Analysis of phytosterol by MALDI-TOF mass spectrometry†

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β -Sitosterol is the most abundant phytosterol lipid in plants. This study demonstrates a method for analyzing β -sitosterol using a laboratory-built MALDI-TOF mass spectrometer. The matrix compound, solvent system, laser intensity, alkali ion, and analyte concentration for sample preparation were optimized to acquire reproducible and informative mass spectra of the analyte. The best MALDI spectrum of β -sitosterol was obtained by mixing the analyte solution with an equal volume of 2,4-DHB/iPrOH-H₂O (9 : 1) solution (60 mM) at 13.5 mJ cm⁻² laser intensity. Under the optimized experimental conditions, β -sitosterol generated intense peaks consisting of the initial lipids and their oxidation products. The former included mass peaks at m/z 396.6, 397.6, 436.9, and 452.6, which corresponded to the [M - H₂O]⁺, [M + H - H₂O]⁺, [M + Na]⁺, and [M + K]⁺ ions of β -sitosterol, respectively. The latter included mass peaks at m/z 412.7, 413.7, and 431.9, which corresponded to the [M' - H₂O]⁺, [M' + H - H₂O]⁺, and [M' + H]⁺ ions of mono-oxidized β -sitosterol, respectively. A quantitative analysis of β -sitosterol was also conducted using a matrix peak. Calibration curves obtained for β -sitosterol show a good linear range of three orders of magnitude with a regression coefficient square of 0.992. The linear ranges were between 12.1 and 1207.5 μ M. The detection limit was 10 nM. These results demonstrate the potential application of MALDI mass spectrometry for analyzing phytosterols.

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1. Introduction

Phytosterols are sterol lipids synthesized only in plant cells.¹ More than 250 different phytosterol molecules have been identified to date.² β -Sitosterol is the most abundant and comprises 45–95% of the total sterol present in plants.³ Phytosterols are famous for their low-density-lipoprotein-cholesterol-lowering effect and have been suggested as safe and effective functional ingredients offering various beneficial effects.^{4–6} In the field of medicine, phytosterols have been suggested as useful clinical complexes in the study of human disorders.^{7–9} Phytosterols also act as fingerprints to identify the source of fecal contamination in rivers.¹⁰ Therefore, it is important to establish rapid analytical methods for the identification of phytosterols and other sterol lipids to assist in diagnosing diseases, analyzing environmental samples, and providing reliable tools for nutrition and food science.

Gas chromatography-MS (GC-MS) is the “gold” standard for sterol profile analysis.¹¹ This method has been applied to analyze sterols derived from various sample matrices^{12,13} with high sensitivity (subpg).¹⁴ GC-electron impact (EI)-MS is the most commonly used because EI provides valuable fragment-

ion information.¹⁵ However, GC-MS cannot directly analyze polar and thermally labile sterols. It requires the removal of any conjugating groups and the derivation of free acid and any alcohol groups to methyl esters and trimethylsilyl ethers,¹⁶ which reduces their polarity and simultaneously increases the volatility of the derivatives.¹⁷ Aside from the requirement of derivatization, the second disadvantage of GC-MS for sterol analysis is that the molecular ions for polyhydroxylated molecules may be weak or not observable.¹⁸ Consequently, several efforts to develop other MS techniques, such as liquid chromatography MS (LC-MS) and LC-MS/MS, have been exerted using well-established methods for sterol analysis.^{19,20} In practice, atmospheric pressure chemical ionization (APCI)^{17,21,22} and electrospray ionization (ESI)^{20,23} with LC-MS are the most frequently used methods for sterol analysis. Although both GC-MS and LC-MS are reliable for the accurate determination of sterols, they often require derivatization and chromatographic separation, which makes these methods time consuming.²⁴

Conversely, matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS) has fast and easy sample preparation, making it a valuable technique for the detection of lipid molecules.²⁵ Sterols are poor candidates for analysis because of their poor ion yields.²⁶ However, the improvements in the sensitivity, mass accuracy, and resolution of MALDI MS have opened the possibility to detect and unambiguously identify sterols.²⁷ Efforts have been exerted to analyze cholesterol, a phytosterol-like molecule found in animals.²⁸ MALDI MS has

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already been successfully used to detect cholesterol extracted from various biological samples^{29–31} or commercial cholesterol materials.^{32–34} Intense fragment ions (m/z 369) are formed by protonation and consecutive neutral loss of water moiety $[M + H - H_2O]^+$.³⁵ Phytosterol is structurally similar to cholesterol; they only differ in an additional ethyl group present at C-24.²⁸ However, the qualitative and quantitative analyses of phytosterols by MALDI MS has seldom been reported.

