# Aziridination-assisted mass spectrometry of nonpolar lipids with isomeric resolution

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**ABSTRACT:** Characterization of nonpolar lipids is of significance, as they serve a variety of key biological functions and can naturally exist in isomeric forms. Electrospray ionization mass spectrometry (ESI-MS) is a powerful tool for most lipid analysis, but nonpolar lipids do not easily ionize in electrospray, complicating their analyses. In this work, we use the Du Bois catalyst ($Rh_2(esp)_2$) for aziridination of carbon-carbon double bonds (C=C bond) of six nonpolar sterol lipids, simultaneously increasing ionization efficiency of nonpolar lipids and facilitating C=C bond identification. The incorporation of nitrogen expands the lipid categories detected by MS, and higher-energy C-trap dissociation of the aziridines generates diagnostic ions that can be used to locate C=C bond positions.

## INTRODUCTION

Lipids play many important biological roles in cellular barriers, membrane matrices, energy storage, and signaling due to their great diversity of structures and physiochemical properties. In recent years, nonpolar lipids such as cholesterol and its derivatives have gained increased recognition for their importance in disease detection, progression, and treatment.[1,2,3] Because of its necessity in membrane biogenesis, it has become a hallmark for many types of cancers due to increased cholesterol biosynthesis and uptake. Further, recent studies have demonstrated that tumor growth can be inhibited through the manipulation of cholesterol metabolism.[1] These discoveries have led to an increased need for the study of cholesterol metabolism and thus an increased need for dynamic, specific, and accurate methods for the study of cholesterol and its derivatives.

Mass spectrometry (MS) has emerged as a powerful tool for lipidomic studies that measure special and temporal alterations in the composition of lipids due to its high specificity and sensitivity. Recent efforts in MS-based lipid analysis—including ion activation methods[4], chemical derivatization,[5,6] and ion mobility spectrometry[7]—focus on identification of lipid isomers with the same molecular formulas but different structures and functions. Polar lipids, such as glycerophospholipids, often dominate the mass spectrum, as they can be pre-charged or are more effectively ionized in electrospray. However, nonpolar lipids, which account for over a third of the total lipids in human epidermal cells,[8] cannot be ionized well, thus their species and isomeric forms are more challenging to be detected by MS. Current methods for improving ionization efficiencies of nonpolar lipids include radical cation formation by ultraviolet photodissociation (UVPD) which requires specific instrumentation.[4,6] Another approach is derivatization through reactions with specific functional groups such as carboxyl[6] or hydroxy[5] groups. While these derivatizations are effective, they are limited in their application, as many important nonpolar lipids do not contain these groups. Additionally, they do not inform the isomeric structures of these lipids, creating a demand for a method that is versatile in its application and capable of simultaneously increasing ionization efficiency of nonpolar lipids while providing isomeric structural differentiation. The functionalization of carbon-carbon double (C=C) bonds via aziridination offers both benefits. C=C bonds are found in unsaturated lipids, and tandem MS (MS/MS) analysis can fragment the aziridine to produce diagnostic ions that inform the C=C bond location.

Recent development in aziridination of isolated C=C bonds by Cheng and coworkers used Du Bois catalyst ($Rh_2(esp)_2$) to functionalize unactivated olefins via nitrogen transfer.[9] Previous work by our group used this reaction to develop an aziridine-based isobaric tag labeling strategy on fatty acids, glycerophospho lipids, cholesteryl esters, and glycerides, using aziridination of C=C bonds to enable reactions with isobaric mass tags.[10] This finding inspired the investigation of the method presented here, in which the functionalization of C=C bonds in nonpolar lipids is used to improve their ionization efficiencies and inform their isomeric structures. The method was investigated with cholesterol and other sterols as model lipids, given recent developments in their importance in disease progression and diagnostics. We successfully increased the ionization efficiencies of the studied nonpolar lipid sterols and determined C=C bond position for double bonds on the 17-position chain.

## EXPERIMENTAL

### *Materials*

Water (HPLC), methanol (MeOH) (HPLC), stigmasterol, ergosterol, and 7-dehydrocholesterol were purchased from Sigma-Aldrich (MO, USA). Hydroxylamine-O-sulfonic acid (HOSA) was purchased from Combi-Blocks (CA, USA). Hexafluoro-2-propanol (HFIP), cholesterol, and β-sitosterol were purchased from Fisher Scientific (NH, USA). Pyridine was purchased from Millipore Sigma (MA, USA). Bis[rhodium(α, α, α', α'-tetramethyl-1,3-benzenedipropionic acid)] was AmBeed (IL, USA). All solvents and chemicals were used without further purification.

