charge is eventually localized at the electronegative oxygen. The same mechanism drives the formation of ions at *m/z* 151 and 123 as well as 191 and 177 for the β-/γ-, and δ-tocopherols, respectively. These ions represent similar ions, generated from various precursor ions (Supplementary materials, Table S4).

The need for high-resolution MS/MS data, obtained from the Quadrupole-Orbitrap instrument, 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 the ions with exact *m/z* values of 69.0705, 83.0860, and 97.1015 that are subsequent losses of CH_2 moieties. Such observation confirmed that these product ions are generated from the carbon side chain with a $C_xH_y^+$ formula.

In a final note, β- and γ-isomers cannot be distinguished in their MS/MS since the only difference between the two isomers is the position of the methyl groups on the chroman ring (Figure 1b) and as such, they share the exact MS/MS fragmentation pattern.

Identification of Phytosterols in Oil Samples Among the numerous product ions observed, four common product ions,

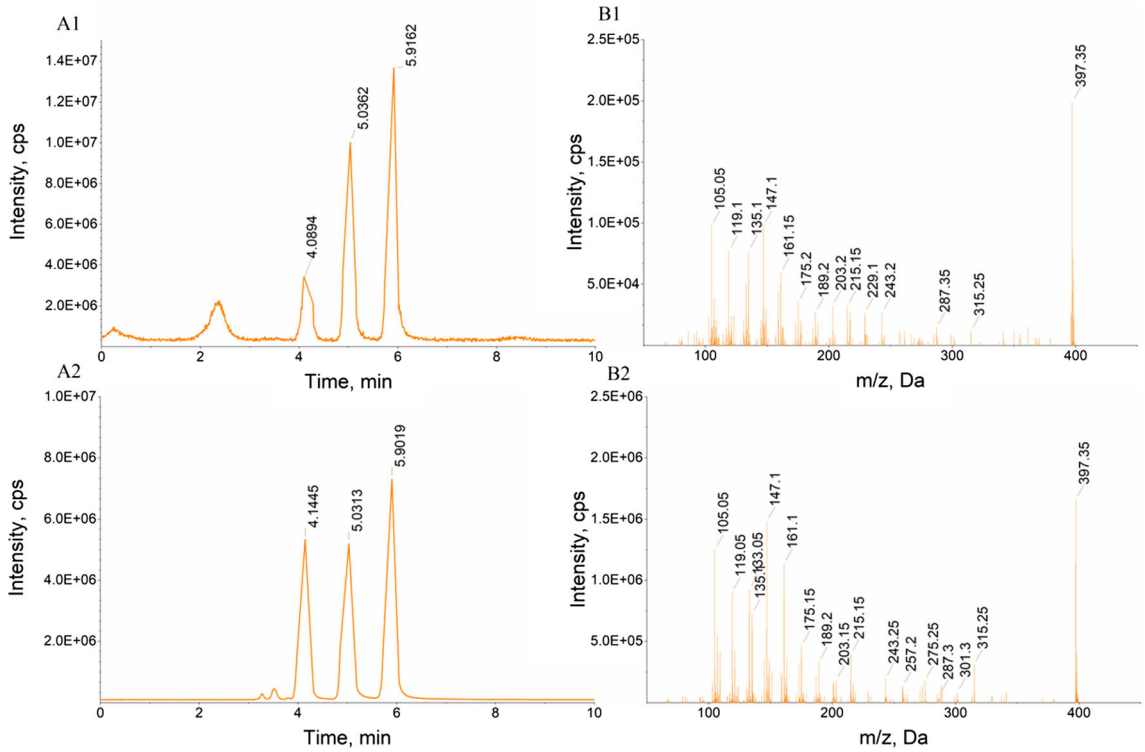


Figure 6. Comparison of PIS-EPI and MIM-EPI in profiling of phytosterols and MS/MS data for stigmasterol. Profiling data obtained by PIS-EPI (A1) and MIM-EPI (A2); MS/MS spectrum of stigmasterol from PIS-EPI (B1) and MIM-EPI (B2). Data was acquired using Qq-LIT instrument

