This is the post-print version of the following article: George N. Gachumi and Anas El-Aneed. (2017), Mass Spectrometric Approaches for the Analysis of Phytosterols in Biological Samples. J Agric Food Chem. 2017 Nov 29;65 (47):10141-10156. Published in final form at doi: 10.1021/acs.jafc.7b03785.

REVIEW ARTICLE

MASS SPECTROMETRIC APPROACHES FOR THE ANALYSIS OF PHYTOSTEROLS IN BIOLOGICAL SAMPLES.

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ABSTRACT

Plant sterols (phytosterols) are important structural components of plant cellular membranes and they play a major role during development and metabolism. They have health-associated benefits, especially in lowering blood cholesterol levels. Due to their many health claims, there is a growing interest in their analysis. Although various analytical strategies have been employed in analyzing phytosterols, chromatography linked to mass spectrometry (MS) is superior due to its sensitivity. Furthermore, specificity and selectivity are enhanced by utilizing tandem mass spectrometry (MS/MS). This article reviews the various mass spectrometric strategies used for the analysis of phytosterols. It highlights the applications and limitations associated with each MS strategy in various sample matrices such as plant, human, animal, food, and dietary supplements. GC-MS was historically the method of choice for analysis; however, the derivatization step rendered it tedious and time-consuming. On the other hand, liquid chromatography coupled to MS (LC-MS) simplifies the analysis. Many ionization techniques have been used namely electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). APCI showed superiority in terms of ion intensity and consistency in ion formation, primarily forming $[M+H-H_2O]^+$ ions rather than $[M+H]^+$. In addition, matrix assisted laser desorption ionization (MALDI) as well as ambient mass spectrometry such as direct analysis in real time (DART) have also been evaluated.

Keywords: Phytosterols, GC-MS, plant sterols, LC-APCI-MS, LC-ESI-MS

INTRODUCTION

Phytosterols represent a family of lipids present in plant cells, structurally and biosynthetically similar to cholesterol[1]. While cholesterol molecular structure contains 27-carbon atoms with one alcohol functional group, phytosterols typically consist of 28 or 29-carbons with one alcohol functional group. The main difference is within the branched hydrocarbon side chain of phytosterols that contain 9-10 carbon atoms instead of 8. In addition to the presence of a

double bond in the sterol nucleus, an additional double bond is sometimes present in the alkyl side chain of phytosterols (Figure 1).

Phytosterols are responsible for the stabilization of the phospholipid bilayer of the cell membrane by regulating the fluidity and permeability properties of the membrane as well as controlling other membrane-associated metabolic processes such as modulating the activity of membrane-bound enzymes[2, 3]. Although more than 200 plant sterols have been identified, they can be broadly classified as 4-desmethylsterols, 4-methylsterols, or 4,4'-dimethylsterols (Figure 1) with the majority of plants showing a high abundance of the 4-desmethylsterols group[4, 5]. The vast majority of phytosterols are unsaturated; however, fully saturated phytosterols (stanols) are present in much lower concentrations[1]. Stigmasterol, campesterol, brassicasterol, and β-sitosterol are the major sterols found in plants, however, their content and composition may vary depending on the plant species. For example, the total phytosterols content in canola oil (4590-8070 µg/g) was approximately twice that in sunflower (2100-4540 µg/g) or soybean (2340-4660 µg/g) while brassicasterol is only found in canola oil[6]. It has also been shown that factors such as genetics and growing locations affect phytosterols amount and composition. High phytosterol content was found in the oilseed rape variety, Sansibar, compared to the Oase variety, while the ratio of 24-methyl to 24-ethyl phytosterols was higher in the case of the Oase variety[7].

Phytosterols have received a wide attention due to their various health-associated claims such as, anticancer[8, 9], anti-inflammatory[10, 11], blood cholesterol lowering properties[12-14], antifungal[15], and antibacterial properties[16]. Their blood cholesterol lowering abilities are well-established as the uptake of a diet with moderate-to-high amount of phytosterols enhanced excretion of biliary and dietary cholesterol on tested human subjects compared to those on phytosterols-deficient diet[12, 17-22]. These studies employed placebo controlled and/or randomized crossover feeding trial on healthy, mildly hypercholesterolemic, and hypercholesterolemic subjects. Sample size ranged 11-153 subjects on a dose of 1-18 g/day of phytosterols for a treatment duration of 4 weeks to 1 year. A 4% decrease in serum cholesterol was reported on a retrospective cohort study based on pharmacy dispensing records (3829 subjects, male and female) in statin and non-statin users when they were fed phytosterol/phytostanol enriched margarine[23]. Similary, a randomized, double blinded study on hypercholesterolemic men and women (n=37) showed a reduction of low-density lipoprotein cholesterol (LDL-C) by 32% and 8% during a 4 week period when statin (cerivastatin) and phytosterols (2 g/day) were administered, respectively[24]. However, when cerivatstin and phytosterols were administered together, an additive effect was observed where LDL-C was lowered by 39%. Due to the favorable properties of phytosterols, they are incorporated in functional foods, cosmetics, and pharmaceutical products[25, 26]. Phytosterols are "Generally recognized as safe" and this has led to their wide acceptability as nutraceutical ingredients resulting in their increased use in food products. The U.S Food and Drug Administration (FDA) has approved the use of phytosterols for their health benefits[27].

Phytosterols are mainly obtained via extraction using organic solvents[28-31] although other green extraction techniques such as the use of supercritical carbon dioxide have been investigated[32, 33]. High purity phytosterols via solvent extraction are obtained by employing additional techniques such as crystallization, molecular distillation, column chromatography, thin layer chromatography and/or combination of more than one method[33-35]. These techniques have been applied to samples ranging from low to high content of phytosterols. However, solid-phase extraction (SPE) and solid-phase micro-extraction (SPME) are ideal when dealing with samples whose phytosterols contents are very low[36-39]. In addition, SPME offers an additional advantage as both extraction and derivatization can be performed simultaneously when the microfiber is coated with the derivatizing agent[37, 38]. The use of enzymes has also been utilized for the extraction of phytosterols, particularly lipases but more research is being carried out to identify enzymes that are selective to enhance recovery[40-42]. Analytical characterization of extracted phytosterols is needed for the identification and assessment of their composition and purity. Similarly, pharmacokinetic evaluation requires the measurement of phytosterols in plasma/serum. Therefore, accurate, sensitive, and reliable analytical methods for the qualitative and quantitative analysis of these active metabolites are needed. Rigorous analysis of phytosterols is critical as these nutraceuticals must conform to regulatory requirements. Phytosterol analysis will be key in recognizing and identifying their health benefits and unfolding any future beneficial applications.

Different strategies of analysis of phytosterols have been explored with gas chromatography and liquid chromatography being the mainly applied techniques[43-46]. Other analytical strategies, such as capillary electro-chromatography (CEC) have also been used but its application have received limited interest[47, 48]. This is due to the inability to produce reproducible CEC packed columns leading to low analytical precision and sensitivity. Most of the detection systems such as ultraviolent/visible light (UV/Vis), evaporative light scattering detector (ELSD), and flame ionization detector (FID) are not very specific and the inherent chromatographic problem of co-elution represents a great challenge. However, Mass spectrometry (MS) can address many of the challenges observed in other analytical strategies. Therefore, it has been widely adapted in conjunction with other separation techniques due to its sensitivity and its ability to identify co-eluting compounds by the application of tandem mass spectrometry (MS/MS). However, there are various MS-based strategies that can be employed, particularly by varying the separation mode, the ionization source, or by using low resolution (LR) versus high resolution (HR) MS instruments. In this paper review, the various MS strategies used for the analysis of phytosterols in biological samples are discussed and reviewed.

Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography is a physical separation method where an inert mobile phase is used and separation is based on the variation in the volatility of the analyte and their interaction (intermolecular forces such as hydrogen bonding, dispersion, and dipole-dipole) with a stationary phase within a capillary column. Modification of stationary phase functionality, such as the

addition of cyanopropyl and phenyl groups have a confounding effect on the separation efficiency and therefore the choice of the stationery phase (i.e polar vs non-polar) stationary phase is crucial during GC analysis. Phytosterols are hydrophobic compounds and non-polar stationary phases are employed for their separation. However, variations amongst non-polar stationary phases will exhibit varying interactions and the choice will be based on the analytes or desired resolution. GC is ideal for the analysis of volatile compounds although some non-volatiles can be chemically modified to form volatile and stable derivatives that are amenable for GC-MS analysis[4, 49]. Electron impact (EI) is the widely-employed ion source in GC-MS and ionization is achieved from a high voltage electron bombardment. The availability of a mass spectral library makes this technique attractive as qualitative analysis can be performed despite the lack of a commercially available standards.

The presence of a hydroxyl group in phytosterols renders them less volatile and hence a capillary column that can withstand high temperatures should be employed especially for routine analysis where derivatization is omitted. However, high temperatures can affect the stability of the analyte and degradation should be carefully assessed. Many studies have profiled phytosterols in plant and biological samples without the use of any derivatization strategy. For example, Naz et al.[50] determined the constituents of a deodorizer distillate directly from both canola and palm oil. These samples were saponified with ethanolic potassium hydroxide for 60 min at 80°C, unsaponifiables extracted using n-hexane and analysis conducted on a GC-MS in full scan mode. Through a comparison of retention times and relative abundance with standards as well as mass spectrum match from NIST library, brassicasterol, ergostanol, and lanosterol were found in canola while stigmasterol and 4,22-stigmastadiene-3-one were present in palm[50]. Campesterol, sitosterol, and stigmast-4-en-3-one were identified in both distillates[50].

GC-MS/MS was utilized in the analysis of cholesterol and phytosterols in plant oil and foods after their extraction and saponification[51]. The use of multiple reaction monitoring (MRM) provided a reliable method that could simultaneously confirm and quantify cholesterol and phytosterols[51]. Similar results were obtained in the analysis of phytosterols in tobacco where a short column (10 m) was used to correct for broad and tailing peaks associated with the relatively low volatility of phytosterols[52]. Campesterol, stigmasterol, and sitosterol were identified and quantified by monitoring one or more *m/z* transition(s) as quantifier and/or qualifier ion highlighting the advantage of using MS/MS as compared to conventional single-stage GC-MS. In single stage analysis, results are based on retention times of standards and NIST mass spectral library match. Thus, single stage GC-MS may suffer from the inability to distinguish analytes especially in cases of co-elution or the presence of a structurally similar compound. MS/MS therefore introduces selectivity in the identification process and contamination within a peak is reduced. Although the analysis of underivatized phytosterols is favorable due to shorter sample preparation time and low cost, it suffers from poor peak shape, resolution and sensitivity, especially within a complex matrix system. In fact, it was reported that when phytosterols were analyzed in their underivatized form, the sterol content was 6.0-8.7% lower compared to the use

of derivatization [53]. It can be speculated that degradation may have been a major factor as the high injection and column temperatures involved in GC will result in dehydration or decomposition. In addition, derivatization was found to mainly influence sitosterol (9.4-11.8% increase) which elutes at the end of the chromatographic run[53]. Derivatization, therefore, enhances volatization and resolution due to reduced surface adsorption, and most importantly stabilizing thermally labile compounds.

Phytosterols can be analyzed as acetate derivatives although the silylated derivatives are more common[54]. Acetylating reagents are known to be highly reactive and any excess must be removed (usually via evaporation of the solvent in reaction mixture) since interfering artifacts might be produced causing damage to the chromatographic column and the system[55, 56]. This is usually via the production of side products as well as modification of the column stationary phase via hydrolysis (column bleed) or side reactions leading to change in stationary phase chemistry. In addition, acetylated derivatives have been reported to be more stable when compared to trimethylsilyl derivatives[57, 58]. However, caution should be observed when dealing with low molecular weight compounds as they will be lost during the removal of the excess derivatizing reagent. The most common silylating reagents, alongside few applications highlighting the reaction conditions as well as figures of merit where reported are shown in Table 1. Although the majority of these silylating agents have similar mechanism to donate the silyl group, there are significant differences in terms of kinetics, stability of the formed derivative(s), as well as variations in volatility of the products[59]. N-O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) has a bulky fluorinated side group (Table 1) and is usually used in combination with a catalyst that increases the silyl donor strength, hence speeding up the reaction[60, 61]. Volatile products are formed by using BSTFA leading to less chromatographic interferences although N-Methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) has been shown to perform similarly as BSTFA but with the formation of highly volatile derivatives[62]. In fact when MSTFA and BSTFA were compared during the analysis of phytosterols in pumpkin, the results were not statistically different[63]. Other catalysts, such as Trimethylchlorosilane (TMCS), Dithioerythritol (DTE), and Trimethyliodosilane (TMIS) have also been used in combination with MSTFA in enhancing the silyl donor strength[64, 65]. Trimethylsilyl derivatives are, however, sensitive to moisture and will readily undergo hydrolysis. Thus, the derivatizing agent, reaction mixture, as well as the derivatized product/s should be kept free of moisture.

Profiling phytosterols via derivatization using GC-MS was conducted on selected seed oils by analyzing the acetylated derivatives where the relative percentage as well as absolute amount of sterols were determined[57]. It was found that acetylation was preferred to the use of trimethylsilyl (TMS) due to their stability against hydrolysis and the ease with which the side chain unsaturation can be detected in the sterol molecules[57]. Xu et al.[66] however, employed a multi-dimensional gas chromatography (GC-GC-MS) to create a phytosterol profile for characterizing, classifying, and detecting oil adulteration. Phytosterols were analyzed as trimethylsilyl derivatives and quantification was achieved in the MRM mode. Chemometrics was

then applied to build a classification model for peanut, soybean, rapeseed, and sunflower oil and it was effectively used to detect peanut oil adulteration with soybean oil. Phytosterol identification was also reported, using high resolution (HR) GC-MS. Eduardo et al.[67] analyzed underivatized phytosterols present in *Miconia* species while Novotny et al.[68] qualitatively analyzed the trimethylsilyl derivatives in tobacco and marijuana. While both studies assigned structures based on comparison to retention time of authentic standards, the latter applied mass spectral interpretation where reference standards were unavailable. However, only scarce information was provided and no conclusion could be made based on these studies as to whether the use of HRMS offered an advantage over low resolution (LR) MS.

Analysis of phytosterols using GC-MS has been widely applied in human and animal studies, particularly for elucidating their health benefits. Human blood cholesterol and phytosterols from normal healthy adults were analyzed by comparing the efficiency of different silylating reagents such as MTBSTFA:NH_4I, BSTFA:TMCS, and MSTFA:DTE:TMIS[69]. The results obtained by using MTBSTFA:NH_4I failed in repeatability with a reported coefficient of variation exceeding 70%, while both derivatized and underivatized peaks were observed in the chromatogram when MTBSTFA:NH_4I and BSTFA:TMCS were used. The use of MSTFA:DTE:TMIS proved to be simple, fast, selective, and sensitive (LLOQ 100 ng/mL) largely due to the potency and selectivity of TMIS as a trimethylsilyl donor as well as the catalytic activity of DTE. No interference from underivatized sterols was observed[69]. Such analytical approach was geared towards the development of better cholesterol-lowering treatments either through prescription of therapeutic drugs or provision of dietary plans. Generally, the percentage of cholesterol absorption decreases with an increased intake of phytosterols[12].

GC-MS has also been widely utilized for qualitative analysis as it serves as a complimentary analytical strategy to gas chromatography with flame ionization detector (GC-FID) which is preferred[54] (largely due to its wide linear dynamic range) for quantitative analysis. GC-MS was used post GC-FID quantification to confirm the identity of phytosterols present in serum of eight healthy postmenopausal female volunteers. This was done via comparison of phytosterol spectra with those of standards and the NIST library[70] confirming the identity of the peaks in GC-FID chromatogram. In another study, GC-MRM-MS was developed to analyze phytosterol and cholesterol in the plasma of healthy normalipidaemic subjects as these precursors serve as biochemical markers of cholesterol intestinal absorption and liver biosynthesis[71]. A simultaneous determination of cholesterol, cholestanol, desmosterol, lathosterol, campesterol, and sitosterol was achieved but at the expense of a long run time (65.80 min), primarily due to the use of a long column (60 m) which was needed for good resolution[71]. Identification of the observed peaks was done by comparing the retention times to that of standards as well as the MS/MS fragmentation pattern.

