Analytical separations for lipids in complex, non-polar lipidomes using differential mobility spectrometry

Sarah E. Hancock¹,²†, Berwyck L. J. Poad³, Mark D. P. Willcox⁴, Stephen J. Blanksby³* and Todd W. Mitchell¹,²*

¹School of Medicine and Molecular Horizons, University of Wollongong, Wollongong NSW, AUSTRALIA
²Illawarra Health and Medical Research Institute, Wollongong NSW, AUSTRALIA
³Central Analytical Research Facility, Institute for Future Environments, Queensland University of Technology, Brisbane QLD AUSTRALIA
⁴School of Optometry and Vision Science, University of New South Wales, Sydney, NSW, AUSTRALIA

*Please address correspondence to:
toddm@uow.edu.au
stephen.blanksby@qut.edu.au

†Present address: School of Medical Sciences, University of New South Wales, Sydney AUSTRALIA
1. Abstract

Secretions from meibomian glands located within the eyelid (commonly known as meibum) are rich in non-polar lipid classes incorporating very-long (22-30 carbons) and ultra-long (>30 carbon) acyl chains. The complex nature of the meibum lipidome and its preponderance of neutral, non-polar lipid classes presents an analytical challenge, with typically poor chromatographic resolution, even between different lipid classes. To address this challenge, we have deployed differential mobility spectrometry-mass spectrometry (DMS-MS) to interrogate the human meibum lipidome and demonstrate near-baseline resolution of the two major non-polar classes contained therein, namely wax esters and cholesteryl esters. Within these two lipid classes, we describe ion mobility behaviour that is associated with the length of their acyl chains and location of unsaturation. This capability was exploited to profile the molecular speciation within each class and thus extend meibum lipidome coverage. Intriguingly, structure-mobility trends in these non-polar lipids show similar trends and inflections to those previously reported for other physicochemical properties of lipids (e.g., melting point and phase-transition temperatures). Taken together, these data demonstrate that differential ion mobility provides a powerful orthogonal separation technology for the analysis of neutral lipids in complex matrices, such as meibum, and may further provide a means to predict physicochemical properties of lipids that could assist in infering their biological function(s).
2. Introduction

Meibomian glands, located within the eyelids, produce a waxy, lipid-rich substance known as meibum. This meibum is thought to supply the outermost tear film lipid layer, which helps prevent the evaporation of the aqueous layer beneath it. The composition of the tear film lipid layer influences its stability (1), and changes in the ratio of lipid classes present are thought to be involved in the development of dry eye syndrome (2–4). The meibum lipidome is quite complex, and is unusual in that it contains mostly neutral, non-polar lipid classes that contain very-long (22-30 carbons) to ultra-long (>30 carbons) acyl chains. The two most abundant of these, the wax esters (WE) and cholesteryl esters (CE), make up around 75 mol% of total meibum lipid (5–7). Other lipids present within meibum include triacylglycerols (TG), diacylated α,ω-diols (8, 9), the recently characterised (O-acyl)-ω-hydroxy fatty acids (OAHFA) (10, 11) and a small amount of amphiphilic lipids including phospholipids (6, 12).

Two mass spectrometry (MS) strategies have previously been used to identify and quantify meibomian lipids, namely, direct infusion with tandem MS (also known as shotgun lipidomics) and MS coupled to liquid chromatography (LC-MS). Both approaches employ soft ionisation techniques, such as atmospheric pressure chemical ionisation or electrospray ionisation (ESI). Shotgun lipidomics has been successful in identifying and quantifying a range of lipid species within meibum (6, 12–14), but the presence of isobaric species (i.e., lipids with the same nominal mass but differing chemical composition) can complicate spectra and impede the discovery of new compounds (e.g., cholesteryl esters of the form CE x:2 are isobaric with wax esters WE [x+26]:0). LC-MS based workflows have also been successful in mapping much of the meibum lipidome (2, 15–20) including uncovering the presence of OAHFA in meibum (10). Nevertheless, LC is unable to completely resolve the neutral meibum lipid classes. Meibum separation by normal phase LC typically displays a single broad peak under which all of the non-polar lipid classes elute (10, 16, 19, 21). In contrast, reversed-phase LC separates meibum lipids by their acyl chain length and degree of unsaturation (18, 21, 22). Under these conditions, significant overlap remains between the two major meibum lipid classes, CE and WE (5).

