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Sequential collision- and ozone-induced dissociation enables assignment of relative acyl chain position in triacylglycerols

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Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Marshall, D. L., Pham, H. T., Bhujel, M., Chin, J. S. R., Yew, J. Y., Mori, K., Mitchell, T. W. & Blanksby, S. J. (2016). Sequential collision- and ozone-induced dissociation enables assignment of relative acyl chain position in triacylglycerols. Analytical Chemistry, 88 (5), 2685-2692.

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Sequential collision- and ozone-induced dissociation enables assignment of relative acyl chain position in triacylglycerols

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ABSTRACT: Unambiguous identification of isomeric lipids by mass spectrometry represents a significant analytical challenge in contemporary lipidomics. Herein, the combination of collision-induced dissociation (CID) with ozone-induced dissociation (OzID) on an ion-trap mass spectrometer is applied to the identification of triacylglycerol (TG) isomers that vary only by the substitution pattern of fatty acyl (FA) chains esterified to the glycerol backbone. Isolated product ions attributed to loss of a single FA arising from CID of $[TG + Na]^+$ ions react rapidly with ozone within the ion trap. The resulting CID/OzID spectra exhibit abundant ions that unequivocally reveal the relative position of FAs along the backbone. Isomeric TGs containing two or three different FA substituents are readily differentiated by diagnostic ions present in their CID/OzID spectra. Compatibility of this method with chromatographic separations enables the characterisation of unusual TGs containing multiple short-chain FAs present in *Drosophila*.

INTRODUCTION

Lipid structure plays an important role in biological function; for example, defining the interactions between lipids and their binding proteins.[1,2] Complete structural characterisation of lipids in complex biological extracts is an on-going challenge in contemporary lipidomics. Mass spectrometry at least partially answers this challenge and has therefore become the method of choice for modern lipidomic analysis. The sum composition (total number of carbons and double bonds) of fatty acids (FAs) comprising a triacylglycerol (TG) may be readily obtained from an accurate mass measurement of a $[TG + M]^+$ (M = Li, Na, K, NH_4) cation formed *via* electrospray ionisation (ESI). Further, the composition of the individual FAs carried by the TG may be identified by collision-induced dissociation (CID) of the same $[TG + M]^+$ cation; the dissociation mechanisms of which are increasingly well understood.[3,4]

Although powerful, liquid chromatography coupled to mass spectrometry (LC-MS) is unable to routinely and unequivocally resolve structural isomerism commonly encountered in TGs. Unambiguous identification of lipid isomers that differ in chirality about the glycerol backbone; double bond position or stereoisomerism; and/or the substitution pattern of FAs on the glycerol backbone pose a significant challenge to conventional tandem mass spectrometry. Here we focus on elucidating the relative positions of acyl chain substitution on the glycerol backbone; a structural feature which influences FA bioavailability.[5] For a TG with two different fatty acyls A and B, there are two possible substitutional isomers, namely AAB and ABA. When three different fatty acyls are bound to the glycerol backbone, three substitutional permutations arise: ABC, ACB, and BAC. Recent review of the literature finds only limited reports of successful chromatographic separation of TG positional isomers.[6] When employing either silver ion high-performance liquid chromatography (HPLC)[7] or reversed-phase HPLC,[8] complete separation may require multiple columns and/or prohibitively long retention times, particularly when the FAs are similar in number of carbons and degree of unsaturation.[9] Separation of these TG isomers may instead be achieved by employing differential mobility spectrometry (DMS).[10] Resolution of enantiomeric TGs (*i.e.*, AAB and BAA) by chiral HPLC has also been recently demonstrated.[11] Even if separated, however, assignment of molecular structure remains challenging and relies on subtle differences between tandem mass spectra. For example, in the CID mass spectra of $[TG + M]^+$ ions the relative abundance of product ions associated with FA loss is dependent on the position of each acyl chain on the glycerol backbone.[12-15] Loss of the *sn*-2 FA is disfavoured relative to loss of the outer chains and thus the resulting product ions are less abundant than ions arising from loss of the terminal substituents.[16] Product ion abundances can be used to assign the regiochemistry of a single TG, however, in isomeric mixtures that are common in biological extracts, careful calibration of ion abundances with isomeric

composition is required.[12,15] Further complications arise where the FA identity influences the product ion abundance.[17-19]

Rather than relying on ion abundance ratios in CID spectra, an explicit marker ion for assigning positional isomerism in TGs is desirable. This approach was first demonstrated with high energy (> 1 keV) CID of $[TG + M]^+$ ions on a multi-sector instrument and has since been replicated on tandem time-of-flight platforms.[20,21] High energy CID diagnostic marker ions identify the relative positions of each FA on the backbone. Hsu and Turk have similarly demonstrated that MS^3 of first generation $[TG + M - R_nCOOH]^+$ product ions give rise to characteristic spectra for TG positional isomers.[22,23] Central to this chemistry is the proposed mechanism of unimolecular dissociation whereby loss of the initial acyl chain is affected by nucleophilic substitution driven by the adjacent ester. This process results in a five-membered 1,3-dioxolane ring with the FA adjacent to that lost now linked to the glycerol backbone by a newly formed carbon-carbon double bond. If this mechanism is correct, it presents the opportunity to use gas phase ozonolysis to selectively cleave this bond and thus establish the sequence of neighbouring FAs on the glycerol backbone.

Combinations of CID and ozone-induced dissociation (OzID) have previously been used to distinguish isomeric phospholipids that vary only by the *sn*-positional isomerism of the two FAs esterified to the glycerol backbone.[24] In these experiments CID drives loss of the phosphate headgroup involving substitution by the ester moiety of the adjacent *sn*-2 FA substituent. In a subsequent OzID step, CID product ions are isolated in the presence of ozone resulting in selective cleavage of the *sn*-2 FA. Advantageously, the target carbon-carbon double bond is activated toward ozonolysis, improving compatibility with a chromatographic timescale.[25] Given the success with which this method is able to resolve *sn*-positional isomerism in phospholipids, CID/OzID is here applied to uncovering the substitution pattern of FAs in TGs. The compatibility of this technology with HPLC is demonstrated by structure elucidation of unusual TGs extracted from *Drosophila*.