In this study, β -sitosterol is analyzed with a laboratory-built MALDI-TOF MS. Five experimental factors including matrix compound, solvent system, laser intensity, alkali ion, and analyte concentration for sample preparation were optimized to obtain the maximal signal in the experiment. The reliability and reproducibility of the rapid identification method for β -sitosterol was tested under different experimental conditions. This study aims to develop a qualitative and (semi)-quantitative method to rapidly analyze phytosterol using MALDI-TOF MS.

2. Materials and methods

2.1 Materials

2,4-Dihydroxybenzoic acid (2,4-DHB), 2,5-dihydroxybenzoic acid (2,5-DHB), α -cyano-4-hydroxycinnamic acid (CHCA), dithranol, and β -sitosterol (70%) were purchased from Sigma-Aldrich (St. Louis, MO). Methanol (MeOH), isopropanol (iPrOH), ethanol (EtOH), sodium chloride (NaCl), and potassium chloride (KCl) were obtained from Beijing Shiji (Beijing, China). All substances were of analytical grade and were used without further purification. Deionized water was supplied by the RCEES support laboratory.

2.2 Samples preparation

The method of sample preparation is shown in Table 1. Samples A–E were investigated in Sections 3.1–3.5, respectively.

2.3 MALDI-TOF Analysis

Mass spectra of β -sitosterol were obtained by a laboratory-built MALDI-TOF MS. The method has been described in detail elsewhere³⁶ and is reviewed briefly in the current study. The

mixture containing the analyte and matrix was pneumatically nebulized into suspended droplets at the liquid consumption of nebulization ($\sim 0.2 \text{ mL min}^{-1}$). The droplets were introduced into the MS with a nozzle of $\sim 0.12 \text{ mm}$ orifice combined with an aerodynamic lens assembly and deposited continuously on a removable stainless steel target stick. The spot of the deposit was $\sim 0.5 \text{ mm}$ in diameter. A 10 Hz pulsed 266 nm laser (New Wave Research, Polaris III) was utilized to dissociate and ionize the deposit. The pulse length of the laser was 3 ns. The generated ions were detected with a reflectron MS. Each spectrum was obtained by averaging 128 laser shots. The external calibration was carried out with polyethylene glycol 400 cationized by Na^+ . The calibration error of the mass spectra was within $\pm 0.3 \text{ Da}$.

Statistical analysis was performed using Origin 8.5. Results from three independent MALDI MS analyses for each sample were used to calculate the mean $\pm$ SD.

3. Results and discussion

3.1 Matrix compounds

The careful selection of the matrix enables the detection of the compounds of interest.³⁷ In the present study, a series of matrices that included proton-donating (2,4- and 2,5-DHB, CHCA) and neutral matrix (dithranol) were investigated in positive-ion MALDI-TOF MS. MALDI-TOF mass spectra of β -sitosterol using 2,4-DHB (A), 2,5-DHB (B), CHCA (C), and dithranol (D) as the matrices are shown in Fig. 1. Samples A(1–4) shown in Table 1 are used. The solvent system is chosen based on the comparative experiments conducted previously. All matrices behaved similarly under experimental conditions. Compared with ESI-MS that produced protonated molecular ions $[M + H]^+$ at m/z 415.7,¹⁷ MALDI-TOF MS yielded signals $[M - H_2O]^+$ at m/z 396.6. This characteristic fragment that resulted from water loss was also previously observed in mass spectra generated by EI-MS³⁸ and APCI-MS.²¹ The other peak at m/z 436.9 with low intensity was observed extensively and corresponded to $[M + Na]^+$, which would be clearly demonstrated later. Peaks at m/z 396.6 and 436.9 corresponded to $[M - H_2O]^+$ and $[M + Na]^+$ ions of β -sitosterol. In addition, Fig. 1A–C are