Ion mobility spectrometry (IMS) coupled to MS has recently been applied to the separation of a range of isomeric and isobaric compounds in the gas phase (23). This technology is rapidly being incorporated within existing shotgun and LC-MS lipidomics workflows to enable resolution of lipid classes (24–33), isobars (27, 31, 34–36), and isomers
(25, 28, 30, 36–42) in both standard mixtures and biological extracts. Conventional ion mobility such as drift time ion mobility spectrometry (DTIMS) measures the migration time of ions through an inert buffer gas under low electric field conditions, with the mobility of a given ion being inversely related to its collisional cross-section (CCS), a function of the mass-to-charge ratio ($m/z$) of the ion, its shape and size and its interactions with drift gas (23). Travelling-wave ion mobility spectrometry (TWIMS) is a variation on DTIMS where ions are subjected to an electric field that is applied to one section and set to sequentially sweep through the length of the ion mobility cell in the direction of ion migration. In both approaches, libraries of drift time and CCS are generated from standards to aid in identification of isomeric or isobaric lipids. Separation of isomeric and/or isobaric lipids by DTIMS or TWIMS is limited by the resolving power (i.e., a function of drift tube distance and pressure), with even high-resolution DTIMS being unable to separate several isomeric phospholipids (30, 33, 34). More recently, an ultra-high resolution TWIMS platform known as Structures for Lossless Ion Manipulations (SLIM) achieved baseline separation of phosphatidylcholine double bond positional isomers and near-baseline separation of stereoisomers, a feat that was enabled by two passes through the serpentine ion path of the SLIM platform, equivalent to a drift tube distance of 30.6 m (41).

In contrast to ion mobility measured with DTIMS or TWIMS, differential mobility spectrometry (DMS, also known as field asymmetric ion mobility), separates ions in a given population by exploiting their differential mobility under low- and high-field conditions when exposed to an alternating asymmetric waveform known as a separation voltage (SV). Under the influence of this SV ions will oscillate and drift towards one of the electrodes unless their trajectory is corrected by a superimposed DC compensation voltage (CV). By scanning through CV one can derive an "ionogram" based on the mass spectrometric detection of the ion populations being transmitted through the DMS cell for a given SV. The separations observed in DMS are often found to have lower $m/z$-dependence and thus greater structural sensitivity than either DTIMS or TWIMS. DMS has been used as a filter to separate lipid classes and/or isomers/isobars in both synthetic mixes and biological extracts prior to structural determination by tandem MS (26, 31, 36), ozone-induced dissociation (38, 40, 42) or electron impact excitation of ions from organics (29, 43). These combination technologies have been applied for separation of lipids based upon within-class structural characteristics such as chain length and unsaturation (28, 36), $sn$-position (36, 38, 39), stereochemistry (36, 37), and double bond position (36, 40, 42).
Herein, we show the ability of DMS coupled to MS (DMS-MS) to separate the two main lipid classes present in meibum, WE and CE, with near-baseline resolution, a feat that is not achievable using common chromatographic strategies. The DMS-MS workflow applied here reveals several characteristics driving this separation by DMS-MS, including the influence of carbon chain length and double bond position on mobility behaviour. By combining DMS with OzID, 123 unique WE and CE species were characterised within meibum, including four double bond isomers within each of seven CE sum compositions. In addition, the relationship between ion mobility behaviour and carbon chain length in both WE and CE and double bond position in WE follows a similar trend to that observed for their phase transition temperature (T_M). Therefore, DMS may not only provide an orthogonal approach to the separation of non-polar lipids that can be readily incorporated into shotgun or LC-MS lipidomic workflows, but may also be a way of predicting the physiochemical properties of newly discovered lipid molecular species within complex lipidomes.
3. Experimental methods

Materials

LC/MS grade methanol and HPLC grade chloroform were obtained from VWR International (Brisbane, QLD, Australia). Ammonium acetate and lithium acetate of the highest purity available was purchased from Sigma-Aldrich (Sydney, NSW, Australia). All experiments were conducted in compliance with the tenets of the Declaration of Helsinki (2013) and was approved by the University of New South Wales Human Research Ethics Advisory Panel (HC15609). The meibum sample was a pooled sample from 170 human donors collected and extracted by approved protocols as described previously (6). Briefly, meibum samples were collected from patients by meibomian gland evaluator, with no topical anaesthesia used. The meibomian gland evaluator was placed below the eyelash line of the lower eyelid and was held in this position for 10 s. A stainless steel ethanol-cleaned and heat-sterilized spatula was used to collect the expressed meibum by gently sliding across the orifices. Meibum collected on the spatula was dissolved in a glass vial containing ~1 mL of chloroform. This sample was then transferred to a sleeved glass vial, dried under N₂, and resuspended in 200 µL chloroform. Samples were pooled prior to analysis.

Nomenclature

Lipid nomenclature used throughout is based on general literature recommendations (44, 45). WE are denoted as X₁:Y₁/X₂:Y₂ where X₁:Y₁ define the number of carbons and double bonds present on fatty acid and X₂:Y₂ the fatty alcohol. CE are described as X:Y, where X is the total number of carbons and Y is the number of double bonds in the fatty acyl chain. Double bond position is described by either “n-ₓ” or “Δᵧ” nomenclature, where x and y are the number of carbon-carbon bonds from the methyl end of the fatty acid (i.e., omega position) or carboxylate moiety, respectively.