### *Aziridination of nonpolar lipids*

Lipid aziridination was performed as previously reported by our group.[10] Briefly, lipid standards were dissolved in HFIP to achieve a final concentration of 10 mM. HOSA (1.5 equiv C=C bond), pyridine (3 equiv) and $Rh_2(esp)_2$ (5 mol %) were mixed in, and the reaction was stirred at room temperature for 3 hours.

### *MS analysis*

Samples were diluted twenty-fold with 2:1 MeOH:$H_2O$ prior to analysis. Lipid analysis was performed on a Q-Exactive Plus hybrid quadrupole-orbitrap mass spectrometer from Thermo Fisher Scientific (San Jose, CA). NanoESI emitters were made from borosilicate glass tubing with filament on a P-100 micropipette puller, both from Sutter Instrument Company (Novato, CA) using the following parameters: heat 545, pull 20, velocity 25, time 250, and pressure 500. Samples were analyzed in positive ion mode. The following MS parameters were set for data acquisition: spray voltage 1.3 kV, capillary temperature 275 °C, S-lens RF level 60.0, *m/z* range 50-500. Higher-energy C-trap dissociation (HCD) was used to obtain tandem mass spectra at normalized collision energy (NCE) of 23-55.

## RESULTS AND DISCUSSION

### *Characterization of cholesterol*

Cholesterol was used as a model compound for the experiment, as all other examined sterols share the same sterol backbone. When unmodified cholesterol was analyzed (Figure 1a), only background peaks from the solvent system were shown in the spectrum. Analysis of aziridinated cholesterol yielded the anticipated protonated peak at *m/z* 402.37 in high abundance (Figure 1b). MS/MS analysis of the aziridinated product via HCD with an NCE of 53 produced a "fingerprint" series of ions that were found to be diagnostic of the sterol backbone (Figure 1c). The charge introduced by the aziridine helped the dissociation in MS/MS, allowing the backbone to be visualized in detail in the series of ions corresponding to varying degrees of fragmentation off of the charged ring.

### *Analysis of sterols*

Following the successful analysis of cholesterol, the method was applied to stigmasterol, ergosterol, 7-dehydrocholesterol, and β-sitosterol. ESI-MS analysis of the aziridinated products all yielded ions of the protonated products in high abundance. In MS/MS analysis, the fingerprint visualized with cholesterol was consistently found in all of the aziridinated sterols studied. It was consistent in relative ion intensities and only varying in mass in correspondence to the number of C=C bonds present on the backbone rings. For each aziridinated C=C bond, the peaks for C=C bond-containing regions shifted to reflect a mass loss of 2, which reflected the presence of the second C=C bond.

The β-sitosterol protonated aziridine product was abundant in full MS (Figure 2b), however the spectrum also showed stigmasterol (222.20 *m/z*) and campesterol (416.39 *m/z*) derivatives as well, which was a result of impure standard. MS/MS analysis of the β-sitosterol peak at 430.40 *m/z* yielded a sterol backbone identical to that of cholesterol (Figure 2c). The 17-position ring of β-sitosterol exhibited radical-directed dissociation, exhibiting both the individual and simultaneous losses of ethyl and isopropyl groups from carbon 24. The chain fragmentation pattern was different from that of cholesterol,

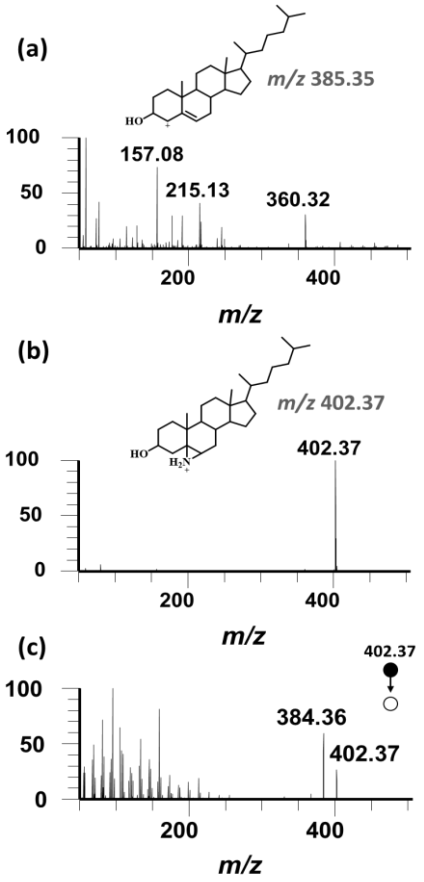


**Figure 1.** Mass spectra of cholesterol (a) before and (b) after aziridination. Structures of the anticipated analyte are shown above the spectra and their expected *m/z* values are in red. (c) MS/MS analysis of aziridinated cholesterol at *m/z* 402.37.