EXPERIMENTAL SECTION

Materials. Methanol, chloroform (HPLC grade) and sodium acetate (analytical grade) were purchased from Thermo Fisher Scientific (Waltham, MA). Compressed oxygen (high purity, 99.99%) and helium (ultra-high purity, 99.999%) were obtained from Coregas (Sydney, Australia). TG standards with three long-chain FAs were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. TG standards with at least one short chain FA were synthesised as previously described.[26] Extracts from *Drosophila* were also obtained as previously described.[27]

Chromatography and mass spectrometry. An HPLC system (Dionex UltiMate 3000 RSLC) comprising a quaternary pump, split-loop autosampler, and column oven was used for all experiments. Samples were dissolved in MeOH (with up to 50% $CHCl_3$ if needed) at *ca.* 0.1 μM. A 5-10 μL aliquot of synthetic lipid solutions or lipid extract was injected onto a Waters Acquity C_{18} CSH column (100 mm x 2.1 mm x 1.7 μm) at 10 °C. The mobile phase consisted of a gradient beginning with 85% MeOH in water, and (after a 5 minute isocratic period) increasing to 100% MeOH over 55 minutes, at a flow rate of 0.1 mL min^{-1}. The post-column eluent was combined with a sodium acetate solution (0.1 mM in methanol) *via* a syringe pump and T-junction prior to infusion into the mass spectrometer to facilitate the exclusive formation of $[TG + Na]^+$ ions. Alternately, synthetic TG standards were dissolved in 2:1 MeOH:$CHCl_3$ at 1 μM with 0.1 mM sodium acetate, and infused directly into the mass spectrometer at 5 μL min^{-1}.

Mass spectra were recorded on a linear ion trap mass spectrometer equipped with a heated ESI source (LTQ XL, Thermo Fisher Scientific). The instrument has been modified to seed ozone into the helium buffer gas.[28,29] Briefly, the instrument helium splitter has been bypassed to directly connect the supply to the ion trap, with the flow rate controlled by a metering flow valve until the instrument ion gauge pressure reads *ca.* 0.8 x 10^{-5} Torr. Ozone (*ca.* 17% by weight in O_2) was produced online by an ozone generator (HC-30, Ozone Solutions, Hull, IA) and passed through 6.3 mm Teflon tubing connected to a catalytic destruct (IN USA, Norwood, MA) and exhausted from the laboratory. Prior to destruct, the tubing is connected to a 25 μm (i.d.) x 100 mm PEEKsil restrictor *via* a stainless steel union tee. The PEEKsil restrictor is connected to the helium supply line downstream from the flow valve through a shut-off ball valve and union tee (see Figure S-1, Supporting Information). For safety, an ambient O_3 monitor (Aeroqual, Auckland, New Zealand) was deployed in the laboratory.

A data-dependent method was written in the instrument control software (Xcalibur 3.0) to acquire ESI-MS^n spectra across the chromatographic run. When the abundance of $[TG + Na]^+$ ions breached a defined threshold, a CID spectrum was acquired with a normalised collision energy of 25 (arbitrary units). MS^3 (CID/OzID) spectra were acquired for each nominated CID product ion, with a reaction time of 0.25 s and a normalised collision energy of 0.

Nomenclature. Lipid nomenclature used here is guided by literature recommendations.[30-32] If the identity of the individual FAs comprising the TG are known, they are separated by an underscore, *e.g.,* TG(16:0_18:0_18:1). A slash is used to denote the *sn*-position of the FAs, *e.g.,* TG(16:0/18:0/18:1) indicates the order of the FAs on the glycerol backbone is known. Here we propose a new nomenclature for an intermediate level of structural detail. The chirality of the glycerol backbone was not determined in these experiments and thus the *sn*-1 and *sn*-3 positions are considered equivalent, *i.e.*, TG(16:0/18:0/18:1) is not differentiated from TG(18:1/18:0/16:0). This lipid is described herein as TG(18:1_/18:0/_16:0). The slashes specify that the *sn*-2 FA is defined as 18:0, and the underscores indicate that the *sn*-1 and *sn*-3 FAs are interchangeable. Synthetic lipid standards have fully defined regiochemistry and thus the TG(A/B/C) nomenclature remains appropriate.

RESULTS AND DISCUSSION

AAB-type triacylglcerols. For TGs with two different fatty acyl substituents on the glycerol backbone (*i.e.*, AAB-type) CID of $[TG + Na]^+$ ions results in two pairs of product ions. Example spectra recorded on an ion-trap mass spectrometer are provided in Figure 1(a) and 1(b), corresponding to the synthetic lipids TG(16:0/16:0/18:1) and TG(16:0/18:1/16:0), respectively. Each CID spectrum features the same product ions at *m/z* 599 and 573 corresponding to loss of the fatty acids with retention of sodium (*i.e.*, $[TG + Na - R_nCOOH]^+$); and *m/z* 577 and 551 corresponding to loss of the fatty acyl sub-

stituents as sodium salts ($[TG + Na - R_nCOONa]^+$). The only difference observed between the two CID spectra is the variation in product ion abundances. For example, the lower abundance of ions at *m/z* 551 and 573 corresponding to neutral loss of the 18:1 FA chain in Figure 1(b) is consistent with the known location of the substituent at the *sn*-2 position.