Differential mobility spectrometry-mass spectrometry

DMS-MS was conducted using a QTRAP 5500® equipped with a SelexION™ DMS interface and TurboV™ ESI source controlled by Analyst™ 1.5.2 (SCIEX, Concord, Canada). ESI voltage was set at +5.5 kV, with the source equilibrated to room temperature. Nitrogen was used for nebulizing (15 psi), curtain (20 psi), and collision (9 arb units) gas. The pooled meibum extract was diluted 100-fold in methanol spiked with 7.5 mM ammonium acetate, and directly infused into the mass spectrometer at a rate of 5 µL/min. In a typical DMS experiment
separation voltage (SV, +4500 V), resolving gas (25 psi, nitrogen) and DMS cell temperature (225 °C) were held at an optimised value while compensation voltage (CV) was scanned and MS data acquired. No gas-phase modifiers were used. DMS-MS data was acquired from m/z 50 – 1200 with a declustering and exit potential of +100 V and +10 V, respectively. A wide CV range (-20 to +30 V) and large step size (0.5 V) were used initially to determine the optimal transmission range for meibum lipids ionized as [M+NH₄]⁺ cations after which the CV range and step size were narrowed (+6 to +22, step size 0.1 V). Precursor ion scans for cholesteryl and wax esters were obtained using settings reported previously (6). DMS-multiple reaction monitoring experiments (DMS-MRM) used optimised Q1/Q3 masses for previously reported meibum CE and WE species (6, 13, 17), and the transitions used for these experiments are detailed in the supplementary material (Tables S1-S4).

**Ozone-induced dissociation**

The QTRAP 5500 used for DMS-MS experiments was modified for ozone-induced dissociation (OzID) as illustrated in Figure 1, in a manner similar to that previously described (38, 46). An ozone generator (Titan 30, Absolute Ozone, Alberta, Canada) was used to produce ozone (~14.7% w/w O₂) from oxygen supplied at a constant flow rate (0.2 L/min), pressure (20 psi) and power setting 30%, which was then delivered to the collision cell of the mass spectrometer via a variable leak valve (Nenion, Lustenau, Austria) through the collision gas line. For OzID experiments, meibum extract was diluted 50-fold in methanol spiked with 2 mM lithium acetate and infused into the mass spectrometer at a rate of 5 µL/min. DMS settings were as described above, while experimental details for DMS-MRM of [M+Li]⁺ monounsaturated CE are described in the supplementary material (Table S5). For DMS-OzID a modified time-delayed fragmentation scan was used to mass-select monounsaturated CE [M+Li]⁺ ions in Q1, which were then transferred into q2 with minimal collision energy (e.g., 5 eV). Target ions were trapped in q2 with ozone and allowed to react for 1 s before being ejected into Q3. OzID fragment and residual precursor ions were then scanned out for mass analysis at 1,000 Th/s. The observed neutral losses in the resulting OzID mass spectra were used to assign the site(s) of unsaturation in the corresponding lipids using the previously predicted transitions listed in Table 1 (47). DMS-OzID of lithiated CE species were acquired using SV, temperature and pressure settings described above while ramping CV from +6 to +12 V (step size = 0.01 V, n = 4)
Data analysis

DMS-MS, MRM and OzID data were processed using PeakView® software (SCIEX, Concord, Canada) and the statistical package R (47). DMS ionograms were processed in R using the smoother package (49). Gaussian filtering was applied with a window size determined by a combination of the normalised standard deviation and number of data points across the full-width at half maximum (i.e., CV step size), and were visualised using the ggplot2 package (50). Data presented were averaged across 4-5 measurements from the same sample on differing days, and are presented as mean plus or minus the relative standard deviation (RSD). Piecewise regression between carbon chain length and CV was conducted in R using the segmented package with statistical significance set at \( p < 0.05 \) (51, 52).
4. Results