Table 1 Method of sample preparation in the experiment

Sample ^a	β -Sitosterol	Matrix	Solvent mixture (v/v)	Alkali ions
A1	24 μM	2,4-DHB, 60 mM	iPrOH–H ₂ O (9 : 1)	—
A2		2,5-DHB, 60 mM		—
A3		CHCA, 50 mM		—
A4		Dithranol, 40 mM		—
B1	24 μM	2,4-DHB, 60 mM	iPrOH–H ₂ O (9 : 1)	—
B2			MeOH–H ₂ O (9 : 1)	—
B3			EtOH–H ₂ O (9 : 1)	—
C	24 μM	2,4-DHB, 60 mM	iPrOH–H ₂ O (9 : 1)	—
D1	24 μM	2,4-DHB, 60 mM	iPrOH–H ₂ O (9 : 1)	—
D2				NaCl, 3 mM
D3				KCl, 3 mM
E	2 mM	2,4-DHB, 60 mM	iPrOH–H ₂ O (9 : 1)	—

^a Samples A–E were investigated in Sections 3.1–3.5, respectively.

Fig. 1 MALDI-TOF mass spectra of β -sitosterol using 2,4-DHB (A), 2,5-DHB (B), CHCA (C), and dithranol (D) as the matrices. The preparations for sample A are shown in Table 1. M or M' represents β -sitosterol or mono-oxidized one, respectively.

dominated by a peak at m/z 412.7 assigned to $[M' - H_2O]^+$ ions of mono-oxidized β -sitosterol. Similar to unsaturated lipids, β -sitosterol can undergo autoxidation by various oxygen species, including reactive oxygen species, hydrogen peroxide, and hydroxyl radical ($OH^\bullet$).³⁹ During MALDI irradiation, the free radical $OH^\bullet$ could be produced by the decomposition of DHB⁺⁺.⁴⁰ The highly reactive radical $OH^\bullet$ (diffusion-controlled, *i.e.*, $k \approx 10^9 \text{ M}^{-1} \text{ s}^{-1}$)⁴¹ frequently attacks lipid molecules by abstracting hydrogen, predominantly at C-7 for sterols, usually setting off free-radical chain reactions.⁴² Consequently, $OH^\bullet$ could react with molecules forming the fatty acid, alkoxy and peroxy radicals⁴³ and finally forming primary products (hydroperoxides), which decompose into secondary oxidation products with different chemical functional groups, such as hydroxy, epoxy, and keto groups.^{3,42,44} However, the ions that could be attributed to the hydroperoxides were not observed. MALDI MS can not detect the expected primary products; thus, previous studies usually focused on secondary products.⁴⁵ Smith⁴⁴ concluded that $5\alpha,6\alpha$ -epoxystanol with a molecular weight of 430.7 Da is a predominant secondary oxidation product formed by $OH^\bullet$ attack on sterols. The derived lipid $5\alpha,6\alpha$ -epoxystanol containing a hydroxyl group at C-3 loses water moiety leading to the formation of a detectable peak at m/z 412.7. In conclusion, under experimental conditions, β -sitosterol generated intense peaks consisting of the initial lipids and their oxidation

products. Similar peaks of mono-oxidized β -sitosterol were previously observed in heated olive oil mass spectra.⁴⁶

Although four matrices produced similar detectable peaks of β -sitosterol, they did not perform equally well. The most informative example was 2,4-DHB, which is consistent with previous reports.^{45,47} Excluding the shared peaks at m/z 396.6, 412.7, and 436.9 as discussed above, two extra peaks appeared at m/z 402.6 and 420.6, which were assigned to potassium adducts and are clearly demonstrated later. The use of 2,5-DHB or CHCA produced similar results. However, the intensity of peaks with 2,5-DHB was fivefold lower than that with CHCA. The β -sitosterol analyzed in dithranol had the lowest overall peak intensity with extremely high chemical noise, *i.e.*, peaks appeared at every m/z value in this range. However, dithranol revealed similar results with a few exceptions, especially at m/z 416.7, which was previously observed in the EI spectrum.⁴⁸