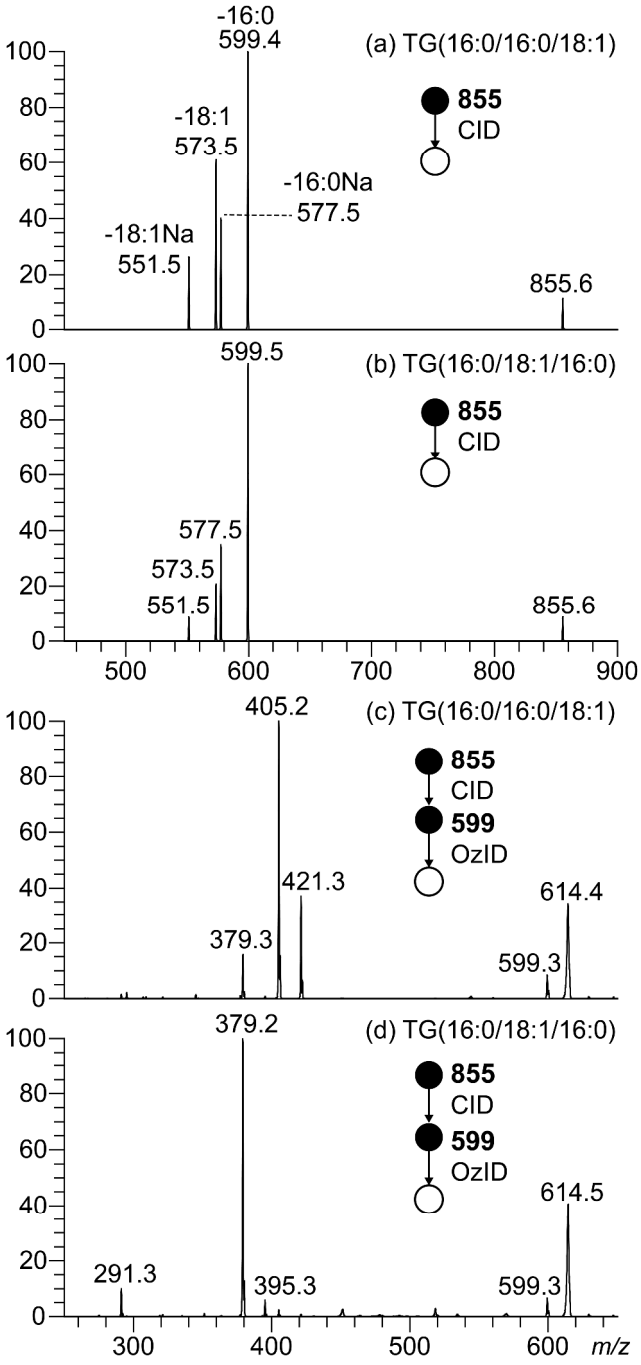
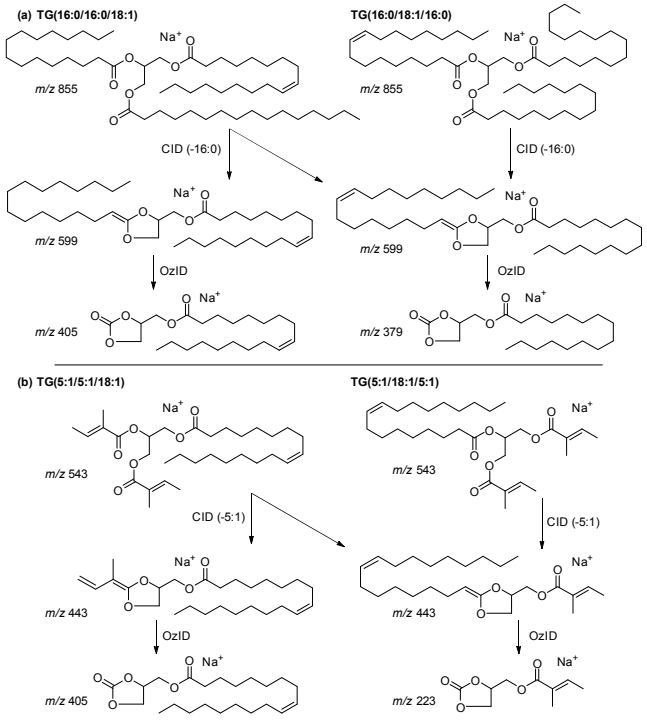


Figure 1. CID spectra of $[TG + Na]^+$ ions acquired by infusion of synthetic lipids into a modified ion-trap mass spectrometer: (a) TG(16:0/16:0/18:1); (b) TG(16:0/18:1/16:0). CID/OzID mass spectra (O_3 reaction time: 1 s) of $[TG + Na - 18:1]^+$ product ions from: (c) TG(16:0/16:0/18:1); (d) TG(16:0/18:1/16:0).

Nonetheless, the spectral differences are subtle and the use of such data for the unequivocal assignment of relative substitution positions in an unknown lipid or mixture would be challenging without reference to relevant standards.

The CID spectra in Figure 1(a) and (b) were recorded with ozone present in the ion trap. As such, without altering the instrument configuration, each of the $[TG + Na - R_nCOOH]^+$ product ions formed upon CID can be isolated and allowed to react with ozone in a formal MS^3 sequence. Example CID/OzID spectra, obtained by allowing the $[TG + Na - 16:0]^+$ CID product ion at *m/z* 599 to react with O_3 are shown in Figure 1(c) and (d). The base peak in the CID/OzID spectrum obtained from TG(16:0/16:0/18:1) is *m/z* 405 representing a neutral loss of 194 Da (Figure 1c), while the base peak for the TG(16:0/18:1/16:0) regioisomer is *m/z* 379 corresponding to a neutral loss of 220 Da (Figure 1d). The spectrum from TG(16:0/16:0/18:1) also features the *m/z* 379 product ion but at low abundance relative to *m/z* 405. Importantly, when compared to the CID spectra (Figure 1a and b), the CID/OzID spectra are dramatically different for the two regioisomers and, if these trends are general, could readily be deployed to assign relative acyl chain positions in TGs.



Scheme 1. Proposed mechanism for CID/OzID in TGs.

The CID/OzID product ions observed in Figure 1(c) and (d) can be rationalised by the reactions shown in Scheme 1(a). As proposed by Hsu and Turk, CID of $[TG + Na]^+$ ions leads to FA loss *via* a substitution reaction driven exclusively by the adjacent ester moiety and resulting in a 1,3-dioxolane ring.[22,23] The structure of this CID product ion is such that the acyl chain adjacent to the FA leaving group is anchored to the dioxolane by a new carbon-carbon double bond. Rapid ozonolysis of this activated alkene during OzID leads to selective loss of the second acyl chain, leaving a glycerol carbonate ester with a single terminal FA substituent (*i.e.*, *sn*-1 or *sn*-3). Figure 1(d) illustrates the selectivity of this reaction sequence whereby neutral loss of the 16:0 fatty acyl chain to form the *m/z* 599 ion in the CID of $[TG(16:0/18:1/16:0) + Na]^+$ is driv-