Mobility separation of cholesteryl and wax esters

Lipid class separation by DMS has so far been exploited in only a few studies of biological extracts which are dominated by amphiphilic lipids (24, 26, 43). Given the challenge of separating the neutral lipid-dominated lipidome of meibum by LC, we sought to determine if DMS could achieve greater separation of the major two meibum lipid classes, CE and WE. To that end, we subjected a methanolic solution of meibum spiked with ammonium acetate to ESI to generate abundant [M+NH$_4$]$^+$ ions from WE and CE species. In initial tests, DMS-MS were acquired while ramping CV from +6 to +22 V (step size 0.1 V). The resulting plot of CV against total ion current detected by the mass spectrometer (hereafter referred to as an ionogram) is shown in Figure 2A. This ionogram displayed a bimodal distribution, suggesting the separation of two distinct ion populations within the gas phase by DMS. We probed the identity of each ion population by holding CV at a given voltage and acquiring the resulting mass spectrum (Figure 2B&D). The mass spectrum obtained at CV +11.2 V (Figure 2B) displayed a number of ions with mass-to-charge ratios ($m/z$) that closely matched the pattern of [M+NH$_4$]$^+$ ions obtained from a precursor ion scan targeting CE ($m/z$ 369, Figure 2C). Likewise, the masses observed at CV +16.1 V (Figure 2D) were similar to those of [M+NH$_4$]$^+$ WE obtained by a precursor ion scan for a protonated 18:1 fatty acid ($m/z$ 283, Figure 2E). Taken together, these findings indicate that DMS-MS can separate the CE and WE lipid populations present within meibum as gas phase ions.

To confirm the separation of CE and WE classes in meibum by DMS-MS, we ran a series of DMS-MRM experiments targeting known meibum CE and WE species as [M+NH$_4$]$^+$ ions, using transitions described by previous studies (Table S1-S3) (6, 13, 17). Examination of the total ion chromatogram for each class of lipid revealed near-baseline resolution of CV between the two lipid classes (Figure 3). CE migration through the DMS cell occurred between +7.5 to +15.3 CV, with maximum transmission of CE occurring at +11.6 V (0.5% RSD). WE migrated through the DMS cell between +12.5 and +20.1 CV, with maximum transmission at +16.7 V (±0.6% RSD). The low RSDs reported for CV maxima for both CE and WE suggest a high degree of repeatability between DMS-MS experiments ($n = 4$). This wide range of CV for the transmission of CE and WE species suggests some DMS separation of lipids within each class.
Effect of mass and degree of unsaturation on ion mobility separation

To determine what might be driving the separation of lipid species within the two lipid classes, WE and CE DMS-MRM transitions were examined independently. More than 130 discrete WE ester species were surveyed by DMS-MRM (Tables S1-S3), and WE species present within meibum at an abundance greater than 0.5% of total WE are shown in Figure 4. Meibum WE generally had maximum transmission through the DMS cell at CVs that were inversely proportional to their carbon chain length as might be expected (26, 28, 36), with each carbon increase resulting in ~0.3 V decrease in CV (Figure 4). Unsataturated WE with a double bond in either the fatty acid or fatty alcohol domain were typically transmitted through the DMS cell at a lower CV compared with their saturated counterparts. For example, saturated WE 16:0/24:0 had a maximum CV of +17.7±0.1 V, while monounsaturated WEs 16:1/24:0 and 16:1/24:1 had maximal CVs of +17.2±0.5 V and +15.7±0.4 V respectively. Interestingly, for unsaturated WEs the location of the double bond within the fatty acid or fatty alcohol also influenced its mobility. Unsaturated WEs characteristically displayed lower maximum CVs than their saturated equivalents, with a double bond in the fatty alcohol portion of the WE decreasing maximum CV by ~1.3 V on average compared to a reduction of only 0.6 V when the double bond was located in the fatty acid (Figure 3).

For CE, close to 60 discrete [M+NH₄]⁺ species present in meibum were surveyed by DMS-MRM (Table S4), with the ionograms for CE present at >0.5% abundance of total CE are shown in Figure 5. The ion mobility behaviour seen with CE in many ways paralleled that observed for WE (vide supra), where transmission by DMS was strongly associated with the mass of the molecule and its degree of unsaturation. Like WE, the CV for maximum transmission of a given saturated CE species was inversely related to its mass (Figure 5A). Saturated CE migrated through the DMS cell at CVs ranging from +8 V to +16 V, with a noticeable plateau in this relationship for very-long chain CE (i.e., 22 to 30 carbons). For long-chain saturated CE 16 to 21 carbons in length, maximum CV decreased on average by 0.5 V for each single carbon increase in chain length. In contrast, for the very-long chain saturated CE the relationship between CV and chain length attenuates to less than 0.1 V per carbon-number increase. Piecewise regression confirmed this breakpoint seen in the relationship between saturated CE mass and maximum CV after 21 carbons (R² = 0.992).