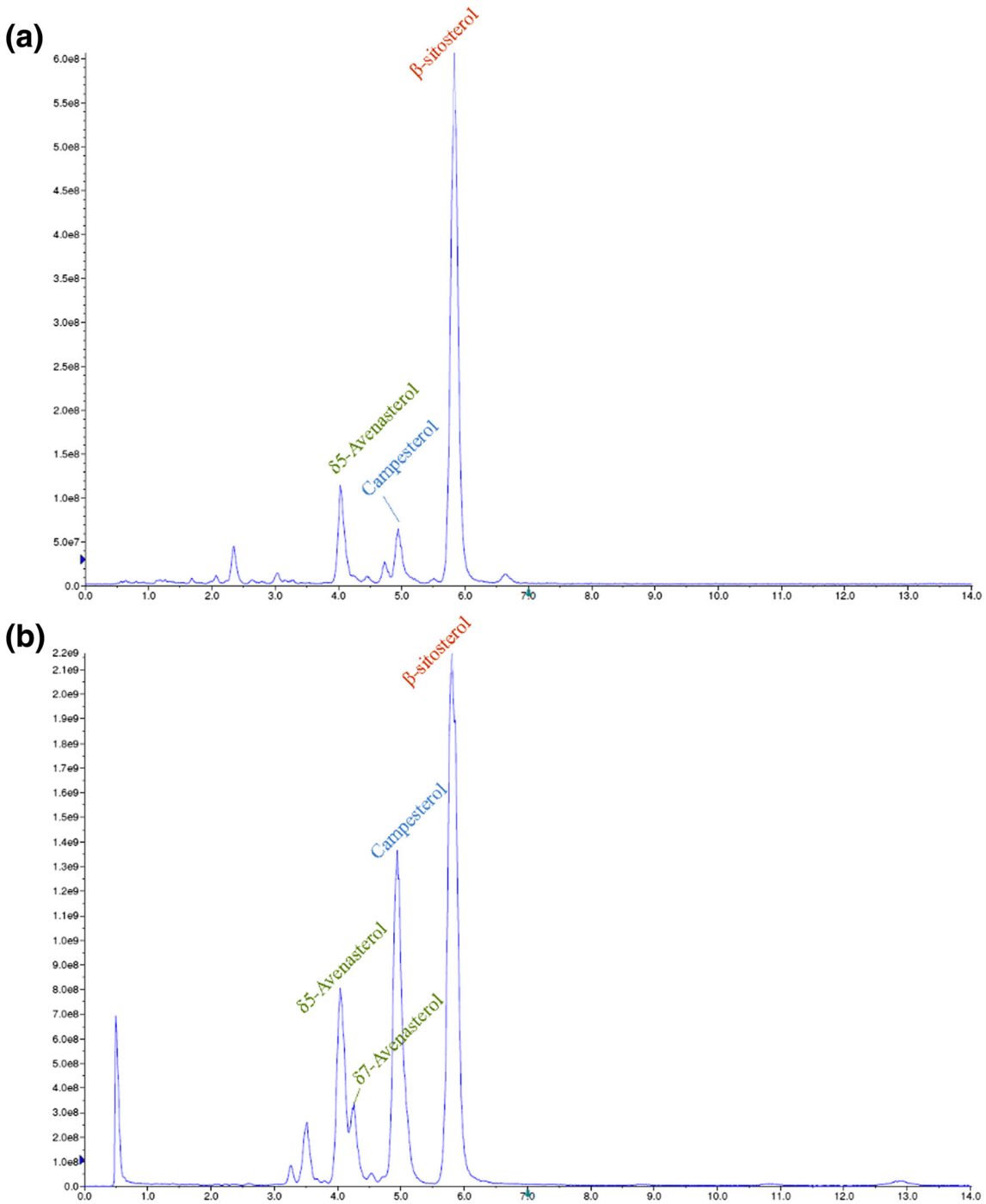


Figure 7. Comparison of precursor ion scan spectra in phytosterols profiling for (**a**) olive oil and (**b**) sesame oil, using Qq-LIT instrument

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; and (3) they maintain the core structures which make them characteristic product ions for phytosterols (i.e., diagnostic ions). Two other product ions, S2 and S2' at *m/z* 297 and 301 (Supplementary materials, Table S5) were also selected as an indicator of the presence or absence of double bond on the site c22-c23 of the side chain.

Various phytosterols, reaching up to 16, have been reported as components of vegetable oils [51–55]. The developed generalized MS/MS fragmentation behavior (Table 2, Figure 4b) can theoretically be applied to other naturally existing phytosterol structures.

The various phytosterols in tested vegetable oils should theoretically yield common product ions listed in Supplementary materials, Table S6. 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 of sesame oil and olive oil is summarized in Supplementary materials, Table S7 [23, 33, 55]. All metabolites yielding common product ions at *m/z* 109, 147, 161, and 215 in MS/MS were considered as potential phytosterols. In fact, all phytosterols could theoretically 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.

A PIS-EPI scan, using the Qq-LIT instrument, was employed, showing three major chromatographic peaks whose signal strength varied substantially (Figure 6). This may be due to the fact that the collision energy was not optimized for the various phytosterols, existing in oils (Supplementary materials, Table S7). Therefore, a multiple ion monitoring-triggered-enhanced product ion scan (MIM-EPI) method was adopted to profile and acquire MS/MS for the target compounds. Compared with PIS-EPI, MIM-EPI displayed a better profiling performance with stronger signal for each possible phytosterol, with a more detailed MS/MS spectrum (Figure 6b). This is due to MIM-narrowed ion scan range in Q1, which helps to acquire more data points. MIM-EPI was reported to be a powerful tool for metabolite profiling, especially for identifying metabolites present in low concentrations [56, 57].