en exclusively by the adjacent 18:1 substituent. Subsequent ozonolysis leaves only the 16:0 FA attached to the backbone as the carbonate ester at *m/z* 379. Conversely, loss of 16:0 FA upon CID of $[TG(16:0/16:0/18:1) + Na]^+$ can be driven by either the adjacent 16:0 or 18:1 FA and thus both the 16:0 (*m/z* 379) and 18:1 (*m/z* 405) FAs can remain following ozonolysis. For the TG(16:0/16:0/18:1) isomer, formation of *m/z* 379 requires a CID-initiated substitution at the secondary *sn*-2 carbon of the glycerol. This is likely to be energetically less competitive than analogous processes occurring at the terminal glycerol positions and thus explains the lower relative abundance of *m/z* 379 compared to *m/z* 405 in Figure 1(c).[16] All major CID/OzID product ions can be explained by the same mechanism (see full reaction scheme, Supporting Information Scheme S-1). Ions observed at *m/z* 395 and 421 are 16 Da more massive than the carbonate ions at *m/z* 379 and 405, respectively. While the structure of these ions remains to be established, the observation is consistent with the general mechanism of ozonolysis, which can deposit one or two oxygen atoms following cleavage of the carbon-carbon double bond.[33] These ions are a common feature of OzID spectra and are generically referred to as Criegee ions.[28] The product ion at *m/z* 291 in Figure 1(d) is consistent with retention of sodium on an oxidised fragment of the 18:1 chain, while the broad peak at *m/z* 614.5 has been assigned as a fragile epoxide ion of actual *m/z* 615.[24] The ability to rationalise all CID/OzID processes stemming from a single mechanism enables the prediction of diagnostic neutral losses for any acyl chain (*e.g.*, Supporting Information Table S-1), which combined with information obtained from CID, should enable *de novo* assignment of acyl chain substitution patterns in unknown TGs.

TGs containing multiple short chain FAs and a single long chain FA are present in milk from ruminant animals,[34] and play an important role in the mating behaviour of *Drosophila* (*e.g.,* TG(5:1_5:1_18:1).[27] The positional isomers of such TGs are difficult to differentiate by infrared spectroscopy or electron ionisation (EI) MS.[26] Moreover, the 5:1 tiglic acid moiety presents an interesting structure to study the dissociation mechanisms of ionised TGs during tandem mass spectrometry, due to the absence of an α-hydrogen adjacent to the carbonyl.

Unlike TGs with three long chain fatty acids – such as those typically found in plant oils[35,36] – the presence of one or more short chain FAs assists the separation of *sn*-positional isomers by reversed-phase HPLC.[9] Using a C_{18} column with a methanol/water gradient, a synthetic standard of TG(5:1/5:1/18:1) eluted *ca.* 1 min earlier than isomeric TG(5:1/18:1/5:1) (see Supporting Information, Figure S-2). Electrospray ionisation and subsequent CID produced spectra shown in Figure 2(a) and (b), respectively. The familiar pattern of $[TG + Na – R_nCOOH]^+$ (*i.e.*, *m/z* 443 and 261) and $[TG + Na – R_nCOONa]^+$ (*i.e.*, *m/z* 421 and 239) ions are observed, although the $[TG + Na – 18:1]^+$ ion at *m/z* 261 is present only in low abundance in both spectra. The diminished abundance of this product ion is consistent with the absence of an α-hydrogen in the tiglic acid moiety. The fact that an ion at *m/z* 261 is observed at all must indicate the action of an alternative mechanism, for example hydrogen abstraction from the terminal allylic position, generating a 1,3-diene as the ionic product (see Supporting Information, Scheme S-2).

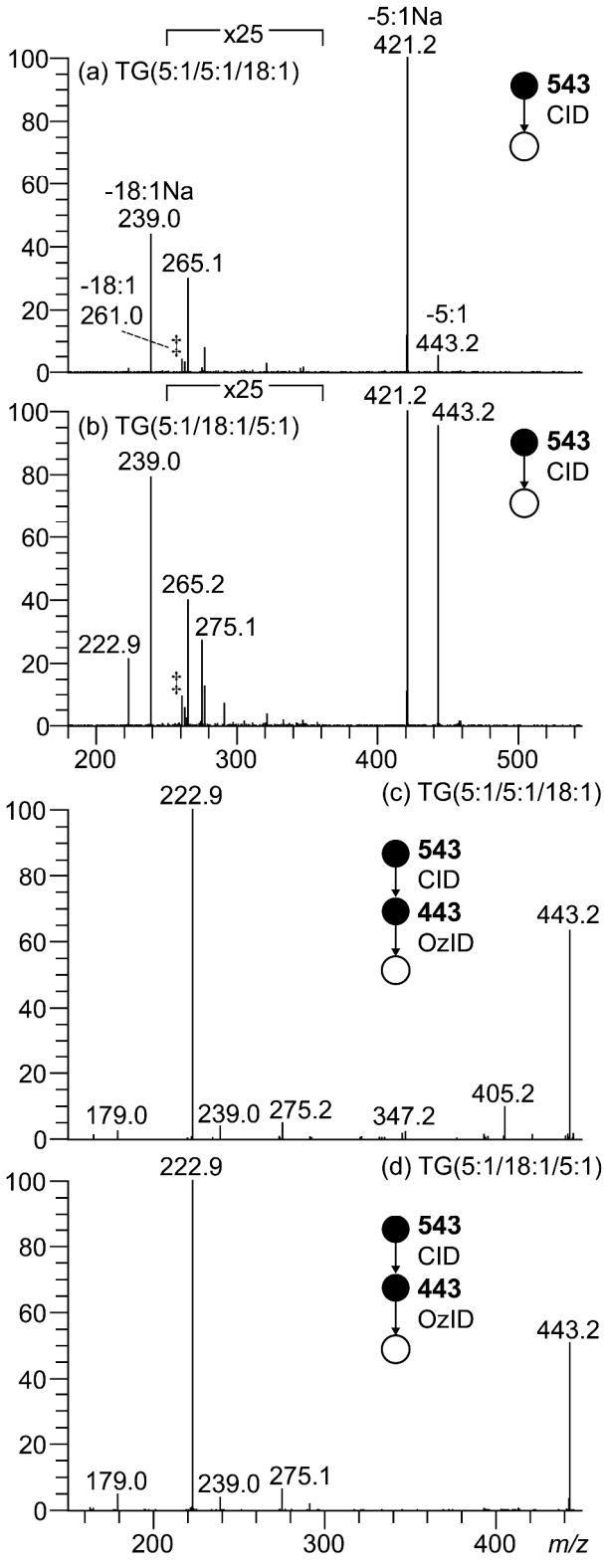


Figure 2. CID spectra of isomeric $[TG + Na]^+$ ions (*m/z* 543) separated by HPLC: (a) TG 5:1/5:1/18:1; (b) TG 5:1/18:1/5:1. LC-CID/OzID spectra of $[TG + Na – 5:1]^+$ product ions (*m/z* 443) from the isomers: (c) TG(5:1/5:1/18:1) and (d) TG(5:1/18:1/5:1). Spectra are the average of 20 scans across the chromatographic peak. O_3 reaction time = 0.25 s. ‡ = $[TG + Na – 18:1]^+$, *m/z* 261.