Monounsaturated CE (i.e., CE with one double bond in their acyl chain) were transmitted through the DMS cell at a slightly lower CV range than their saturated counterparts
(+7.5 to +13.7 V, Figure 5B). However, an unusual U-shaped relationship between carbon chain length and mean maximum CV was observed for monounsaturated CE, with piecewise regression identifying two breakpoints: the first between monounsaturated CE 22 and 24 carbons in length, and second for CE with acyl chains 28 carbons and longer ($R^2 = 0.990$). For long-chain CE with monounsaturated fatty acyl chains between 18 and 22 carbons in length, the average maximum CV decreased by 0.9 V for each two-carbon addition. This trend reversed entirely for monounsaturated CE with 24 to 28 carbons in their acyl chain, with mean maximal CV increasing by 0.7 V for every two-carbon elongation. For very-long to ultra-long chain monounsaturated CE with fatty acids longer than 28 carbons there was little observable change in maximal CV with each two-carbon increase, with CE 28:1, 30:1, and 32:1 averaging a similar maximum CV as CE 18:1. As monounsaturated CE are known to exist as a number of double bond positional isomers in meibum (7), it follows that the site of unsaturation in the fatty acid of the CE could be driving the discontinuous relationship seen between mass and maximum CV for monounsaturated CE.

**Influence of double bond position on CE separation by DMS**

To test our hypothesis that the position of the double bond on the acyl chain of monounsaturated CE was affecting its ion mobility, we characterised monounsaturated CE double bond isomers by OzID. During OzID, mass-selected CE ions were trapped within the collision cell of the mass spectrometer in the presence of ozone, which resulted in characteristic pairs of product ions indicative of double bond position (i.e., Criegee and aldehyde ions spaced by 16 Da, Table 1). The [M+NH$_4$]$^+$ ions formed from monounsaturated CE produced few neutral loss fragments indicative of double bond position, even after up to 5 s trapping with ozone (Figure S1A). However, cationization of monounsaturated CE with lithium resulted in abundant diagnostic Criegee and aldehyde ions within 1 s of trapping time with ozone (Figure S1B). Accordingly, DMS-MRM transitions of [M+Li]$^+$ monounsaturated CE were acquired ($n = 5$, Table S5) to confirm similar ion mobility behaviour, with the resulting ionogram displaying a analogous U-shaped relationship between mass and CV to that observed for [M+NH$_4$]$^+$ monounsaturated CE acquired under the same DMS-MS conditions (Figure S2). Some small differences in maximal CV transmission for specific CE species were seen between the two ion adducts, including a lower CV range for [M+Li]$^+$ adducts (+6.7 to +11.9 V) and a higher maximum CV for CE 30:1 versus CE 18:1 (cf. Figure 5B).
Subsequently, DMS-OzID spectra were acquired on mass-selected monounsaturated CE [M+Li]$^+$ ions while ramping CV from +6 to +12 V ($n = 4$). Monitoring the extracted ion chromatograms (XICs) for diagnostic ions indicative of double bond position revealed the presence of four different double bond isomers within each monounsaturated CE, including $n$-5, $n$-7, $n$-9 and $n$-11 (see Figure S3 for example using CE 20:1). These XICs for each CE double bond isomer were then used to produce an ionogram that compares maximal CV between monounsaturated CE species with the same double bond position (Figure S4). Surprisingly, monounsaturated CE with the same $n$ double bond position had distinct mean maximal CV, suggesting that double position from the methyl end was not a major factor driving the separation of monounsaturated CE by DMS (Figure S4). Double bond position in lipids can also be referenced from the carboxylate moiety of the acyl chain (i.e., $\Delta$). When $\Delta$ double bond position was plotted against the mean maximal CV of isomeric monounsaturated CE species (Figure 6), lipids with a common position of the double bond with respect to the carboxylate moiety were found to group together suggesting this to be a better predictor of CE differential ion mobility behaviour.
5. Discussion

Herein we have shown the application of DMS-MS to the separation of lipids present within meibum (i.e., secretions from human meibomian glands), a substance that is composed of a principally nonpolar lipidome. Using this approach, we detected 123 unique meibum WE and CE species in total (Table 2). Analysis of lipidomes that contain predominately nonpolar lipid species typically present an analytical challenge, as the lipid species contained therein are inadequately separated by commonly used LC-MS methods. While DMS-MS separation achieved near baseline resolution of the two major lipid classes present in meibum, WE and CE, individual lipids within these two classes were also dispersed based on carbon chain length and the position of carbon-carbon double bonds.