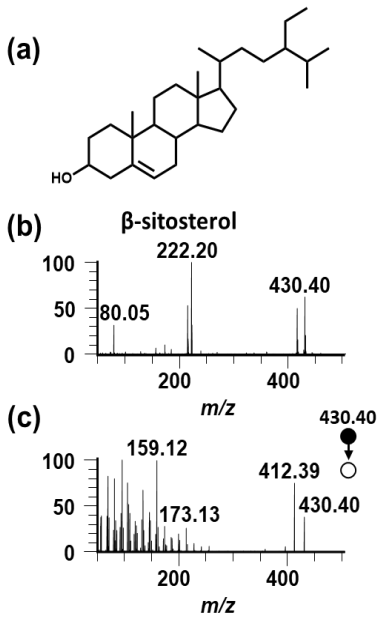


**Figure 2.** (a) Structure of β-sitosterol with (b) full MS of aziridinated sample and (c) MS2 at 430.40 *m/z*, its most abundant aziridine product ion.

indicating that the dissociation enabled by the presence of the aziridine can help to inform structure of sterols.

For 7-dehydrocholesterol (Figure 3a), both the mono- (400.36 *m/z*) and the diaziridine (208.19 and 415.37 *m/z*) products were visualized in full MS (Figure 3b), however the mono-aziridine product was far more abundant. This, when compared to the results for stigmasterol and ergosterol, suggests that the aziridination of conjugated C=C bonds on the B-ring is disfavored. MS/M analysis of the most abundant ion, the monoaziridine, yielded the diagnostic sterol backbone pattern, although peaks for ions containing the B ring were shifted to reflect a mass loss of 2, the result of the second C=C bond.

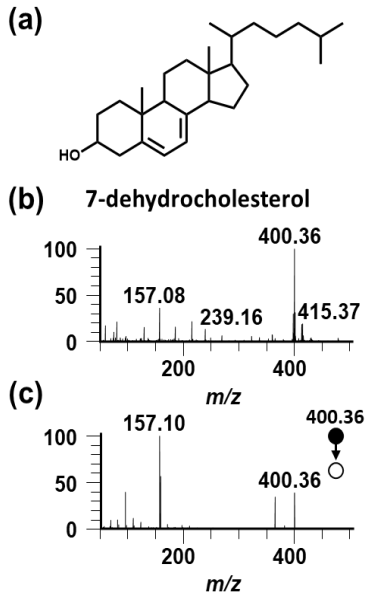


**Figure 3.** (a) Structure of 7-dehydrocholesterol with (b) full mass spectrum of aziridinated sample and (c) MS/MS at 400.36 *m/z*, its most abundant aziridine product ion.

Stigmasterol (Figure 4a) overwhelmingly formed its diaziridine product, as indicated by the 2+ diprotonated peak at *m/z* 222.20 on the full mass spectrum (Figure 4b). The monoaziridine product was visualized as well but in significantly lower abundance. The abundance of the diaziridine product relative to that of 7-dehydrocholesterol suggests that complete aziridination of C=C bonds is favored when they are not located close to each other. MS/MS analysis of the most abundant product peak reflected the same loss patterns shown in the other sterols, with the neutral loss of $H_2O$ favored over that of the $NH_3$ from the aziridine and a diagnostic sterol backbone pattern.

The aziridination and ionization behavior of ergosterol (Figure 5a) followed that of stigmasterol more closely than 7-dehydrocholesterol. The 2+ diaziridine product (*m/z* 214.19) was overwhelmingly the most abundant ion in the full mass spectrum (Figure 5b). The 1+ diaziridine and 1+ monoaziridine products were still seen, but in significantly low abundance. The triaziridine product could be seen in its 2+ form as well, but no 3+ triaziridine peak was seen. MS/MS analysis of the most abundant ion, the diprotonated diaziridine product at *m/z* 214.19, demonstrated identical behavior to that of stigmasterol in HCD (Figure 5c).