Similarly, Bordoni et.al utilized GC-MS to determine the distribution of phytosterols in rat cardiomyocytes after they were fed different concentrations of phytosterols with the aim of

studying the effects of phytosterols in reducing metabolic activity and slowing down cell growth in cardiac cells[72]. Although campesterol, stigmasterol, and sitosterol were used as standards, most of the peak assignments were accomplished by comparing mass spectra with data reported in the literature. In addition to using GC-MS for studying the distribution of phytosterols in animal cells, it can also provide information on the fate of these compounds after ingestion. Song et.al[73] investigated the metabolism of phytosterols by identifying metabolites in rat feces. The mass spectrometer was operated in the selected ion-monitoring mode (SIM) and peaks were identified by their fragmentation pattern. MSTFA containing 5% ammonium iodide was used as the silylating reagent and the results showed that, oxidation at 3-position, saturation at 5- and 6-position, and 17-side chain cleavage were the predominant routes of phytosterols oxidation[73]. In another study, the influence of phytosterols consumption on the excretion of endogenous boldione in human urine was conducted using GC-MS/MS as phytosterols are precursor to anabolic steroids[74]. Boldione is listed as an exogenous anabolic steroid by the World Anti-Doping Agency, although it can also be produced endogenously[75]. There is an increased urgency for the differentiation of exogenous and endogenous anabolic steroids in the world of professional sports and particularly in elucidating their sources. However, there was no evidence for the excretion of phytosterol-related anabolic steroids in human subjects that were fed with phytosterols-enriched food[74].

Despite its wide application in the analysis of phytosterols and related compounds, GC-MS still suffers from major setbacks. It is only applicable to volatile compounds which are thermally stable. Where necessary, nonvolatile compounds, such as phytosterols, must be converted into derivatives that will enhance their volatility as well as promote their thermal stability. This derivatization process is time consuming, requires high temperature and although it enhances peak shape and resolution, it is not ideal for a complex matrix such as biological samples where phytosterols should maintain their integrity in the injection port and through the column. The high temperatures involved may cause degradation and thus pose a challenge in differentiating free versus esterified phytosterols. . These drawbacks have been addressed by the application of liquid chromatography-mass spectrometry (LC-MS), which can analyze both volatile and nonvolatile compounds without the need for derivatization.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

LC-MS is an analytical technique that is widely used in the separation and detection of both volatile and nonvolatile analytes. Volatility of the analyte is not essential to make it amenable for LC-MS analysis, hence the technique is widely applicable in the analysis of biological samples. A liquid mobile phase either as a single solvent or mixture is used and separation occurs at mild temperatures (most often at room temperature). Thus, the analyte integrity remains intact making LC-MS an ideal platform for the analysis of thermally labile compounds. Unlike GC-MS where derivatization is needed for nonvolatile compounds, LC-MS does not require derivatization. However, derivatization have been shown, in some occasions, to improve the separation and the ionization efficiency, enhancing MS response[76]. Some analytes

cannot be easily ionized when soft ionization techniques are applied leading to their low sensitivity during LC-MS analysis. In addition, limited MS/MS fragmentation will usually hinder structural determination[77]. Such drawbacks can be addressed via derivatization.

In LC-MS instrumentation, different types of MS configuration are in existence but they can be broadly categorized based on their ion source or mass analyzer[78, 79]. Application of MS for the analysis of phytosterols is discussed below where the subcategories are grouped based on the ionization source.

Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

Electrospray ionization allows for the ionization of analytes in solution under atmospheric pressure conditions[80]. It is ideal for molecules that bear charges (e.g. quaternary amines), those with electronegative atoms, or compounds carrying basic or acidic functional groups. However, it is challenging to effectively ionize neutral, low polarity and nonpolar compounds. Alternatively, such compounds can be derivatized or ionized using alternative ionization sources. Berkel et.al[81] have used ferrocene based "electrochemically ionizable" derivatives for the enhancement of ESI-MS performance for the analysis of low polarity analytes. The approach was successfully employed in the analysis of saw palmetto fruit extract and plant oils where alcohols, phenols, and sterols were analyzed as ferrocenecarbarmate ester derivatives while pinacol and diol were analyzed as ferroceneboronate derivatives[81, 82]. Derivatization using dansyl chloride was similarly reported to significantly increase the sensitivity in the determination of estradiol-17β in human serum, with limit of detection (LOD) of 1 ng/L[83] while in another study, a lower limit of quantification (LLOQ) of 1 pg/mL was reported.[84]. Shou et.al[85] reported a 1000-fold improvement in sensitivity of dansylated ethinyl estradiol versus underivatized in human plasma where LLOQ of 2.5 pg/mL was achieved[86].

Phytosterols are nonpolar molecules and hence poorly ionized using ESI[87]. Canabate-Diaz etal.[88] evaluated the efficiency of ESI versus atmospheric pressure chemical ionization (APCI) in both positive and negative ionization to analyze a series of phytosterols in olive oil, namely stigmasterol, β-sitosterol, sitostanol, fucosterol, erythrodiol, and uvaol. Satisfactory ionization (solvent system containing 0.01% acetic acid) was achieved in the positive ionization and SIM mode was employed. Whereas APCI consistently formed $[M+H-H_2O]^+$ ions, ionization in ESI was varied among these compounds. Fucosterol and cholesterol formed $[M+H-H_2O]^+$, β-sitosterol and fucosterol $[M+H]^+$, cholesterol, erythrodiol, and uvaol formed $[M+Na]^+$ while β-sitosterol, erythrodiol, and uvaol produced $[2M+H]^+$. In addition, stigmasterol and sitostanol did not produce detectable ions using ESI[88]. Similar results were obtained when ESI and APCI were compared in positive ionization for the development of an analytical method for the determination of oxysterols, phytosterols, and cholesterol intermediates in human serum[89]. It was found that, in ESI (where solvent system contained ammonium acetate), the majority of these analytes (β-sitosterol, desmosterol, campesterol, lanosterol, 24-hydroxycholesterol, and 27-hydroxycholesterol) formed $[M+H]^+$, stigmasterol formed an ammonium adduct, cholestanol and

sitostanol formed $[M+H-H_2O]^+$ while in APCI, $[M+H-H_2O]^+$ was consistently formed for all the compounds. Although $[M+H]^+$ is observed during electrospray, it has been suggested not to arise from gaining a proton but rather as a result of losing gaseous ammonia (NH_3) from the ammoniated adduct[90, 91]. Even though ESI showed reproducible MRM transitions, APCI ionization was eventually adapted for the analysis as ion count intensity was at least 4 times higher than ESI.

In addition to conventional ESI, nano LC-ESI has also been adapted due to reduced analysis time, high efficiency, small sample size, and low consumption of solvents. A nano-LC-MS operated in the positive ion mode was used for the accurate identification of phytosterols present in extra-virgin olive oil; however, quantitative analysis was performed using HPLC linked to an ultra-violet (UV) detector[87]. Unlike previous studies in which the produced ion varied among various phytosterols, an $[M+H-H_2O]^+$ ion was observed for all tested phytosterols (stigmasterol, campesterol, brassicasterol, β-sitosterol, and cholesterol)[87]. ESI was sufficient for the identification but it failed in quantification as a mixture of standards at the LOD of the LC-UV method could not be detected using nano LC-MS due to the low ionization efficiency of the targeted phytosterols[87]. Similaraly, $[M+H-H_2O]^+$ ions were observed during the quantification of free phytosterols, namely cholesterol, ergosterol, stigmasterol, and β-sitosterol present in tobacco leaves[92]. Ionization was carried out via ESI in the positive ionization with MS/MS being applied for quantification. Satisfactory results were achieved with a limit of quantification (LOQ) in the range of 4.8-9.7 ng/mL which was three orders of magnitude lower compared to GC-MS analysis of derivatized phytosterols [93].