Ozonolysis of $[TG + Na - 5:1]^+$ ions (*m/z* 443) generated from CID of TG(5:1/5:1/18:1) results in product ions at *m/z* 223 and *m/z* 405, arising from the loss of 18:1 and 5:1 FA substituents, respectively (Figure 2c). As demonstrated in Scheme 1(b), these two product ions can be rationalised only for an AAB-acyl chain arrangement. Figure 2(d) displays the analogous CID/OzID spectrum obtained from TG(5:1/18:1/5:1). Notably, in this spectrum only a single ozonolysis product ion is present at *m/z* 223. This observation is consistent with an ABA-type substitution pattern according to Scheme 1.

These data support the hypothesis that CID of ionised TGs generally proceeds *via* a 1,3-dioxolane. The presence of multiple ozonolysis product ions in the CID/OzID spectrum leads to straightforward assignment of the *sn*-2 FA and by extension, the relative arrangement of FAs on the glycerol backbone. The presence of abundant diagnostic CID/OzID ions increases analytical sensitivity and produces simpler spectra compared to CID/CID of $[TG + Li]^+$ ions (see Figure S-3).[23]

CID/OzID of ABC-type TGs. Figure 3 displays the region of interest in the CID spectra of isomeric $[TG(16:0\_18:1\_18:0) + Na]^+$ ions obtained from direct infusion of synthetic standards. Each spectrum features the expected product ions at *m/z* 627, 601 and 599 ($[TG + Na - R_nCOOH]^+$) and *m/z* 605, 579 and 577 ($[TG + Na - R_nCOONa]^+$) differing only in relative ion abundances, with a diminished ion abundance providing tentative evidence for the identity of the *sn*-2 FA.

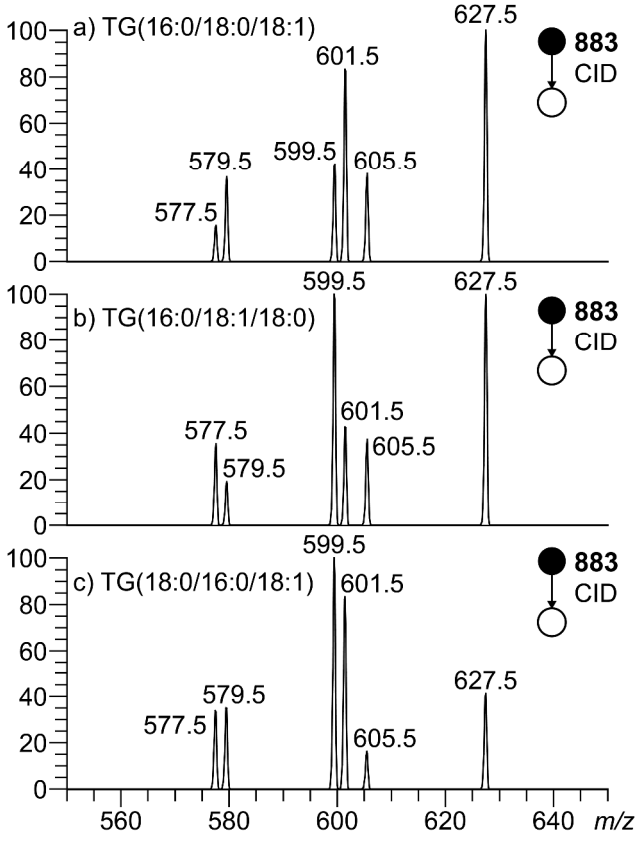


Figure 3. Region of interest in CID spectra of $[TG + Na]^+$ ions of TG(52:1) *sn*-positional isomers: (a) TG(18:1/18:0/16:0); (b) TG(16:0/18:1/18:0); (c) TG(18:0/16:0/18:1).