The relationship between the size of a lipid (as a function of the number of carbons in its acyl chain) and its time of arrival during low field ion mobility is well documented (25, 27, 32, 33, 55–58). In conventional IMS such as DTIMS and TWIMS, larger or more extended molecules have a greater CCS, and therefore interact more with the drift gas resulting in longer arrival time distributions. When DMS is conducted without any gas-phase modifiers, lipids typically exhibit type-C differential ion mobility behaviour where the CV at which gas-phase ions successfully traverse the DMS cell rises with increasing SV (42, 59). Type-C behaviour is dominated by hard-sphere interactions, with minimal ion clustering during the low-field portion of the waveform. Under these conditions, physical characteristics of the molecule such as CCS largely drive the mobility of the ion (59, 60). Consequently, a general trend between ion mobility behaviour and mass has been reported for DMS of lipids with no gas-phase modifiers, where larger lipids typically have lower CVs (28, 36). Thus far, linear relationships between CV and mass for several lipid classes have been reported in the literature with the exception of TGs, which show a slight attenuation of the relationship between m/z and CV with increasing size (28). In the present study, we observed an inverse but primarily linear relationship between WE mass and CV (Figure 4), whereas both saturated and unsaturated CE had a discontinuous relationship between size and CV (Figure 5). For saturated CE, we observed a distinct plateau in the relationship between CV and size for CE with very-long acyl chains (Figure 5A). This change in differential ion mobility behaviour cannot be solely attributed to the mass of the lipid, given that meibum CE are, on average, up to about two-thirds the mass of the largest TG. However, the fatty acyls found in meibum CE are substantially longer than those usually found in other animal tissues, which typically have a maximum of 24 carbons in any given fatty acid. Interestingly, the relationship between
saturated CE carbon chain length and CV bears a resemblance to the relationship between acyl chain length and melting point for long chain and ultra-long chain free fatty acids (61), and show reasonable agreeance when the two values are correlated (Figure S5). This suggests that the same physiochemical properties that determine the temperature at which saturated lipids transition from the solid to liquid phase may also affect their ion mobility behaviour under an asymmetric waveform. Of note, increased rotamer disorder within the lipid hydrocarbons as the acyl chain lengths would lead to a larger cross-sectional area, thus influencing its ion mobility. We hypothesize that at a certain hydrocarbon length the difference in CCS between the ordered and disordered state would become comparatively smaller, resulting in the attenuated differential ion mobility behaviour seen for very-long chain CE (Figure 5A).

Unsaturated lipids also display a reduction in ion mobility transit time during both DTIMS (25, 35, 55, 56) and TWIMS, (27, 57), which is a product of the smaller CCS imparted by the double bond. Similar behaviour has also been reported for a number of other lipid classes during DMS, in which unsaturated lipids have lower CVs when compared with their saturated counterparts, including phosphatidylcholines (but not lyso-phosphatidylcholines), phosphatidylethanolamines, triacylglycerols and diacylglycerols (26, 28). In the present study, we observed that the location of the carbon-carbon double bond within a WE had a significant impact on differential ion mobility, with a double bond being present in the fatty alcohol of the WE resulting in a greater reduction in CV than a double bond in the fatty acid (Figure 4). This behaviour might be partially explained by the extreme asymmetrical structure of meibum WE, where the carbon chain length of fatty alcohol can be anywhere from 2 to 16 carbons longer than the fatty acid. Indeed, variability in ion mobility behaviour for asymmetric versus symmetric phospholipids with saturated acyl chains has been well-documented with DMS, with greater differences in CV seen when the discrepancy between the two acyl chains is larger (28). No differences in arrival time have been observed for symmetric versus asymmetric saturated phospholipids during conventional DTIMS (57). Greater differences in CV can be seen between isomers when the longer acyl chain contains a double bond, allowing the resolution of sn-positional isomers as [M+Ag]⁺ ions by DMS (38) and high resolution DTIMS (34). This long carbon chain might impart greater flexibility to the molecule, thus affecting the CCS of the ion and its ability to interact with any background gases as the ion moves through the high and low field conditions of the DMS cell. A similar relationship has also been described between transition temperature ($T_M$) and double bond position in wax esters, where a wax ester with a double bond present in the fatty alcohol has a lower $T_M$ than a wax ester
with a double bond in the fatty acid (66). This lends further support to our hypothesis that the same physicochemical properties that influence lipid physical properties such as \( T_M \) also direct their mobility as type-C ions under an asymmetric waveform.

The most striking ion mobility behaviour, was observed for the DMS-MS of monounsaturated CE species. Monounsaturated CE displayed a U-shaped relationship between their carbon chain length and the CV at which they migrated through the DMS cell (Figure 5B). Moreover, this U-shaped relationship was best correlated with the position of the double bond from the carboxylate moiety (Figure 6). Atomistic modelling has shown that liquid-phase cholesteryl oleate exists on a spectrum of folded to unfolded ensembles, with the greatest flexibility being present between carbons 3 and 5 on the acyl chain (67). In previous work, we determined that an interaction exists between the site of charge localisation on the carbonyl and both the endocyclic and acyl carbon-carbon double bonds in cholesteryl oleate, which potentially aids folding of the structure in the gas-phase resulting in a smaller CCS and enhanced ion mobility (68). Therefore, it stands to reason that the distance between the sterol head group and double bond may also affect the folding of the steryl ester in the gas-phase, with elongated distances between the sterol and double bond resulting in compact structures when the double bond is located >15 carbons from the carboxylate moiety. To our knowledge no measurement of melting point or \( T_M \) for lipids with ultra-long monounsaturated fatty acids like those typically found in meibum have been reported in the literature, preventing more definitive conclusions from being drawn on the relationship between differential ion mobility behaviour and this property of ultra-long chain monounsaturated CE.
6. Conclusions