As discussed above, it is apparent that the ionization behavior observed in ESI for the various phytosterols in olive oil, tobacco leaves, extra-virgin oil and human plasma was not constant despite structural similarities. For example, as seen in Table 2, stigmasterol and sitostanol did not show detectable ions in olive oil, however, they were ionized in human serum[88, 89]. While stigmasterol formed $[M+NH_4]^+$ in human serum[89], it formed $[M+H]^+$ in both extra-virgin olive oil and tobacco leaves despite the fact that they all contained ammonium acetate as an additive.[87, 92]. β-sitosterol formed $[M+H]^+$ in the analysis of both olive oil and human serum, however, $[M+H-H_2O]^+$ was formed in extra-virgin olive oil and tobacco leaves[87-89, 92].

To enhance ESI ionization, phytosterols are chemically derivatized, particularly as ester derivatives. Fatty acid steryl esters have been analyzed in corn and phytosterol enriched foods such as spreads[94, 95]. Plant sterols are added to food as fatty acid steryl esters to enhance oil solubility and residence time in the small intestine[96]. It was observed that when ammonium acetate was used as an additive, ammonium adducts $[M+NH_4]^+$ were formed during ESI-MS[95]. In Pro-Activ® margarine, sitosteryl-oleate (SO) ester and its oxidation products in simulated cooking conditions and after exposure to sunlight were analyzed; although SO formed $[M+NH_4]^+$, some of the oxidation products did not[94]. However, when lithium formate was used, lithiated adducts $[M+Li]^+$ were formed for both SO and its oxidation products. Quantification

was accomplished using MRM, showing that, SO formed many oxidized products (oxidation on sterol and/or oleic acid moiety) when exposed to high temperatures and cooking time. On the other hand, sunlight had very little influence in forming oxidized products[94]. Similarly, steryl esters were identified in margarine enriched with sterols derived from soybean by monitoring $[M+NH_4]^+$ and it was found that, β-sitosterol and campesterol were esterified to stearic, oleic, and/or linoleic acid. This consistent formation of $[M+NH_4]^+$ was also observed during the identification of steryl esters present in corn where β-sitosterol, campesterol, and stigmasterol were found to be esterified to oleic and/or linoleic acid. Structural confirmation of steryl ester isomers was successfully accomplished by performing second generation MS analysis, i.e.MS^3. In fact ESI was superior in analyzing intact sterol esters compared to APCI that predominantly formed $[M+H-HOOCR]^+$ ions resulting from in-source fragmentation [95].

Another derivatization strategy was the use of 4'-carboxy-subsitituted rosamine and N-alkylpyridinium derivatives in the analysis of phytosterols in vegetable oils, functional foods, and medicinal herbs[97, 98]. The derivatized sterols possess a permanent charge due to the presence of a quaternary amine, leading to enhanced ionization. The tested derivatized phytosterols showed $[M]^+$ ion and the MRM mode was used for quantification. Both derivatization showed improved detection limits, N-alkypyridinium in the range 0.02-0.05 ng/mL while 4'-carboxy-subsitituted rosamine was 0.005-0.015 ng/mL[97, 98] when compared to 4.8-9.7 ng/mL[92] for underivatized phytosterols. The latter lower detection limits have been attributed to improved sample preparation, where microwave assisted derivatization combined with dual ultrasound-assisted dispersive liquid–liquid micro-extraction helped in reducing matrix effects although the former employed HRMS[97]. In another study utilizing HRMS, *N* -chlorobetainyl chloride was used as a derivatization agent in the analysis of free and esterified sterols in *Arabidopsis* leaves or roots[99]. Whereas the free sterols were derivatized and formed $[M+Betainyl]^+$, SE, SG, and ASG were observed as ammoniated adduct, $[M+NH_4]^+$. MS/MS was applied for quantification with an LOQ of 0.01 nmol where the MRM transitions, $[M+Betainyl]^+ \rightarrow [Betainyl]^+$ for FS and $[M+NH_4]^+ \rightarrow [M-NH_4-FA+H]^+$ for conjugated phytosterols were monitored.

Derivatization has also been applied in biological samples and phytosterols were first converted into their picolinyl ester prior to MS analysis[100-102]. An acetonitrile-sodium adduct of the picolinyl ester $[M+Na+CH_3CN]^+$ was formed and the sodiated product ion $[M+Na]^+$ was used for MRM analysis[100-102]. Eleven sterols were quantified where the MRM transition $[M+Na+CH_3CN]^+ \rightarrow [M+Na]^+$ was employed . Through derivatization, excellent sensitivity (LOD of 0.259 nmol vs 1000 nmol for derivatized and underivatized cholesterol, respectively) was achieved making the analytical approach ideal for the diagnosis of inherited disorders in cholesterol metabolism. In fact, all the 11 sterol compounds that were quantified showed an LOD of less than 2.59 nmol[101]. In addition, it was possible to quantify lathosterol, desmosterol, campesterol, and sitosterol which are serum biomarkers involved in the synthesis and absorption of cholesterol in humans. It is worth noting that the physico-chemical properties of sterols often influence their ionization and fragmentation pattern. Picolinyl ester derivatives of mono-hydroxy

sterols formed $[M+Na+CH_3CN]^+$ ion which in MS/MS showed the transition $[M+Na+CH_3CN]^+ \rightarrow [M+Na]^+$ and $[M+Na+CH_3CN]^+ \rightarrow$ [Picolinic acid+Na]$^+$ when collision energy of 10-15V and 25-30V were applied, respectively[101, 102]. However, di-, tri-, and tetra-hydroxysterols formed $[M+Na]^+$ ion which showed transitions to [M-Picolinic acid+Na]$^+$ or [Picolinic acid+Na]$^+$ at collision energy of 10-30V[102]. Therefore, collision energy was maintained at 10-15v for phytosterols (monohydroxy). In addition, these analytical methods were validated for precision, accuracy, linearity, recovery and matrix effect[101, 102] which ensure the validity of the obtained quantitative data.

Although derivatization of phytosterols have been shown to enhance and promote uniform ionization, factors such as the reaction conditions, derivatization efficiency, selectivity, and the stability of the derivatives need to be considered. However, this information is scarce in the literature and not usually reported, apart from few studies highlighted below. The synthesis of 4'-carboxy-substituted rosamine derivative was performed under microwave (750W) at 60 °C for 5 mins, with a yield more than 40% and a 98% purity[97]. The derivative was stable when compared to rosamine's structural isomer, rhodamine B, which undergoes transformation to form a lactone and loses the intramolecular positive charge.

N-alkylpyridinium derivative synthesis was done at room temperature by vortexing the reaction mixture for 5 minutes while the synthesis of beitanyl chloride required 4 h at 42 °C. The beitanyl chloride derivative was stable for at least 48 h at 18 °C[99]. Picolinyl esters synthesis, on the other hand, was performed at 25 °C for 1h or at room temperature for 30 min[100, 102]. Picolinyl esters seem to be highly favored for the ESI-MS analysis, likely due to the relatively moderate reaction conditions. Besides, picolinyl esters were reported to be stable in acetonitrile for at least 6 months[101]. Generally speaking, it can be speculated that the derivatized products need to be analyzed immediately unless information relating to stability have been reported. In summary, ESI is a widely-used ionization technique in MS analysis. However, it shows deficiency in its ionization efficiency for underivatized phytosterols with better results achieved via derivatization, which is rather a laborious process. ESI did not produce observable ions for some phytosterols and due to the observed inconsistency, alternative ionization techniques have been adapted for the identification and quantification of phytosterols[88, 89]. These techniques include APCI, atmospheric pressure photoionization (APPI), matrix assisted laser desorption ionization (MALDI) and ambient mass spectrometry; direct analysis in real time (DART).

Liquid Chromatography-Atmospheric Pressure Chemical Ionization-Mass Spectrometry (LC-APCI-MS)

Unlike ESI, APCI involves a desolvation process followed by reaction of the solvent molecules with already ionized nitrogen gas in the source[103]. Depending on proton affinity, charged analyte species are formed either by charge transfer, addition, or removal of a proton. This technique is ideal for low polarity or neutral low molecular-weight compounds, and it is widely applied in lipid analysis[104].