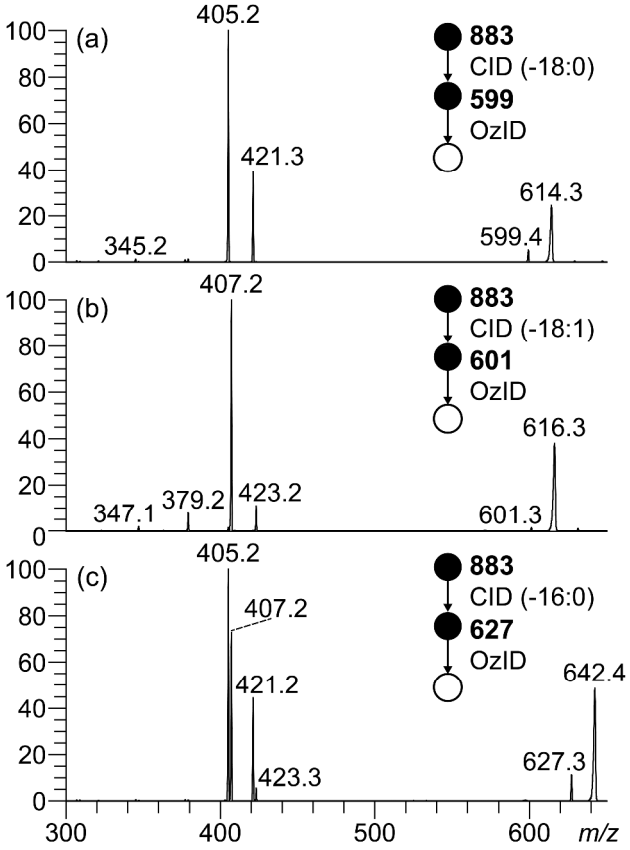
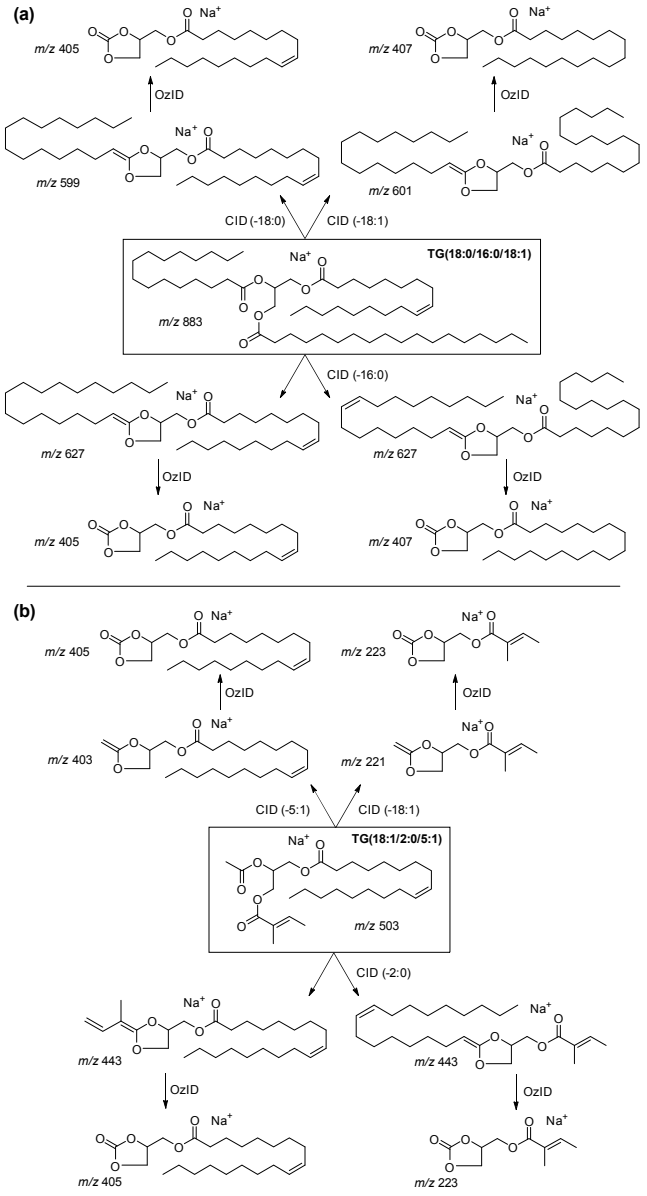


Figure 4. CID/OzID (MS^3) spectra obtained from CID product ions of TG(18:0/16:0/18:1): (a) 18:0 FA loss (*m/z* 599); (b) 18:1 FA loss (*m/z* 601); (c) 16:0 FA loss (*m/z* 627). The ion at *m/z* 379 in panel (b) arises from CO_2 loss from the Criegee ion at *m/z* 423.

Subsequent OzID (MS^3) interrogation of each $[TG + Na - R_nCOOH]^+$ product ion unequivocally identifies the FA substitution pattern. CID/OzID spectra for TG(18:0/16:0/18:1) are displayed in Figure 4. Equivalent CID/OzID spectra of isomeric TG(18:1/18:0/16:0) and TG(16:0/18:1/18:0) are presented as Supporting Information (Figure S-4 and S-5). In Figure 4(a), the 18:0 FA is eliminated during CID, and the predominant OzID product ion at *m/z* 405 represents a loss of 194 Da. Similarly *m/z* 407 dominates the spectrum shown in Figure 4(b), which represents the same 194 Da neutral loss from the precursor ion $[TG + Na - 18:1]^+$. As in Figure 1(c), a 194 Da neutral loss indicates loss of the 16:0 FA during ozonolysis, thus this FA was connected through a new carbon-carbon double bond to the dioxolane. The 16:0 FA can only be involved in the elimination of both 18:0 FA (Figure 4a) and 18:1 FA (Figure 4b) during CID if the 16:0 FA is adjacent to both, and therefore occupies the *sn*-2 position, as shown in Scheme 2(a). Both *m/z* 405 and *m/z* 407 are present in nearly equal abundance in the CID/OzID spectrum of $[TG + Na - 16:0]^+$ shown in Figure 4(c). These ions represent neutral losses of 222 Da and 220 Da, corresponding to loss of 18:0 and 18:1 FA, respectively. Both possible ozonolysis products are observed in this spectrum due to the presence of multiple isomers of *m/z* 627, arising from either outer chain displacing the *sn*-2 16:0 FA during CID (Scheme 2a). To reiterate, OzID of ions arising from *sn*-1 or *sn*-3 FA loss during CID results in a single carbonate ester product ion and the *sn*-2 FA is exclu-

sively cleaved by ozonolysis. When the *sn*-2 fatty acid is lost during CID, both possible OzID product ions are observed.



Scheme 2. CID/OzID mechanism in ABC-type TGs.

Pheromone TGs containing 3 different FAs have been previously identified in *Drosophila*, including TGs incorporating short chain acetic (2:0), and tiglic (5:1) acids.[27] Gas chromatography resolved 3 synthetic isomers of TG(18:1_5:1_2:0),[26] however structure elucidation remains incomplete due to the similarities of their EI mass spectra, which is particularly troublesome if synthetic standards with unequivocal structures are unavailable. CID/OzID coupled to HPLC offers significant promise as an analytical technique capable of separation and unambiguous identification of these TG *sn*-positional isomers. Using the same chromatographic conditions, *sn*-positional isomers of TG(18:1_5:1_2:0) standards were separated by *ca.* 0.7 minutes (extracted ion chromatograms are shown as Supporting Information, Figure S-6). Although separation is achieved by chromatography, identification of regioisomers requires subsequent MS interrogation.