In the present study we have described the ability of DMS to achieve near-baseline separation of the two dominant neutral lipid classes present in human meibum, WE and CE. This strategy is shown to be effective in removing isobaric overlaps in direct infusion analysis of non-polar lipidomes. Combined with collision and ozone-induced dissociation DMS-MS/MS revealed 123 individual molecular contributors to WE and CE classes including four double bond isomers within seven monounsaturated CE. DMS separation of discrete molecular species within these two neutral classes revealed the influence of carbon chain length and double bond position on differential ion mobility behaviour, where an inverse relationship between lipid acyl chain length and CV were observed. We also observed a distinct effect of unsaturation position on ion mobility behaviour, where the inclusion of a double bond in either the fatty acid or alcohol in WE influenced the CV at which it migrated through the DMS cell. Unsaturated CE showed the most striking DMS behaviour, with the position of the double bond relative to the carboxylate moiety best correlating with ion mobility. Many of the observed behaviours under the asymmetric ion mobility field bore similar relationships between lipid physical properties and their T$_{M}$, indicating that the same physicochemical properties influence the differential ion mobility behaviour. Taken together, this suggests that DMS-MS may capable of predicting the physicochemical properties of discrete lipid molecular species in complex lipidomes.
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9. Tables

Table 1: Neutral mass losses (in Da) from ionized monounsaturated lipids upon ozone-induced dissociation (OzID) based on predictions of Brown et al. (47). Double bond (DB) position is indicated using the n-x nomenclature indicating the bond position with respect to the omega (or methyl) end of the chain.

| DB position (n-x) | Neutral loss (Da) |
|------------------|-------------------|
|                  | n-hydrocarbon loss | Aldehyde (+O) | Criegee (+2O) |
| 1                | CH₂               | -2            | -18           |
| 2                | C₂H₄              | 12            | -4            |
| 3                | C₃H₆              | 26            | 10            |
| 4                | C₄H₈              | 40            | 24            |
| 5                | C₅H₁₀             | 54            | 38            |
| 6                | C₆H₁₂             | 68            | 52            |
| 7                | C₇H₁₄             | 82            | 66            |
| 8                | C₈H₁₆             | 96            | 80            |
| 9                | C₉H₁₈             | 110           | 94            |
| 10               | C₁₀H₂₀            | 124           | 108           |
| 11               | C₁₁H₂₂            | 138           | 122           |
| 12               | C₁₂H₂₄            | 152           | 136           |
| 13               | C₁₃H₂₆            | 166           | 150           |
| 14               | C₁₄H₂₈            | 180           | 164           |
| 15               | C₁₅H₃₀            | 194           | 178           |
Table 2: Summary of all wax and cholesteryl ester species detected in meibum extracts by DMS-MS