Phytosterols have been identified and quantified from vegetable oils, such as soybean, palm, sunflower and olive using LC-APCI-MS[43, 105, 106]. Hexane or diethyl ether are usually used for extraction after saponification although other additional clean-up steps, such as solid phase extraction (SPE) may be employed[107, 108]. Thin layer chromatography (TLC) has also been employed, as clean-up step, where each of the TLC spots is extracted and analyzed[88]. TLC was used to separate phytosterols prior to LC-MS analysis and quantification was performed in the SIM mode for the ion designated as $[M+H-H_2O]^+$ [88]. Similarly, Carretero et.al[105] employed SIM mode, however, their results after analyzing phytosterols with and without TLC were similar. In fact, the results were comparable to those obtained by employing GC-FID, the American oil chemists' society (AOCS) official method[105]. The various phytosterols identified in vegetable oils were cholesterol, Δ^5 avenasterol, stigmasterol, β-sitosterol, sitostanol, uvaol, erythrodiol, fucosterol, brassicasterol, cycloartenol, lupeol, and campesterol, although the composition and amount were dependent on the source of oil[43, 105]. The LOQ values obtained with and without TLC were similar and ranged from 150-2,515 ng/mL[88, 105].

Quantification using the MRM mode, however, improved the sensitivity in the determination of phytosterols in vegetable oils with LOD values in the range of 2-25 ng/mL[43]. Although base peak separation is not required in the MRM mode, the co-eluting compounds should not share the same MRM transitions. In such a case, either chromatographic separation should be employed or the two species are measured as one mixture. For example, erythrodiol and uvaol have the same MRM transitions *m/z* 425.3 → 407/295 as shown in Figure 3 and were quantified collectively [106]. Thus, despite the selectivity of MRM, it still suffers from the inability to differentiate structurally similar compounds that share exact MRM transitions and retention times; co-eluting compounds have, therefore, to be quantified collectively which can be considered a limitation depending on the intended application. On the other hand, a gradient elution using acetonitrile/methanol/water (Figure 4) allowed for the separation of erythrodiol and uvaol with an LOD of 0.003-0.05 mg/L in SIM mode[109].

In addition to vegetable oils, phytosterols are analyzed in plant samples such as rice, sea weed, and cereals (oat, sesame seed, corn, peanuts) using APCI in positive ionization [45, 107, 110, 111]. The formation of $[M+H-H_2O]^+$ ion is predominant although $[M+H-2H]^+$ and $[M+H-4H]^+$ has also been observed, albeit in low intensity[112]. Identification of desmosterol, cholesterol, campesterol, fucosterol and stigmasterol in seaweed was accomplished in the SIM mode by monitoring the $[M+H-H_2O]^+$ ion[110]. Phytosterols were confirmed by comparing the retention times and MS/MS to that of standards. Similarly, Stigmasterol, β-sitosterol, and campesterol were identified and quantified in seven Oryza sativa L. rice cultivars using 5α-cholestan-3β-ol as internal standard and although their concentrations were found to vary depending on the rice cultivar, sitosterol was always the major component[45].

SIM mode was employed in the analysis of phytosterols present in oats and sesame seeds[107]. However, some phytosterols were either not detected or quantified, probably due to the use of SIM mode rather than MRM, reducing both selectivity and specificity. Further drawbacks

of using SIM are highlighted in an LC-APCI-MS method developed for the identification and determination of phytosterols in two saw palmetto standard reference materials (SRMs) developed by the National Institute of Standards and Technology[113]. Both acid and base hydrolysis was necessary for the saw palmetto fruit while only base hydrolysis was needed for the saw palmetto extract to achieve high yield of phytosterols. Quantification was done by monitoring $[M+H]^+$ for lupenone and $[M+H-H_2O]^+$ for cholesterol, brassicasterol, campesterol, stigmasterol, β-sitosterol, cycloartenol, and lupeol[113]. Baseline resolution however, was not achieved for the pair's cycloartenol/ β-sitosterol and campesterol/stigmasterol and potential interferences were identified due to the presence of common fragments. For example, campesterol produces fragments at *m/z* 395 (stigmasterol monitoring ion) and this bias can only be eliminated if there is chromatographic separation or adaptation of an MS/MS analysis. Two LC methods, therefore, were employed where phenyl column with acetonitrile/water resolved campesterol/stigmasterol and C18 column with methanol/water resolved cycloartenol/ β-sitosterol, permitting quantification[113].

The use of MS/MS increased the sensitivity in the quantification of phytosterols in edible oils with one MRM transition being monitored[43]. However, it is favorable to use more than one MRM to add additional level of confidence in terms of selectivity and specificity. For example, free phytosterols present in tobacco leaves as well as phytosterols intermediates in spinach leaves were analyzed by monitoring two MRM transitions, one as quantifier and the other as qualifier[111, 114]. Millan et al.[115] have developed an MS/MS method for the identification and quantification of phytosterols in oenological matrices by monitoring two MRM transitions. With an LOQ of 8 ng/mL, this analytical method was applied in screening and quantifying phytosterols present in various matrices (pulp, skin, seed, and wine) where β-sitosterol, stigmasterol, campesterol, and fucosterol were identified. However, desmosterol, brassicasterol, ergosterol, and sitostanol were not detected. As shown in Table 3, various product ions are utilized during MRM either because of their high intensity and/or because they serve as diagnostic ions. The $[M+H-H_2O]^+$ ion have similar fragmentation pattern in both ESI and APCI and the product ions are identical[87, 92]. Figure 3 shows the proposed structures of phytosterol ions and some of the product ions formed (cleavage of side chain or sterol nucleus ring) in APCI ionization.

Monitoring two MRM transitions becomes critical when analyzing biological samples to ensure selectivity. LC-APCI-MS/MS was successfully applied in the analysis of phytosterols and cholesterol intermediates in plasma and serum[89, 116]. Rat plasma was used to study the pharmacokinetic properties of phytosterols after rats were fed 500 and 2000 mg/kg doses of Insadol extract (contains β-sitosterol, campesterol, and stigmasterol)[116] while human serum was used in quantifying phytosterols and cholesterol intermediates[89]. In human serum, SPE clean-up step was needed prior to LC-APCI-MS/MS analysis. Deuterated cholesterol was used in both cases as the internal standard and LLOQ of 50-250 ng/mL and 4.05 pM (≈ 0.0017 ng/mL) in rat plasma and human serum respectively, were achieved. Free and bound sterols have also been analyzed in human serum and quantified in SIM mode using deuterated cholesterol as internal

standard, however, even with SPE clean-up, the LLOQ was 8-274 μg/mL[117], which was much higher than methods in which MRM and SPE were employed[89, 116].

In the sections discussed above, phytosterols have been quantified in their free forms or after their hydrolysis using a base. However, acid hydrolysis is required when steryl glycosides (SG) and acylated steryl glucosides (ASG) are present as the acetal bond between the sterol hydroxyl and the sugar moiety is resistant to base hydrolysis[118, 119]. Both free phytosterols (FS) and conjugated phytosterols (SG, ASG, fatty acid steryl esters-SE) have been isolated and quantified in functional foods (spreads, beverages) and cereals (spelt, wheat)[108, 112, 120]. The quantification was achieved in the SIM mode by monitoring $[M+H-H_2O]^+$, $[M-FA+H]^+$, $[M-FA-sugar+H]^+$, and $[M-sugar+H]^+$ for FS, SE, ASG, and SG respectively, where FA represents a fatty acid moiety. In addition to the major ion used in SIM for quantification, other minor ions were observed as shown in Table 4 for selected free and conjugated phytosterol.