3.2 Solvent systems

Fig. 2 shows the MALDI spectra of sample B shown in Table 1. The analyte β -sitosterol behaved similarly in the various solvent systems and generated characteristic peaks at m/z 396.6, 412.7, and 436.9. However, the use of different solvents resulted in the detection of several markedly different peaks. General differences distinguishing $iPrOH-H_2O$ (9 : 1) from $MeOH-H_2O$ (9 : 1) or $EtOH-H_2O$ (9 : 1) were that the former solvent tended to form

Fig. 2 MALDI-TOF mass spectra of β -sitosterol with $iPrOH-H_2O$ (A), $MeOH-H_2O$ (B), and $EtOH-H_2O$ (C) as the matrix solvents, respectively. The preparations for sample B are shown in Table 1. M or M' represents β -sitosterol or mono-oxidized one, respectively.

peaks at m/z 402.6 and 420.6, whereas the latter two solvents showed propensity to form a peak at m/z 406.8, which will be discussed later. Solvents are crucial in facilitating the productive analyte–matrix interactions during crystallization.⁴⁹ Russell and Besson⁵⁰ suggested that the ideal aerosol MALDI solvent should have low viscosity, low surface tension, and high density. The surface tensions and specific gravities of the solvents used in the present study are arranged as MeOH > EtOH > iPrOH at 20 °C, whereas the viscosities are arranged as iPrOH > EtOH > MeOH at 25 °C.⁵¹ A combination of the experimental results and chemical properties of the investigated solvents suggests that iPrOH could provide the most informative mass spectra under experimental conditions.

3.3 Laser intensity

The laser intensity varied from 4 mJ cm^{-2} to 42 mJ cm^{-2} in the tests. Fig. 3 shows the MALDI-TOF spectra of sample C shown in Table 1 acquired at a laser intensity range of 6 mJ cm^{-2} to 13.5 mJ cm^{-2} . A value of 6 mJ cm^{-2} corresponded to a level just above the threshold to desorb the ionic products. In all cases, the mass spectra were dominated by a peak at m/z 396.6 that could be assigned to the molecular ions $[\text{M} - \text{H}_2\text{O}]^+$ of β -sitosterol. The signal-to-noise ratio increased with increasing laser power (Fig. 3B and C). This effect was attributed to the deposition of greater amounts of energy into the matrix at higher laser intensity, leading to enhanced vibrational

excitation, which might promote ablation of higher sample amounts.⁵² At the same time, the peak at m/z 412.7 appeared, which corresponded to molecular ions $[\text{M}' - \text{H}_2\text{O}]^+$ of the mono-oxidized β -sitosterol, 5 α ,6 α -epoxystanol. However, higher intensity decreased the probability of detecting the peak at m/z 431.9, which corresponded to molecular ions $[\text{M}' + \text{H}]^+$ of 5 α ,6 α -epoxystanol. The protonation of sterol molecules was rare. Protonated molecular ions $[\text{M}' + \text{H}]^+$ of their oxidation products were previously observed through MALDI.²⁴ The results of the present study suggest that high laser intensity increases the absolute peak intensity of the initial lipids and their oxidation products, and affects the ionization behavior of mono-oxidized β -sitosterol. Finally, the quality of mass spectra decreases because of extensive fragmentation and other side effects resulting from very high laser intensity (*e.g.*, 17 mJ cm^{-2}). The optimal laser intensity was 13.5 mJ cm^{-2} . A peak at m/z 418.1 (discussed later) was also observed.

3.4 Alkali ions

Natural or added salts in the preparation solutions significantly affected the MALDI spectra.⁵³ Fig. 4 shows the spectra of sample D shown in Table 1. With NaCl addition, the signal intensity of mass peaks at m/z 396.6, 412.7, and 436.9 obviously increased. Especially, the signal peak intensity at m/z 436.9 increased nearly sevenfold. Thus, the peak at m/z 436.9 was assigned to

Fig. 3 MALDI-TOF mass spectra of β -sitosterol with the laser intensity at 6 mJ cm^{-2} (A), 8.5 mJ cm^{-2} (B), and 13.5 mJ cm^{-2} (C). The preparations for sample C are shown in Table 1. M or M' represents β -sitosterol or mono-oxidized one, respectively.