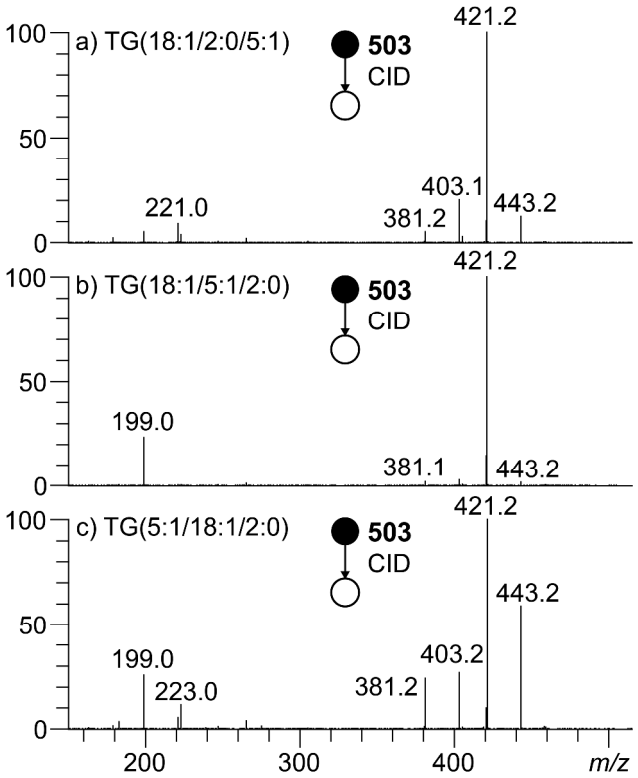


Figure 5. Representative CID spectra obtained from TG(25:2) isomers acquired on an ion-trap mass spectrometer: (a) TG(18:1/2:0/5:1); (b) TG(18:1/5:1/2:0); (c) TG(5:1/18:1/2:0).

CID mass spectra of each individual isomer exhibit remarkable similarity (Figure 5). The expected $[TG + Na - R_nCOOH]^+$ (*m/z* 443, 403, 221) and $[TG + Na - R_nCOONa]^+$ (*m/z* 421, 381, 199) product ions are observed in each case, varying only in their relative abundances. Each spectrum is dominated by an ion at *m/z* 421, representing the loss of sodium acetate from the precursor ion. Unusually for TGs, this product ion is the base peak in the spectrum even when the acetate moiety is in the *sn*-2 position (*i.e.,* TG(18:1/2:0/5:1), Figure 5a). This unusual dissociation pattern further complicates identification of *sn*-positional isomers solely at the MS^2 level.

CID/OzID spectra obtained upon loss of tiglic acid from TG(18:1_2:0_5:1) isomers are presented in Figure 6. Following the logic of Scheme 2, loss of tiglic acid during CID should result in ions at *m/z* 405 from TG(18:1/2:0/5:1) and *m/z* 183 from TG(5:1/18:1/2:0). Both ions are expected to be present when tiglic acid is lost from the *sn*-2 position (*i.e.,* TG(18:1/5:1/2:0)). The CID/OzID spectra shown in Figure 6 do not exclusively follow the predicted mechanisms outlined in Scheme 2. Both possible ozonolysis product ions (*m/z* 183 and *m/z* 405) are observed in each spectrum. Observation of ions at *m/z* 183 in Figure 6(a) and *m/z* 405 in Figure 6(c) is ascribed to an alternate mechanism involving formation of an analogous 6-membered 1,3-dioxane (Scheme S-3, Supporting Information), whereby the *sn*-1 and *sn*-3 FA chains are able to interact during CID because of the compact acetate moiety. Nonetheless, the relative abundance of these product ions indicates that the 5-membered ring mechanism remains the major contributor to fragmentation during CID.

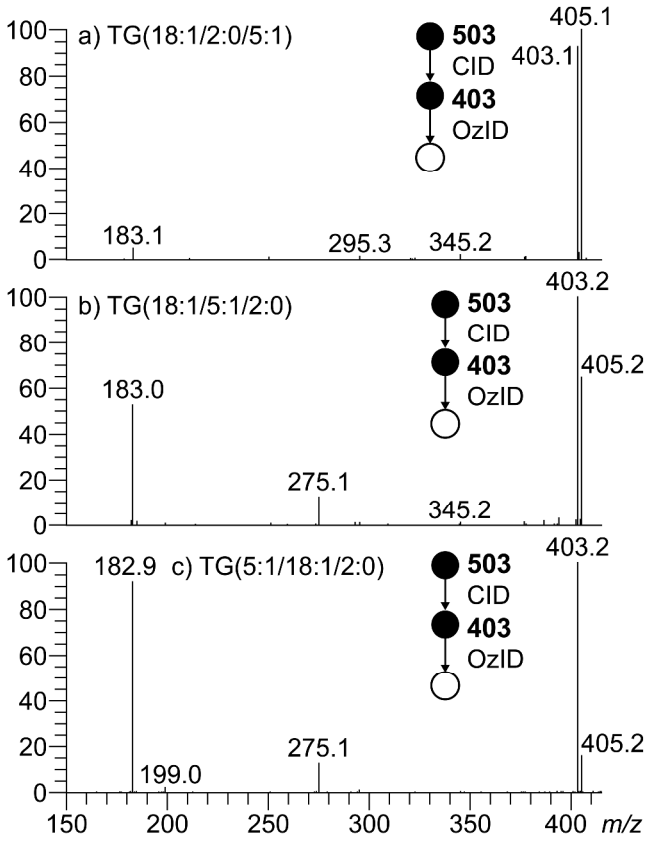


Figure 6. CID/OzID spectra of $[TG + Na - 5:1]^+$ ions from: (a) TG(18:1/2:0/5:1); (b) TG(18:1/5:1/2:0); (c) TG(5:1/18:1/2:0), using an O_3 reaction time of 250 ms.

The interaction between adjacent 5:1 and 18:1 FAs in TG(18:1/5:1/2:0) and TG(5:1/18:1/2:0) is confirmed in Figure 6(b) and 6(c) by the presence of ions at *m/z* 275, assigned as $[C_{16}H_{31}CHO + Na]^+$ arising from ozonolysis of the new carbon-carbon double bond and retention of charge on the long-chain aldehyde (*cf.* Scheme S-1). Analogous ions from the interaction of 2:0 and 5:1 FAs (*i.e.,* $[CH_2O + Na]^+$) may be present but are too low in mass to be observed.

LC-CID/OzID of Drosophila Extract. The same LC-MS^n protocol was applied to separate and identify regioisomer(s) of TG(25:2) and TG(28:3) present in an extract collected from ejaculatory bulbs of *Drosophila*. Both the retention time (peak **2**, Figure 7a) and CID spectrum obtained for TG(25:2) closely resemble those acquired from standard TG(18:1/2:0/5:1) shown in Figure 5(a). Moreover, the CID/OzID spectrum obtained from $[TG + Na - 5:1]^+$ ions (Figure 7b) features the same product ions as the spectrum of the corresponding standard (Figure 6a). The presence of a single chromatographic peak and the demonstrated capability to separate *sn*-positional isomers enables the assignment of TG(18:1_/2:0/_5:1) as the predominant regioisomer of TG(25:2). The CID spectrum obtained from $[TG(28:3) + Na]^+$ (peak **5**) was notably similar to that shown in Figure 2(a), suggesting that this TG is comprised of 2 tiglic acids and a single 18:1 FA. Upon re-isolation of $[TG + Na - 5:1]^+$ ions (*m/z* 443) in the presence of ozone, ions at *m/z* 223 and 405 are observed in the CID/OzID spectrum (Figure 7c, *cf.* Figure 2c), unequivocally assigning TG(28:3) as TG(5:1_/5:1/_18:1).