Although the analysis of conjugated phytosterols is quite challenging due to matrix effects and the lack of authentic standards, statistical approaches can be applied in analyzing the data, allowing for the establishment of relationships between/amongst the samples in relation to the phytosterol profile and their quantity. Millan et al.[121] have reported the use of LC-APCI-HRMS as well as the application of chemometrics for a targeted metabolomics study of grapes based on phytosterol content. The acquired accurate mass data and isotopic distribution of the precursor and product ions were compared to the MS/MS data of reference compounds for confirmation. An accuracy of 93.3%, 100%, and 96.7% in pulp samples, peel, and seeds was achieved, respectively, in discriminating between grape varieties based on phytosterol contents. However, even with high mass accuracy, conjugated phytosterols, although separated on an LC column, undergo in-source fragmentation showing identical *m/z* value for the monitored analyte. However, such in-source fragmentation was not observed with ESI in this case. LC-APCI-MS has been combined with chemometrics in evaluating phytosterol correlations with health disorders like cholesterolemia[122]. Quantification was done in the SIM mode where $[M+H-H_2O]^+$ and $[M-FA+H]^+$ were monitored for FS and SE, respectively. Linear discriminant analysis (LDA) was applied after measuring phytosterols in human plasma and it was found that, certain phytosterol ratios are characteristic and more discriminative of cholesterol metabolism and cholesterol-related disorders[122]. A similar statistical approach was employed in determining the compositional distribution of phytosterol esters in tobacco leaves[123]. Principal component analysis (PCA) was applied and it was found that, the compositional variation observed in a variety of tobacco leaves was due to the curing process rather than the growing district.

In general, APCI showed better ionization efficiency for phytosterols without the need for derivatization. Its application makes the analytical process less laborious, fast, and ideal for the analysis of human, animal and plant samples. Unlike ESI, consistent ionization was observed where FS and SE formed $[M+H-H_2O]^+$ and $[M-FA+H]^+$ ions, respectively. Thus, APCI ionization is widely adapted for the analysis of phytosterols. In fact, APCI was adapted for the analysis after ESI failed to ionize or showed low ion count intensity for phytosterols[88, 89].

Although it is the most preferred ionization technique for phytosterols, additional ionization techniques have also been evaluated but with limited applications, as discussed below.

Other Ionization Techniques Applicable for the Analysis of Phytosterols

APPI is similar to APCI except that, a UV light source is used for ionization instead of a corona needle and $[M]^{\cdot+}$ predominantly forms. The cationic molecular radical is only formed if the ionization potential of the analyte is lower than that of the UV light source[124]. Hence, dopants such as toluene or acetone are often employed to enhance ionization leading to the formation of $[M+H]^+$ and $[M+H-H_2O]^+$ ions[124, 125]. Although APPI has not seen much application for the analysis of phytosterols, it has been shown to be an alternative option for compounds that hardly ionize in ESI. For example, the formation of $[M]^{\cdot+}$, $[M+H]^+$ and $[M+H-H_2O]^+$ ions were observed when LC-APPI-MS was used for the authentication of olive oil [126].

LC-APPI-MS in SIM mode has been employed for the quantification of 27-hydroxycholesterol in human plasma, with toluene as dopant[127]. After saponification, SPE clean-up was performed and quantification using deuterated 27-hydroxycholesterol as internal standard was done by monitoring the $[M+H-H_2O]^+$ ions. The LOQ was 10 ng/mL compared to 25 ng/mL with APCI[128]. In another study, MS/MS was applied for the quantification of both free and esterified brassicasterol, stigmasterol, campesterol, and β-sitosterol in human serum[129]. The efficiency of using toluene, acetone, and anisole as dopant was also evaluated. Free sterols formed $[M+H-H_2O]^+$ while esterified sterols formed $[M-FA+H]^+$. Since phytosterol esters ionize via the ester bond cleavage, the precursor ion for the corresponding free and esterified phytosterol are similar and hence the same MRM transitions were used. Toluene showed better ionization than anisole or acetone and the LOD of both free and esterified phytosterols ranged 0.25-0.68 µg/L[129]. Riffault et al.[130] reported the application of LC-APPI-HRMS for a non-targeted approach in characterizing an ethyl acetate extract from rose flower. Negative ionization was employed and acetone was used as a dopant. Free phytosterols were not identified as they did not ionize in the negative ion mode, however, phytosterol conjugates (SG, and ASG) were detected as $[M-H]^-$ and identification was based on their MS/MS fragmentation.

Ambient mass spectrometry such as direct analysis in real time (DART) was also used in the analysis of phytosterols. Similar to APPI, $[M]^{\cdot+}$ and $[M+H-H_2O]^+$ ions were formed depending on proton affinity and the ionization potential of the analyte, as well as the presence of additives[131]. DART-MS was employed in the qualitative analysis of phytosterols in vegetable oils, animal oils, and phytosterols enriched-margarines where FS and SE were monitored as $[M+H-H_2O]^+$ and $[M-FA+H]^+$ ions, respectively[132, 133]. In addition, statistical analysis of the obtained data (presence of FS, SE, and triacylglycerol's) was conducted to aid in understanding the compositional variation and identify ions or set of ions that can satisfactorily differentiate the samples[132, 133]. Since DART does not require derivatization or laborious sample preparation steps, it can provide instant detection and was successfully utilized for the analysis of β-sitosterol and its oxidation products during a heat-accelerated reactions in vegetable oils[134]. Quantification

with DART is however limited due to the dependence of the analytes signal on its positioning in the gas stream. Thus, if quantification is done, the linear range and sensitivity are extremely compromised.

Finally, MALDI was used for the analysis of phytosterols. MALDI uses a matrix that absorbs laser light energy and acts as a proton donor or acceptor[135]. The choice of matrix and the laser energy employed during MALDI plays a key role for the overall ionization efficiency. Laser energy (4-42 mJ cm^{-2}) and choice of matrix [2,4-Dihydroxybenzoic acid (2,4-DHB), 2,5-dihydroxybenzoic acid (2,5-DHB), a-cyano-4-hydroxycinnamic acid (CHCA), dithranol] were evaluated for the analysis of β-sitosterol[136]. The predominant ion observed was $[M-H_2O]^+$ at *m/z* 396.6 although other ions such as $[M+H-H_2O]^+$, $[M+Na]^+$, and $[M+K]^+$ were also observed, albeit in low intensity. The four matrices produced similar detectable peaks with 2,4-DHB giving the highest intensity (five times that of CHCA), dithranol had the lowest peak intensity and high chemical noise while a high laser intensity was found to induce oxidation. An LOD of 10 nM was achieved with 2,4-DHB under optimized conditions of laser energy and solvent system. Similarly, silver nanoparticles were used as a matrix in the determination of phytosterols in vegetable oils[137]. Brassicasterol, cholesterol, stigmasterol, campesterol, and β-sitosterol were quantified as $[M+Ag]^+$ with 6-ketocholestanol as the internal standard. Better sensitivity was achieved with an LOD of 6-12 fmol when compared to 110-140 pmol for UV detector.

To enhance phytosterol ionization during MALDI-MS analysis, they have been converted into their corresponding picolinyl esters, N-methylpyridyl ethers, and sulfated esters[138]. Picolinyl esters and N-methylpyridyl ethers formed $[M+Na]^+$ and $[M]^+$, respectively in positive ionization while sulfated esters were sensitive in the negative mode and formed $[M-H]^-$. Employing a suitable matrix is critical and the use of 2,5-DHB and 2,4,6-trihydrooxyacetophenone monohydrate (THAP) for picolinyl esters, THAP for N-methylpyridyl ethers, and *p*-nitroaniline and dithranol for sulfated esters were recommended. While the reported LOD for picolinyl esters and sulfated esters were 1.5 µg/mL and 0.2 µg/mL, respectively, quantification using N-methylpyridyl ethers was found unsuitable due to inefficiency in derivatization and further efforts to improve the yield was unsuccessful. MALDI ionization behaviour closely resembles that of ESI especially for the picolinyl esters where $[M+Na]^+$ is formed, however, $[M+H-H_2O]^+$ ion was in low intensity for underivatized phytosterols although it is the predominant ion in both APCI and ESI.

These alternative ionization techniques, although not widely applied for the analysis of phytosterols, could serve as complimentary techniques to EI, ESI, and APCI. The choice of the ionization technique as well as the MS strategy to be used is based on the analytical needs such as sensitivity, accuracy, reproducibility, matrix effects, and simplicity. However, it should be noted that instrument and /or ionization sources from various manufacturers or different models from the same manufacturer may show varying MRM transitions[90], sensitivity, and accuracy. Although APCI will be the most preferred ionization source for the analysis of phytosterols as no

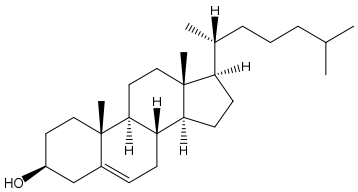
derivatization is required, limitations due to instrument availability can be mitigated by applying alternative MS-based analytical strategies. When derivatization is required, reagents with high conversion efficiency and stable products should be employed.