Fig. 4 MALDI-TOF mass spectra of β -sitosterol with pure 2,4-DHB (A) and 2,4-DHB doped with NaCl (B) and KCl (C). The preparations for sample D are shown in Table 1. M or M' represents β -sitosterol or mono-oxidized one, respectively.

$[M + Na]^+$. In addition, the mass peaks at m/z 402.6 and 420.6 were greatly suppressed. The new peak at m/z 418.1 mentioned in Fig. 3 was observed with NaCl addition. Hence, it was identified as the sodium adduct. When the 2,4-DHB was mixed with KCl, the signal intensity of mass peaks at m/z 396.6, 402.6, and 420.6 decreased moderately, whereas the oxidation peak at m/z 412.7 was greatly suppressed. Hence, these detectable peaks at m/z 402.6 and 420.6 were identified as the potassium adducts. New mass peaks were observed at m/z 444.6 and 452.6. The strongest mass peak at m/z 444.6 shows a plus 38 Da shift to the mass peak at m/z 406.8, which was observed using 2,4-DHB dissolved in MeOH-H₂O (9 : 1) and EtOH-H₂O (9 : 1) (Fig. 2B and C). Therefore, the mass peak at m/z 406.8 was identified as protonated molecular ions. The mass peak at m/z 452.6 showed a plus 16 Da shift to the mass peak at m/z 436.9 (Fig. 4A and B). Therefore, the mass peak at m/z 452.6 could be assigned to molecular ions $[M + K]^+$ of β -sitosterol. Our laboratory-built MALDI-TOF MS currently has no MS/MS function. Hence, after referring to the LIPID MAPS database, we tentatively assigned these sample impurity-derived mass peaks at m/z 402.6, 406.8, 418.6, and 420.6 to steroid + K, sterol + H, ergosterol + Na, and cholesterol + K, respectively. We also tested lithium chloride but yielded no usable results.

3.5 Concentration influence

A stock solution of sample E was freshly prepared. From the stock solution, various final concentrations of β -sitosterol (2 mM to $2 \times 10^{-3}\text{ }\mu\text{M}$) were prepared and mixed with an equal volume of 2,4-DHB solution. All spectra were acquired at a laser intensity of 13.5 mJ cm^{-2} . The results are shown in Fig. 5. Starting at the highest initial β -sitosterol concentration, the signals for the aforementioned peaks at m/z 396.6 and 412.7 decreased relative to the signals obtained at medium concentration. Particularly, the analyte β -sitosterol yielded the peaks with highest intensity at the concentration of $12.1\text{ }\mu\text{M}$ (Fig. 5E). As shown on the low end of the β -sitosterol concentration scale in Fig. 5F–H, the peak intensity initially sharply decreased and then stabilized even when the analyte concentration decreased by tenfold. In addition to a decrease in peak intensity, the new peaks at m/z 397.6 and 413.7 (Fig. 5F–H) show a plus 1 Da shift to the mass peak at m/z 396.6 and 412.7 assigned to $[M - H_2O]^+$ in the high end of the β -sitosterol concentration scale (Fig. 5A–E). The peaks at m/z 397.6 and 413.7 correspond to protonated fragment-ion $[M + H - H_2O]^+$. Schiller³⁵ concluded that water loss is a well-known fragmentation pattern of hydroxylated compounds in MS. Changes in the ionization behavior of β -sitosterol under different concentrations might be related to MALDI ionization. With increasing matrix density, increased absorbance level may favor proton transfer reactions from the DHB matrix.²⁷ In conclusion, the extreme concentrations in Fig. 5 represented situations wherein worse balance existed between β -sitosterol and 2,4-DHB molecules. Thus, lower signals were observed. A higher number of β -sitosterol molecules decreased the M/A ratio; in such cases, the crystal formation of the matrix plus analyte was affected.³⁴ Conversely, a lower number of β -sitosterol molecules with which to react,

Fig. 5 MALDI-TOF mass spectra of β -sitosterol at different concentrations. The concentrations of the samples are labeled in the figure. M or M' represents β -sitosterol or mono-oxidized one, respectively.

results in weak signals. The results of this experiment suggest that the analyte concentration affects the absolute intensity of peaks and the ionization behavior of the analyte.