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 blinded samples prepared by co-author, Gachumi, G. Upon analysis, three samples were successfully identified as a mixture of standard compounds. The other two are phytosterols extracted from sesame and olive oils (Figure 7). The identification was based on the presence and intensity of the ions as well as the retention time of the observed peaks. For example, according to the relative intensity of campesterol, and avenasterol, sample 4 was successfully identified as an extract from olive oil (Figure 7a), while sample 5 was from sesame oil (Figure 7b) as both δ5 and δ7-avenasterol are reported in sesame oil, while only δ5-avenasterol was reported in olive oil [58–62]. 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 and stigmasterol show a significant peak at *m/z* 395 because the only structural difference is the position of the double bond on the side chain. However, the chromatographic retention times will vary among these structures. Therefore, both *m/z* values and retention times were considered to confirm the presence of one specific phytosterol.

Identification of Phytosterols and Tocopherols in the Unsaponifiable Matter of CODD For tocopherols, only MIM-EPI scan was conducted, since only four forms exist in nature. Therefore, tocopherols can be detected through MIM scan method as only four transitions are needed to be monitored.

Tocopherols were, however, not detected in oil samples (data not shown). This could be due to their reported low concentrations in edible oils [59, 61]. The profiling method was then applied to the unsaponifiable matter of CODD. At least three tocopherols were detected in the sample (Supplementary materials, Fig. S2). However, the retention time and *m/z* value of β and γ-tocopherols are identical and cannot be differentiated (co-elute at 3.48 min).

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 8a). However, tocopherols can still be identified (Figure 8b). This experiment has shown the utility of the developed method to simultaneously screen for both phytosterols and tocopherols within an oil sample.

Conclusion

In this study, the MS/MS dissociation behavior of four phytosterols and four tocopherols were established, and the fragmentation pattern was confirmed via MS^3 analysis. The usefulness of the established MS/MS fingerprints was successfully employed to predict the dissociation behavior of other naturally existing phytosterols due to their similar structural features.

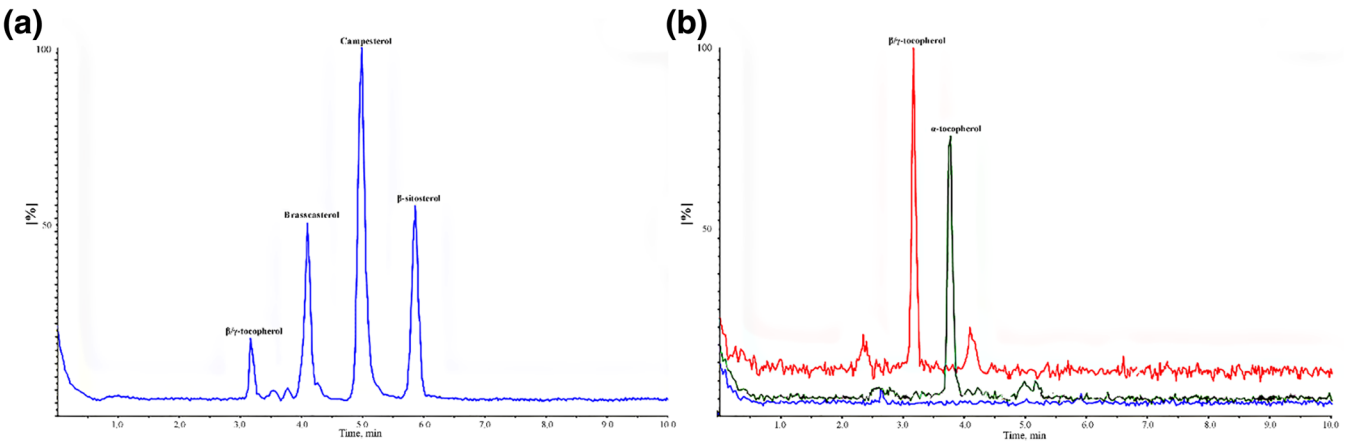


Figure 8. (**a**) TIC chromatogram of MIM-EPI scan for phytosterols and tocopherols in CODD, using Qq-LIT instrument; (**b**) extracted-ion chromatogram (XIC) of tocopherols (red: transition 416 → 416, green: transition 430 → 430, blue: transition 402 → 402)

This finding demonstrates the utility and the value of the established MS/MS data that was successfully extended to other structures within sesame and olive oils. As such, qualitative profiling MS-based methods were developed based on the MS/MS dissociation. 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 demonstrated in the identification of phytosterols and tocopherols present in oil samples (sesame and olive) and CODD. The MS/MS behavior is currently being used to develop targeted LC-MRM-MS/MS quantification method which will be reported upon completion.

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