In conclusion, due to the health-associated benefits of phytosterols, highly sensitive analytical strategies using MS are required for their analysis. The choice of the ionization source is critical as it determines the overall efficiency of the analytical method. ESI ionization showed inconsistency despite structural similarities among the various phytosterols. Thus, there is limitation for the application of ESI, especially if phytosterol concentrations are extremely low. Derivatization, however, increased the ionization efficiency as well as promoted uniform ionization behavior.

APCI, on the other hand, has been widely adapted due to its ionization efficiency without the need for derivatization, primarily forming $[M+H-H_2O]^+$ ions. Despite its wide use, little is done to fully characterize the numerous product ions observed during the MS/MS analysis of phytosterols. These compounds usually produce complex MS/MS spectra and there is a need for a universal MS/MS fingerprint. Such comprehensive analysis will allow for the development of efficient profiling experiments and can aid in identifying new structures. It is anticipated that more validated methods will be needed as phytosterols are being used for health-related applications. APPI is similar to APCI however, it has not found much applications probably due to instrument limitations. Finally, application of other methods is limited. MALDI is highly dependent on the choice of matrix and the laser energy while DART offer a fast analysis approach as the sample preparation steps are eliminated. Although the method performance is highly dependent on the ionization efficiency, sample preparation also plays a key role and it can compromise the analysis even when the best ionization technique and MS strategy has been employed.

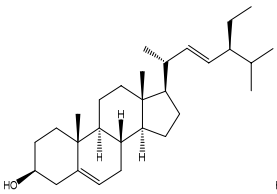
Funding

This work was supported by an Agriculture Development Fund (ADF) grant, Ministry of Agriculture, Government of Saskatchewan, Canada.



Cholesterol

4-desmethylsterols



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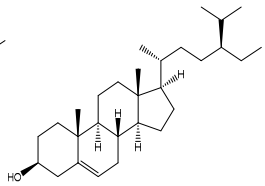
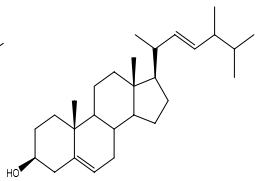


Sitosterol



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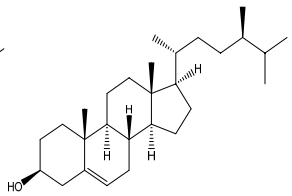
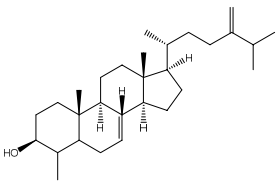
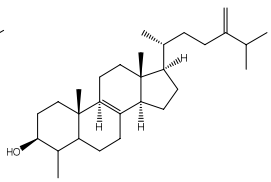


Campesterol

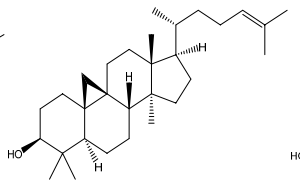
4-methylsterols **4,4’-dimethylsterols**



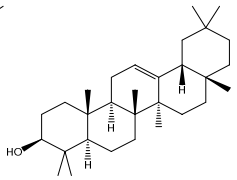
Gramisterol



Obtusifoliol

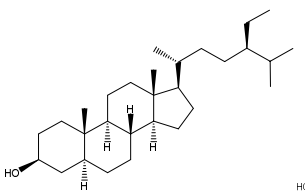


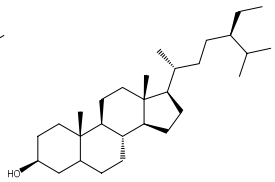
Cycloartenol



Amyrin

Stanols

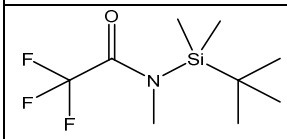
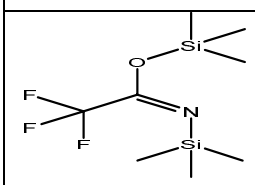
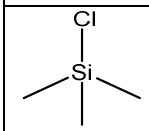
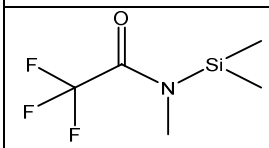
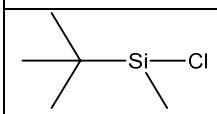
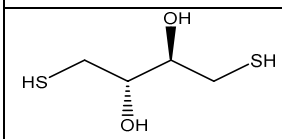
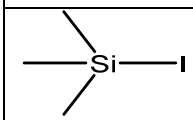




Stigmastanol Sitostanol

Figure 1. Chemical structure of cholesterol and major phytosterols/phytostanols.

Table 1. Structures of commonly utilized silylating reagents.

STRUCTURE/NAME	EXAMPLES OF APPLICATIONS				Refs
	Reagent/s	**Sample analyzed**	**Reaction conditions**	**figures of merit**	
 MTBSTFA	BSTFA/TMCS	Chocolate	70°C, 15 min	Not reported	*139*
		Milk/Yoghurt	60°C, 30 min	LOD 0.1 µg/mL	*140*
		Rice	Room temp, overnight	Not reported	*141*
 BSTFA	BSTFA MSTFA	Pumpkin	70°C, 2h	LOD 0.11-0.19 µg/mL LOQ 0.37-0.65 µg/mL	*63*
 TMCS					
 MSTFA	MSTFA/TMCS	Rhizomes	70°C using 50W microwave, 10 min	Not reported	*64*
 TBDMS	MSTFA/DTE/TMIS	Maquis leaves	60°C, 30 min	LOQ 0.1 µg/mL	*65*
 DTE					
 TMIS					

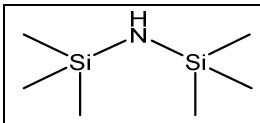
 HDMS						

Table 2. The major ions formed via ESI positive ionization on various phytosterols present in vegetable oils, human serum, and tobacco leaves.

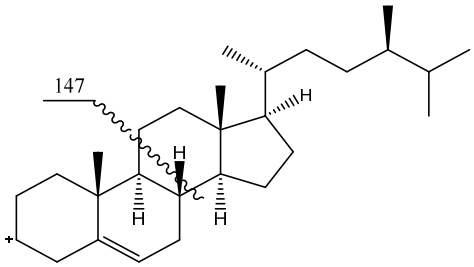
	Major precursor ion						
Sample analyzed	$[M+H]^+$	$[M+H-H_2O]^+$	$[M+NH_4]^+$	$[M+Na]^+$	$[2M+H]^+$	Solvent system	Refs
Olive oil	β-sitosterol, fucosterol	Fucosterol, cholesterol		Cholesterol, erythrodiol, uvaol	β-sitosterol, erythrodiol, uvaol	Acetonitrile/ Water (0.01% acetic acid)	*88*
Extra-virgin olive oil		Stigmasterol, brassicasterol, campesterol, β-sitosterol, cholesterol				Methanol (15mM ammonium acetate)	*87*
Human serum	β-sitosterol, desmosterol, campesterol, lanosterol, 24 & 27-dihydrocholesterol	Cholestanol, sitostanol	Stigmasterol			Methanol/ Water (5mM ammonium acetate)	*89*
Tobacco leaves		Cholesterol, ergosterol, stigmasterol, β-sitosterol				Water (10mM ammonium acetate) /Methanol (0.1% formic acid)	*92*

Table 3. Precursor ion and MRM transitions commonly used for the identification and quantification of phytosterols in plant and biological samples[43, 89, 92, 111].