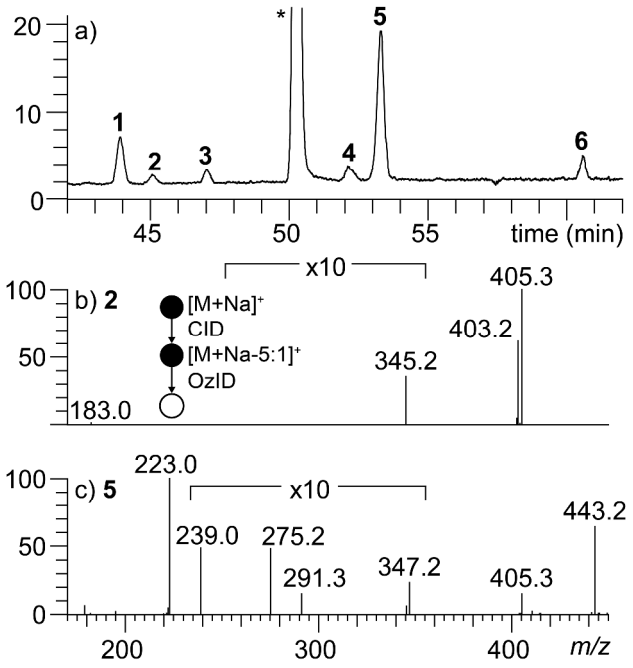


Figure 7. (a) Extracted ion chromatogram of *m/z* 500 – 550 obtained from *Drosophila* extract. The peak at 50.5 minutes does not exhibit TG-like fragmentation. CID/OzID (MS^3) spectra obtained from $[TG + Na - 5:1]^+$ product ions: (b) TG(25:2) (**2**); (c) TG(28:3) (**5**). CID/OzID product ions obtained from peaks **1**, **3**, **4**, and **6** are provided as Supporting Information (Table S-2).

Single peaks corresponding to $[TG + Na]^+$ ions of TG(26:3), TG(28:4), TG(26:2), and TG(28:2) were also observed (peaks **1**, **3**, **4**, **6** in Figure 7a), consistent with previous analysis by laser desorption ionisation MS.[27] Employing the same CID/OzID methodology, these TGs were identified as TG(5:1_/5:1/_X; X = 16:1, 18:2, 16:0, or 18:0, respectively), potentially indicating a common biosynthetic pathway to these structurally related TGs containing a common motif. CID and CID/OzID characterisation details are provided as Supporting Information (Table S-2).

CONCLUSIONS

Successive application of CID and OzID is demonstrated to be a promising method for the determination of relative acyl chain position in triacylglycerols, even when synthetic standards with unambiguous structures are not available. MS^3 spectra obtained upon exposure of $[M + Na - R_nCO_2H]^+$ ions to ozone feature abundant diagnostic marker ions, readily identifying adjacent FAs and thus the substitution pattern along the glycerol backbone. Although the resulting spectra are influenced by the identity of the acyl chains, particularly in the presence of short-chain tiglic acid (5:1) and acetic acid (2:0), the CID/OzID spectra obtained from TGs containing these motifs are nonetheless unique to each isomer. TGs extracted from *Drosophila* were identified as exclusively AAB-type substitutional isomers by the CID/OzID method. Combinations of CID and OzID significantly improve the analytical capability to differentiate isomeric TGs. All experiments described herein involve the same conditions in the ion trap for ozone- or collision-induced dissociation. The set-up is thus compatible with online separation and associated data-

dependent workflows guided by the information required by the analyst. In an extension of this approach, CID/OzID product ions can be further reacted with O_3 in an MS^4 experiment to determine double bond position within each FA (Figure S-7). Ozonolysis of the FA olefin is significantly slower than ozonolysis of the double bond formed by CID, and thus cleavage of the olefin is typically not observed in high abundance in CID/OzID (MS^3) spectra,[24] particularly on the short reaction timescales required for compatibility with chromatography.

Interestingly, the *Drosophila* extracts examined here yielded a single regioisomer. By contrast, multiple regioisomers have previously been observed in TGs extracted from other sources. Relative quantification of isomers using CID/OzID would be most confidently assigned following chromatographic separation (*cf*. Figure S-6). It is acknowledged that such resolution becomes challenging for long chain TGs with similar acyl chain lengths and degrees of unsaturation.[8,9] The recent demonstration of *sn*-positional isomer separation using DMS[10] may enable future development of combined DMS-CID/OzID strategies to facilitate separation, identification and relative quantification of these isomers in complex extracts.

Finally, the CID/OzID spectra reported here are consistent with the unimolecular dissociation mechanisms proposed by Hsu and Turk,[22,23] particularly the 1,3-dioxolane structure of the $[TG + Na - R_nCO_2H]^+$ CID product ion (Scheme 1). Complete reaction of these product ions with ozone by oxidative cleavage of the carbon-carbon double bond (*cf*. reaction of *m/z* 599 in Figure 1c and d) suggests that this ion population consists entirely of the 1,3-dioxolane with no evidence to support the lactone structure recently proposed by Grossert *et al*.[37]

ASSOCIATED CONTENT

Supporting Information

Experimental details, proposed dissociation mechanisms, additional chromatograms and CID/OzID spectra of alternate regioisomers are presented as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

The data reported in this paper were obtained at the Central Analytical Research Facility operated by the Institute for Future Environments (QUT). Access to CARF is supported by generous funding from the Science and Engineering Faculty (QUT). T.W.M. and S.J.B. acknowledge financial support from the Australian Research Council (ARC) through the Discovery Project scheme (DP150101715) with project funding from the ARC and SCIEX (Concord, Ontario, Canada) through the Linkage Program (LP110200648). J.Y.Y. was supported by the Singapore National Research Foundation (NRF-RF2010-06).

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