A multimodal analytical method to simultaneously determine monoacetyldiacylglycerols, medium and long chain triglycerides in biological samples during routine lipidomics

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**A multimodal analytical method to simultaneously determine
monoacetyldiacylglycerols, medium and long chain triglycerides in biological
samples during routine lipidomics**

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Abstract

Introduction: Monoacetyldiglycerides (MAcDG), are acetylated triglycerides (TG) and an emerging class of bioactive or functional lipid with promising nutritional, medical, and industrial applications. A major challenge exists when analyzing MAcDG from other subclasses of TG in biological matrices, limiting knowledge on their applications and metabolism.

Objective and Methods: Here in we demonstrate a multimodal analytical method for resolution, identification and quantitation of MAcDG in biological samples based on thin layer chromatography-flame ionization detection complimentary with C30-reversed phase liquid chromatography-high resolution accurate mass tandem mass spectrometry. We further apply this method to determine the MAcDG molecular species composition and quantity in *E. solidaginis* larvae.

Results and Conclusion: These findings suggest that the proposed analytical method could simultaneously provide a fast, accurate, sensitive, high throughput analysis of MAcDG from other TG subclasses, including the fatty acids, isomers and molecular species composition. We believe this method will allow for MAcDG to be included during routine lipidomics analysis of biological samples and will have broad interests and applications in the scientific community in areas such as nutrition, climate change, medicine and biofuel innovations.

Keywords: Thin layer chromatography, triglyceride analysis, acylated triglyceride, liquid chromatography, mass spectrometry, functional lipids, *Eurosta solidaginis*

1. Introduction

Triglycerides (TG) are neutral lipids which provide primary energy reserves in living organisms ¹. Structurally, TGs are triesters composed of three fatty acid (FA) esterified at the stereospecifically numbered carbon 1 (*sn*-1), 2 (*sn*-2), and 3 (*sn*-3) positions of the glycerol backbone. They are involved in regulation of fatty acid metabolism and plasma levels of lipoproteins in human ^{2,3}. TGs are divided into four subclasses based on their fatty acids. In this regard, monoacyldiglycerides (MAG) are acetylated triglycerides having an acetate moiety (CH₃COO-) at *sn*-3 position and longer fatty acyl chains at *sn*-1 and *sn*-2 positions. Short chain triglycerides (scTG) contain at least one fatty acyl chain composed of 3 – 5 carbons. Medium chain triglycerides (mcTG) contain at least one fatty acyl chain composed of 6 – 12 carbons, while long chain triglycerides (lcTG) are composed of fatty acyl chains containing more than 12 carbon atoms ⁴. lcTG are dietary sources of essential polyunsaturated fatty acids including docosahexaenoic acid (DHA- ω 3), eicosapentaenoic acid (EPA- ω 3), linolenic acid (ALA- ω 3), arachidonic acid (ARA- ω 6), and linoleic acid (LA- ω 6). Several nutritional studies support consumption of these fatty acids and a reduction in risk factors for cardiovascular diseases, cancers, arthritis, diabetes, hypertension and neuropsychiatric disorders ⁵. Furthermore, consumption of mcTG either in the diet or as supplements has been reported to be beneficial for reducing body fat and obesity ⁶, alcoholic liver diseases ⁷, and short bowel syndrome ⁸. MAG have been reported to be effective in treating sepsis ^{9,10}, inflammations, cancers ¹¹, arthritis ¹², and asthma ¹³. Furthermore, MAG is an emerging functional lipid in the dietary and

84 health sector. It is also a useful adjuvant for improving the performance of biofuels under
85 cold climatic conditions ^{14,15}.

86 As such, to fully exploit these benefits from MAcDG, there is a need for fast, accurate,
87 precise and sensitive techniques to identify and quantify MAcDG from other TG
88 subclasses in biological matrices. A major challenge exists in the scientific community with
89 analyzing MAcDG from other subclasses of TG. In most routine lipidomics techniques
90 used, MAcDG is not resolved from the other subclasses of TG, and as such there is limited
91 publications concerning their applications in the scientific community. Furthermore, there
92 is current interest in the biofuel industry to use MAcDG as an adjuvant to resolve viscosity
93 issues with biofuels when used in cold or northern climates. As a result, oil seed crops
94 such as camelina is now successfully engineered to produce high levels of MAcDG for
95 biofuel production and applications in cold climates ^{14,15}. Thus, there is a need for the
96 development of suitable analytical methods to permit the routine analysis of MAcDG to
97 improve our understanding of how this unique lipid function and resolve issues in health
98 and industrial applications. The literature is replete with analytical methods for the analysis
99 of TG in biological samples that could be useful for the analysis of MAcDG ¹⁶. Among
100 these, thin layer chromatography coupled with flame ionization detection (TLC-FID)
101 provides cost effective, accurate, precise, and efficient method for routine quantification
102 of TG subclass composition ¹⁷⁻¹⁹. However, TLC-FID does not provide information on
103 molecular species and fatty acid compositions, which is important for nutritional, medical,
104 and industrial applications of TG. While mass spectrometry-based techniques including
105 gas chromatography coupled to flame ionization detection (GC-FID) and gas
106 chromatography coupled to mass spectrometry (GC-MS) provide higher sensitivity,
107 accuracy, precision, linear range and throughput compared to TLC-FID for quantification
108 of TG fatty acid composition, these methods are not applicable for quantification of intact
109 molecular species composition of TG subclasses including MAcDG ^{19,20}. Furthermore,