The behaviors of the examined sterols followed general patterns. For lipids with multiple C=C bonds—stigmasterol and 7-dehydrocholesterol with two C=C bonds and ergosterol with three—ions corresponding to each degree of reaction completion were visualized. However, the mono-aziridination product was overwhelmingly favored for 7-dehydroxycholesterol, while the di-aziridination products were favored for stigmasterol and ergosterol, suggesting that the aziridination of two bonds that are conjugated to each other is disfavored.

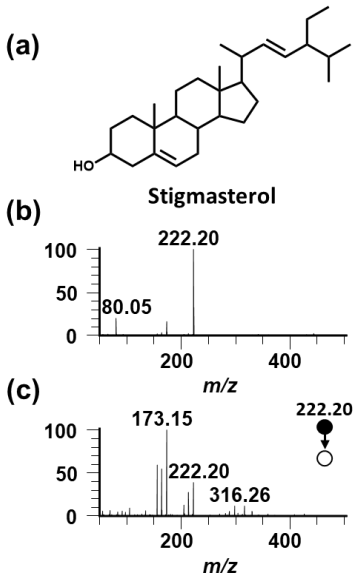


**Figure 4.** (a) Structure of stigmasterol with (b) full mass spectrum of aziridinated sample and (c) MS/MS at *m/z* 222.20, its most abundant aziridine product ion.

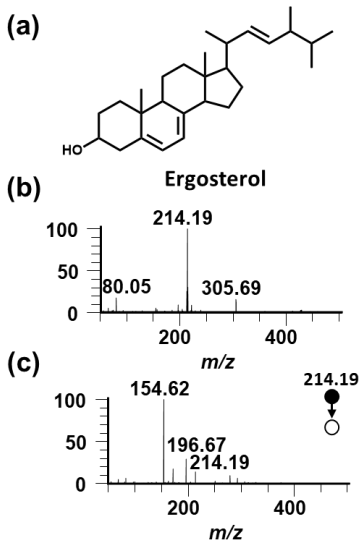


**Figure 5.** (a) Structure of ergosterol with (b) full mass spectrum of aziridinated sample and (c) MS/MS at *m/z* 214.19, its most abundant aziridine product ion.

### *C=C bond location determination*

Stigmasterol and ergosterol, as the two analyzed lipids with a C=C bond on the 17-position chain, demonstrated the proposed

method's ability to inform isomer structure. MS/MS analysis of the aziridination products for stigmasterol and ergosterol produced a set of fragment ions corresponding to the formation of an imine (*m/z* of diagnostic fragment here) along either side of the original C=C bond on the chain. Figure 6 shows the tandem spectrum for the di-protonated stigmasterol diaziridine product. The resulting fragment ions at *m/z* 173.15 and *m/z* 114.13 visualize either side of the original double bond, revealing the C=C bond location.

The products produced by HCD of ergosterol were similar, and the abundance of the diagnostic ions for the fragmentation of ergosterol's mono- and di-aziridine products indicated that the aziridination of the C=C bond on the 17-position chain is favored over aziridination of the C=C bonds on the B ring.

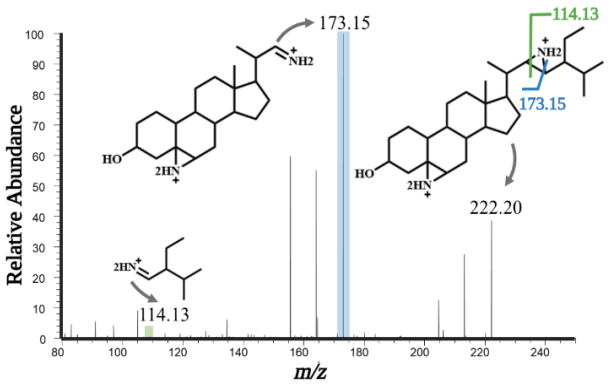


**Figure 6.** Tandem mass spectrum of deprotonated, 2+ diaziridine stigmasterol product at *m/z* 222.20. Highlighted are imine peaks at *m/z* 173.15 (blue) and 114.13 (green) that indicate the double bond position.