3.6 Quantification of mass spectra

Considerable progress has been achieved toward the direct quantification of MALDI spectra, which could be accomplished by comparing the peak of the lipid of interest with a known

Fig. 6 Calibration curve for β -sitosterol using a ratio of the peak height for the analyte and a matrix peak (m/z 273) without any internal standard.

matrix peak.⁵⁵ Compared with the use of a known internal standard, a special advantage of this method is that the sample is not altered by the addition of a known reference compound.⁵⁶ According to the suggestion for quantitative MALDI work,⁵⁷ we simply measured the signal intensity rather than the integrals (peak areas). The lipid peak at m/z 396.6, which corresponded to molecular ion $[M - H_2O]^+$ of β -sitosterol, and a known matrix peak at m/z 273, which was derived from the matrix and corresponded to $[2M + H - H_2O]^+$, were chosen to construct the curve. The laser desorption/ionization-TOF mass spectra of pure DHB included several characteristic matrix peaks: m/z 137 ($[M + H - H_2O]^+$), m/z 155 ($[M + H]^+$), m/z 273 ($[2M + H - 2H_2O]^+$), and m/z 409 ($[3M + H - 3H_2O]^+$).⁵⁸ In the experiment, the ions below m/z 200 were cut off by using an ion gate to avoid the saturation of microchannel plates and the mass peak at m/z 409 was not detected. Hence the matrix peak at m/z 273 was selected as the reference peak. The value of peak height was the average of six mass spectra. The calibration curve for the β -sitosterol is shown in Fig. 6. It was constructed by least-squares regression concentration *versus* the peak height of m/z 396.6 divided by the peak at m/z 273 (Table S1†). The calibration curves obtained for β -sitosterol show a good linear range of three orders of magnitude with a regression coefficient square (r^2) of 0.992. The linear ranges for the β -sitosterol were between 12.1 and 1207.5 μ M. The detection limit or the lowest concentration level that yielded a signal-to-noise ratio of 3 was 10 nM. Compared with previous quantitative MALDI work,²⁴ the upper limit of linear range of the curve for the β -sitosterol was higher by twofold than that for the oxysterol. From the point of analytical features, the intra-day and inter-day variations for β -sitosterol were 20.6% and 23.7%, respectively, which are comparative with previously reported values by MALDI-TOF MS.⁵⁹ Although the analytical conditions (matrix compounds, solvent systems, laser intensities, and analyte concentrations) have been optimized to obtain stable signals, the intra-day and inter-day variations of quantification for β -sitosterol were still large ($\sim$ 20%). We speculated that the variations resulted mainly from complication of the MALDI process which is closely related to the laser intensity and the co-crystallization of matrix and analyte. In this experiment, the deposited layer of the matrix and analyte was not uniform. Any tiny shift of the laser beam could cause an apparent change of ion signals, which might be the main problem for the intra-day and inter-day variations of quantification in this experiment.

4. Conclusions

In this study, β -sitosterol was analyzed with a laboratory-built MALDI-TOF MS. Five experimental factors were optimized to acquire reproducible and informative mass spectra of the analyte β -sitosterol. Under the optimized experimental conditions β -sitosterol generated intense peaks consisting of the initial lipids and their oxidation products. The results demonstrate that MALDI could generate characteristic peaks of β -sitosterol which were previously observed in mass spectra generated by other MS techniques. Additionally, the secondary oxidation product of β -sitosterol was derived from autooxidation irritated

by hydroxyl radicals generated by laser irradiation of the matrix during MALDI. Besides the qualitative analyses, a quantitative analysis of β -sitosterol was performed using a matrix peak. The experimental results show that the matrices, solvent systems, and NaCl ions have no significant influence on characteristic peaks except for signal intensity. However, laser intensities and analyte concentrations affect the ionization behavior of the analyte. These experimental results also show that the MALDI-TOF MS can offer identification and quantification information of phytosterol molecules. The reported method may provide a potential tool for rapid and reliable analysis in nutrition, food science, medicine, and environmental science.

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