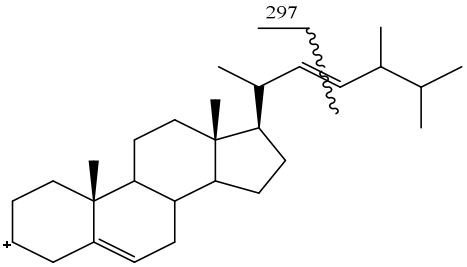
NAME	$[M+H-H_2O]^+$ SIM (*m/z*)	MRM Transitions (*m/z*)
Stigmasterol	395	297, 55 **83.1**/147.3, **83.1**/81.1
Brassicasterol	381	297
B-Sitosterol	397	161, 147 **147.2**/159.2, **161.3**/147.7
Campesterol	383	161, 81 **147.3**/161.1, **146.9**/81.1
Cycloartenol	409	191 **109.0**/94.9
Lupeol	409	137
Cholesterol	369.3	**161.1**/147.1, **147.1**/161.3

Ergosterol	379.3	**159.1**/145.1, **69.3**/159.2

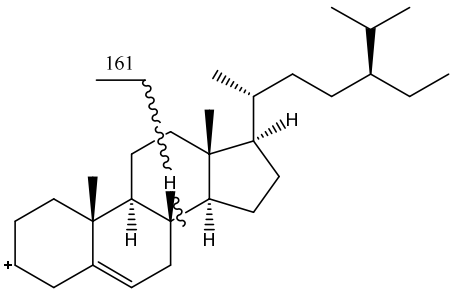
Bolded values represent the quantifier ion when two MRMs have been monitored



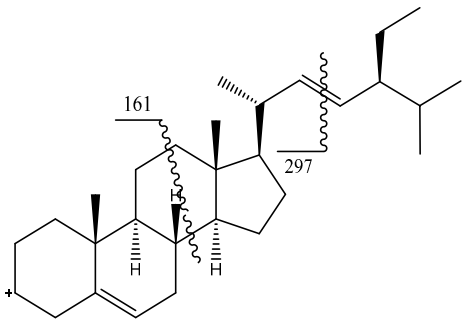
Campesterol 383 *m/z*



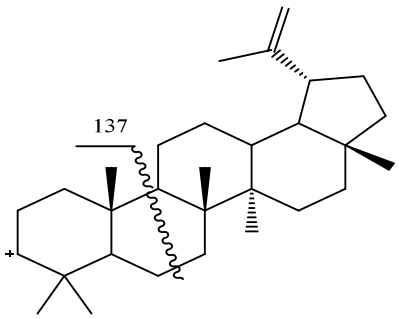
Brassicasterol 381 *m/z*



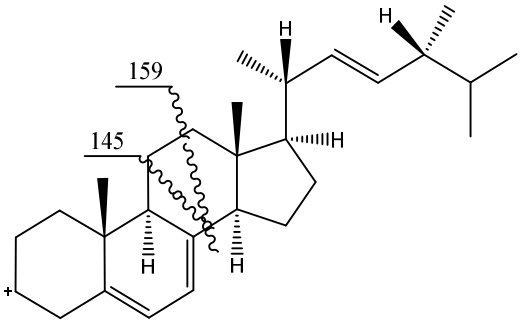
β-sitosterol 397 *m/z*



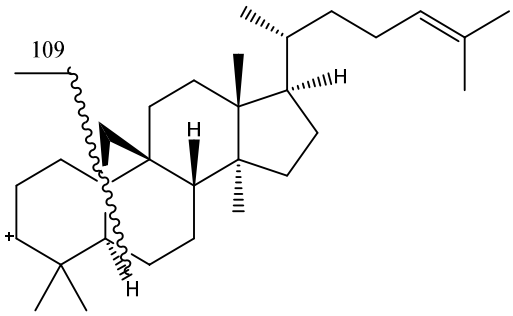
Stigmasterol 395 *m/z*



Lupeol 409 *m/z*

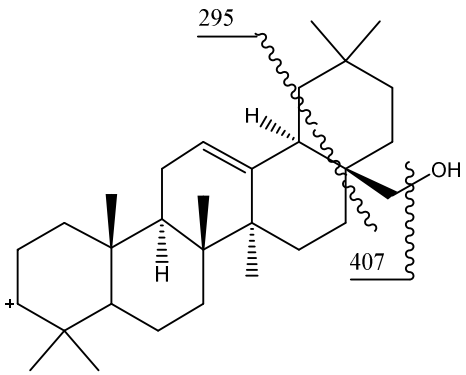


Ergosterol 379 *m/z*

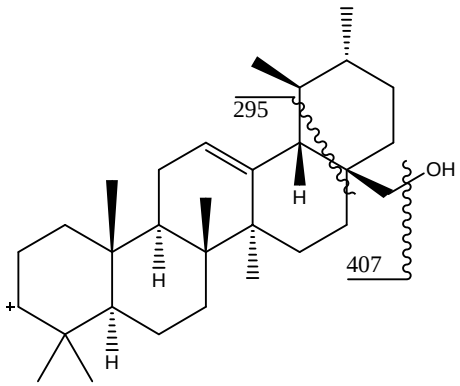


Cycloartenol 409 *m/z*

Figure 2. Proposed structures of $[M+H-H_2O]^+$ ion and the fragmentation pathways for the various product ions formed in ESI and APCI ionization, based on Mo et.al[43].

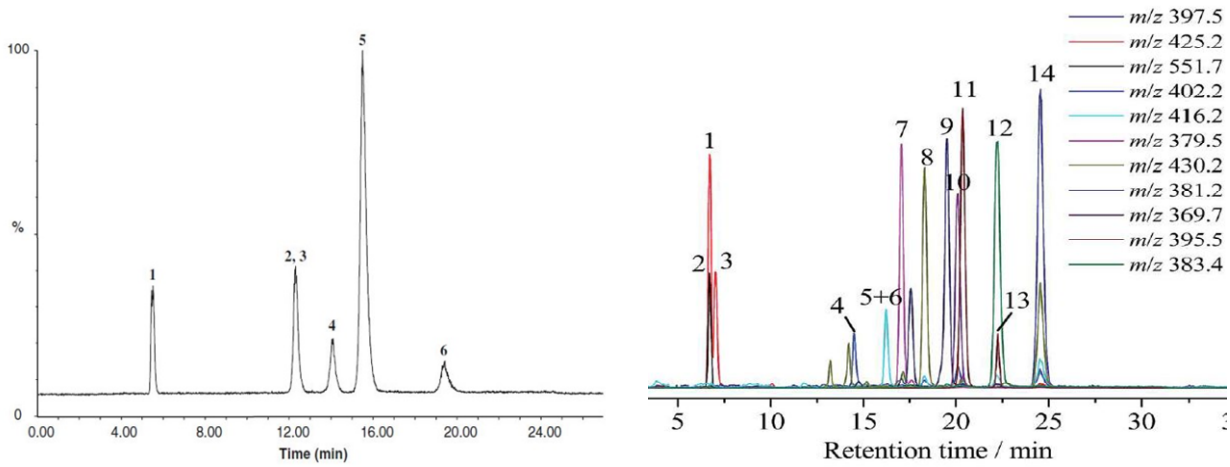


Erythrodiol 425.3 *m/z*



Uvaol 425.3 *m/z*

Figure 3. Structures of the $[M+H-H_2O]^+$ ions of erythrodiol and uvaol at 425.3 m/z and the proposed fragment pathways that give similar product ions, based on Mo et.al[43].



A B

Figure 4. Phytosterols analysis in olive oil using gradient elution where (A) shows total ion chromatogram obtained using acetonitrile/water with peak assigned **1** representing erythrodiol + uvaol, and (B) extracted ion chromatogram obtained using acetonitrile/methanol/water where peaks **1** and **3** represent erythrodiol and uvaol, respectively. Reprinted with authorization from Vidal et.al[106] and Gu et.al[109].

Table 4. Characteristic ions observed during APCI ionization of selected free and conjugated phytosterols[112].

		Fragment ions		
Name	Molecular weight	Major ions (*m/z*)	Minor ions (*m/z*)	
FS: β-sitosterol	414	$[M+H-H_2O]^+$ 397	$[M+H-2H]^+$ 413	$[M+H-4H]^+$ 411
SE: cholesteryl oleate	650	$[M-FA+H]^+$ 369	$[M+H_2O]^+$ 668	$[M+H]^+$ 651
SG: β-sitosteryl β-D-glucoside	576	$[M-sugar+H]^+$ 397	$[M+H_2O]^+$ 594	
ASG: β-sitosteryl (6'-O-palmitoyl) β-D-glucoside	814	$[M-FA-sugar+H]^+$ 397	$[M+H_2O]^+$ 832	

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