### *Relative quantification*

Relative quantification of C=C bond isomers was conducted using a calibration curve of aziridinated cholesterol (Figure 7). The concentration of the stigmasterol aziridine product was maintained at 50 nM, while the concentrations of cholesterol varied from 10 nM, 100 nM, 1 µM, 10 µM, and 100 µM. The calibration curve was constructed by plotting the ion intensity (I) ratio of cholesterol ions to stigmasterol ions against the concentration of cholesterol product. A linear relationship was observed with a good $R^2$ value of 0.999.

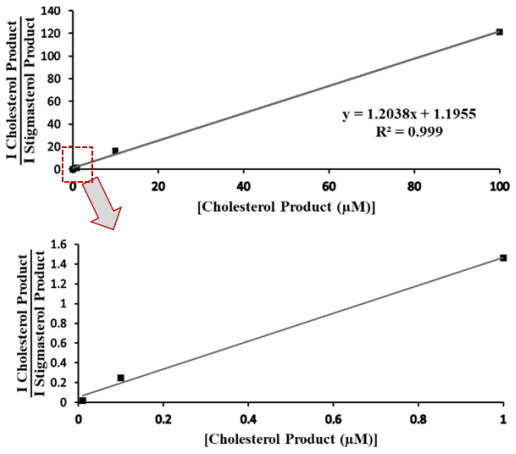


**Figure 4.** Relative quantification of cholesterol and stigmasterol aziridinated products

## CONCLUSIONS

We have developed a method for simultaneously increasing ionization efficiency and informing C=C bond position in non-polar lipids. The method uses $Rh_2(esp)_2$ to catalyze the aziridination of C=C bonds, followed by HCD to generate diagnostic/characteristic fragment ions for the determination of C=C bond positions. The method has great potential due to its ability to simultaneously increase ionization efficiency and inform isomer structure.

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## AUTHOR CONTRIBUTIONS

E.H. and X.Y. designed the experiment. X.Y. supervised the research work. The manuscript was written with the contributions of E.H., M.E., D.F., and X.Y. All authors have given approval to the final version of the manuscript.

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## REFERENCES

1. Huang, B.; Song, B.; Xu, C. Cholesterol metabolism in cancer: mechanisms and therapeutic opportunities. *Nature Metabolism* **2020**, *2*, 132-141.
2. Wang, Y.; Yutuc, E.; Griffiths, W. Cholessterol metabolism pathways - are the intermediates more important than the products? *The FEBS Journal* **2021**, *288* (12), 3727-3745.
3. Mok, E. H. K.; Lee, T. K. W. The Pivotal Role of the Dysregulation of Cholesterol Homeostasis in Cancer: Implications for Therapeutic Targets. *Cancers* **2020**, *12* (6).
4. West, H.; Reed, G. E. Hybrid 213 nm photodissociation of cationized Sterol lipid ions yield [M]þ. Radical products for improved structural characterization using multistage tandem mass spectrometry. *Analytica Chimica Acta* **2021**, *1141*, 100-109.
5. Xia, F.; Wan, J. B. Chemical derivatization strategy for mass spectrometry-based lipidomics. *Mass Spectrometry Reviews* **2021**.
6. Brodbelt, J. S. Photodissociation mass spectrometry: new tools for characterization of biological molecules. *Chem. Soc. Rev.* **2014**, *43* (8), 2757-2783. DOI: 10.1039/c3cs60444f.

7. Tu, J.; Zhou, Z.; Li, T.; Zhu, Z. The emerging role of ion mobility-mass spectrometry in lipidomics to facilitate lipid separation and identification. *Trends in Analytical Chemistry* **2019**, *116*, 332-339.
8. Gray, G. M.; Yardley, H. J. Lipid compositions of cells isolated from pig, human, and rat epidermis. *Journal of Lipid Research* **1975**, *16* (6), 434-440.
9. Cheng, Q.; Zhou, Z.; Jiang, H.; Siitonen, J. H.; Ess, D. H.; Zhang, X.; Kurti, L. Organocatalytic nitrogen transfer to unactivated olefins via transient oxaziridines. *Nature Catalysis* **2020**, *3*, 386-392.
10. Yang, T; Tang, S.; Kuo, S.; Freitas, D.; Edwards, M.; Wang, H.; Sun, Y.; Yan, X. Lipid Mass Tags via Aziridination for Probing Unsaturated Lipid Isomers and Accurate Relative Quantification. *Angewandte Chemie* **2022**.