| Wax esters       | Cholesteryl esters | Wax esters       | Cholesteryl esters |
|------------------|--------------------|------------------|--------------------|
| WE 16:0/20:0     | WE 17:0/29:0       | CE 16:0          | CE 30:1(n-5)       |
| WE 16:0/21:0     | WE 17:1/24:0       | CE 17:0          | CE 30:1(n-7)       |
| WE 16:0/22:0     | WE 17:1/26:0       | CE 18:1(n-5)     | CE 30:1(n-9)       |
| WE 16:0/23:0     | WE 17:1/26:1       | CE 18:1(n-7)     | CE 30:1(n-11)      |
| WE 16:0/24:0     | WE 17:1/27:0       | CE 18:1(n-9)     | CE 32:1(n-5)       |
| WE 16:0/25:0     | WE 17:1/30:1       | CE 18:1(n-11)    | CE 32:1(n-7)       |
| WE 16:0/26:0     | WE 17:2/24:0       | CE 18:0          | CE 32:1(n-9)       |
| WE 16:0/27:0     | WE 18:1/20:0       | CE 19:0          | CE 32:1(n-11)      |
| WE 16:0/29:0     | WE 18:1/21:0       | CE 20:2          |                    |
| WE 16:1/20:0     | WE 18:1/22:0       | CE 20:1(n-5)     |                    |
| WE 16:1/21:0     | WE 18:1/23:0       | CE 20:1(n-7)     |                    |
| WE 16:1/22:0     | WE 18:1/24:0       | CE 20:1(n-9)     |                    |
| WE 16:1/23:0     | WE 18:1/24:1       | CE 20:1(n-11)    |                    |
| WE 16:1/24:0     | WE 18:1/25:0       | CE 20:0          |                    |
| WE 16:1/24:1     | WE 18:1/26:0       | CE 21:0          |                    |
| WE 16:1/25:0     | WE 18:1/26:0       | CE 22:1(n-5)     |                    |
| WE 16:1/26:0     | WE 18:1/27:0       | CE 22:1(n-7)     |                    |
| WE 16:1/26:1     | WE 18:1/28:0       | CE 22:1(n-9)     |                    |
| WE 16:1/27:0     | WE 18:1/28:1       | CE 22:1(n-11)    |                    |
| WE 16:1/28:0     | WE 18:1/29:0       | CE 22:0          |                    |
| WE 16:1/28:1     | WE 18:1/30:0       | CE 23:0          |                    |
| WE 16:1/29:0     | WE 18:1/30:1       | CE 24:2          |                    |
| WE 16:1/30:0     | WE 18:2/16:0       | CE 24:1(n-5)     |                    |
| WE 16:1/30:1     | WE 18:2/18:0       | CE 24:1(n-7)     |                    |
| WE 16:1/32:1     | WE 18:2/19:0       | CE 24:1(n-9)     |                    |
| WE 16:2/18:0     | WE 18:2/20:0       | CE 24:1(n-11)    |                    |
| WE 16:2/20:0     | WE 18:2/21:0       | CE 24:0          |                    |
| WE 16:2/22:0     | WE 18:2/22:0       | CE 25:0          |                    |
| WE 16:2/26:0     | WE 18:2/24:0       | CE 26:1(n-5)     |                    |
| WE 16:2/27:0     | WE 18:2/25:0       | CE 26:1(n-7)     |                    |
| WE 16:2/28:0     | WE 18:2/26:0       | CE 26:1(n-9)     |                    |
| WE 17:0/20:0     | WE 18:2/28:0       | CE 26:1(n-11)    |                    |
| WE 17:0/21:0     | WE 18:2/29:0       | CE 26:0          |                    |
| WE 17:0/22:0     | WE 18:2/30:0       | CE 27:0          |                    |
| WE 17:0/23:0     | WE 18:2/31:0       | CE 28:1(n-5)     |                    |
| WE 17:0/24:0     |                 | CE 28:1(n-7)     |                    |
| WE 17:0/25:0     |                 | CE 28:1(n-9)     |                    |
| WE 17:0/26:0     |                 | CE 28:1(n-11)    |                    |
| WE 17:0/27:0     |                 | CE 28:0          |                    |
| WE 17:0/28:0     |                 | CE 29:0          |                    |
10. Figures and figure legends

Figure 1: A diagram of the QTRAP5500 mass spectrometer illustrating the location and configuration of the DMS device used in these experiments and highlighting the hardware modifications made to the instrument to facilitate OzID.
Figure 2: DMS separation of human meibomian gland secretions results the bimodal ionogram (A). When CV is held at +11.2 V, the resulting mass spectrum (B) closely resembles a precursor ion scan for CE (m/z 369, C). Similarly, holding CV at +16:1 V produces a mass spectrum (D) that resembles a precursor ion scan for WE species containing an 18:1 fatty acid (m/z 283, E).
Figure 3: DMS-MRM of known CE and WE species present within meibum shows near-baseline separation. DMS-MRM were acquired using a separation voltage of +4500 V, a resolving gas pressure of 25 psi, and DMS cell temperature of 225 °C. CV was scanned between +6 and +22 V with a step size of 0.1 V. Settings optimised for DMS-MRM of both WE and CE are available in the supplementary material (Tables S1-S4).
Figure 4: Ionograms of WE detected by DMS-MRM present at an abundance of >0.5% of total WE in meibum. WE are shown grouped by fatty acyl chain (boxes) and fatty alcohols (x axis). WE species are presented as mean maximal CV transmission (closed circles) ±RSD (n = 4). The shaded areas indicate the CV range and relative intensity at which the WE species migrated through the DMS cell.
Figure 5: Ionograms of (A) saturated, (B) monounsaturated, and (C) polyunsaturated meibum CE species (CE 20:2 and CE 24:2) present in meibum at an abundance of >0.5% of total CE. CE species are shown as mean maximal CV transmission (closed circles) ±RSD (n = 4). The shaded areas indicate the CV range and relative intensity at which the CE species migrated through the DMS cell. Solid lines show fitted line(s) from piecewise regression.
Figure 6: Ionograms extracted from DMS-OzID spectra of monounsaturated CE meibum species grouped by their Δ double bond position (i.e., number of carbons present between the double bond and the carboxylate moiety). CE species are shown as mean maximal CV transmission (closed circles) ±RSD (n = 4). The shaded areas indicate the CV range and relative intensity at which the CE species migrated through the DMS cell.