acid/base hydrolysis and chemical derivatization steps required for sample preparation in GC-FID and GC-MS analysis of TG limits the general utility and scope of biological samples which can be analyzed by these methods ²¹. Liquid chromatography-heated electrospray ionization tandem mass spectrometry (LC-HESI-MS/MS) allows identification and quantitation of intact TG molecular species, fatty acid, and subclass composition of biological samples in a single run ^{22,23}. Furthermore, LC-HESI-MS provides higher accuracy, precision, and sensitivity compared to GC-MS and GC-FID, and does not require prior hydrolysis and derivatization steps for mass spectrometric analysis of TG ²⁴. **We herein describe** a multimodal analytical method for routine identification and quantitation of MAcDG as a subclass of TG in biological samples based on TLC-FID complimentary with C30-reversed phase liquid chromatography-tandem mass spectrometry (C30-RPLC-MS/MS). We further applied this method for quantification of the molecular species, and fatty acid composition in biological samples using *E. solidaginis* larvae fat as an example. To the best of our knowledge, the proposed C30-RPLC-MS/MS method is the first to be applied for quantification of MAcDG molecular species, fatty acid and subclass compositions in *E. solidaginis* larvae fat. Furthermore, the proposed TLC-FID method supplements our previous work ²⁰ and demonstrates resolution of hydroxylated MAcDG (OH-MAcDG) in relation to MAcDG, mcTG, and lcTG by TLC-FID. We demonstrate the applicability of the proposed method for the analysis of MacDG during routine lipidomics assessments of biological samples.

2.0. Materials and methods

2.1. Standards and reagents

Triglyceride (TG) standard mix (Splash Lipidomix Standard- 17811-1AMP) was obtained from Supelco (Oakville Ontario, Canada). Synthesized and purified MAcDGs compounds, including 16:0/18:0/2:0, 16:0/9Z-18:1/2:0, 16:0/9Z,12Z-18:2/2:0 and OH-MacDG

16:0/18:0(OH)/2:0, were provided by Chemforce Laboratories (Edmonton, Canada). Calibration standard solutions (0-10 µg/mL) containing these standards were used as spiked internal standards and to prepare standard curves to quantify TG in *E. solidaginis*.

2.2. Collection of *E. solidaginis* larvae

E. solidaginis larvae were extracted from the galls of *Solidago* spp. plants into 2 mL Eppendorf centrifuge tubes and stored at -80 °C²⁰. These plants were from old field habitats in London, Ontario, Canada (43°00'N, 81°15'W). Memorial University Animal Care provided ethics approval [20160041] for this study. All experiments complied with established guidelines and regulations.

2.3. Lipid extraction from *E. solidaginis* larvae

Lipids were extracted in septuplicate (N = 7) from *E. solidaginis* larvae (85 - 170 mg) according to the Folch method using 2.4 mL chloroform/methanol (2:1, v/v) and 0.8 mL of 0.1% v/v butylated hydroxytoluene²⁵. The resulting mixture was vortexed for 10 secs and centrifuged at 10000 ×g for 10 min at -4 °C. The organic layers were pooled into pre-weighed glass vials, and the solvent removed under dry nitrogen stream. Prior to TLC-FID analysis, the dried lipids were dissolved in chloroform (700 - 900 µg/mL).

2.4. Thin layer chromatography-Flame ionization detection (TLC-FID) analysis of MAcDG

A standard mixture containing MAcDG, mcTG, lcTG and OH-MAcDG standards was used for assessing the effectiveness of TLC-FID to resolve MAcDG from other classes of TG. The TLC-FID analysis of TG subclass composition of the complex standard mixture was conducted following the procedures described by Marshal et al. (2014) with minor modifications²⁰. Briefly, 1.7 mL solution of the complex standard mixture in chloroform was resolved on chromarods (5 mm silica gel-coated quartz rod; Shell, Virginia, USA) using 70:30:0.5 benzene:chloroform:formic acid (v/v/v) for chromatographic separation.

TLC-FID analysis was performed using an Iatroscan thin-layer chromatograph (Model MK-6 TLC-FID Analyzer; Shell, Virginia, USA) equipped with a flame ionization detector. The instrument was operated at the following settings: flow rate of 2 L min⁻¹ for atmospheric air, 160 mL min⁻¹ for hydrogen, and scanning speed of 3.0 cm s⁻¹ ²⁰. Identification of TG subclasses (MAcDG, mcTG, lcTG and OH-MAcDG) present in the complex standard mixture was done by comparing retention times and elution order of the individual standards (eg MAcDG) spotted on separate chromarods.

2.5. MAcDG analysis by C30 reversed phase liquid chromatography-heated electrospray ionization high resolution accurate mass tandem mass spectrometry (C30-RPLC-HESI-HRAMS-MS/MS) and application for the analysis of MAcDG in *E. solidaginis* fat

In this section, we demonstrate the application of C30-RPLC-HRAMS-MS/MS for the analysis of MAcDG in a complex standard mixture and a biological sample (*E. solidaginis* fat) containing several different subclasses of TG (MAcDG, mcTG, ScTG, lcTG). LC-MS analysis of *E. solidaginis* fat followed the same procedures described in our previous publications ^{26,27}. Briefly, an Accucore C30 column (150 × 2 mm I.D., particle size: 2.6 µm, pore diameter: 150 Å; Thermo Fisher Scientific, ON, Canada) coupled to a Dionex Ultimate 3000 ultra-high performance liquid chromatography (UHPLC) system and a Q-Exactive Orbitrap high resolution accurate mass spectrometer (Thermo Fisher Scientific, ON, Canada) was used to resolve the lipids present in the complex standard mixture and *E. solidaginis* larvae fat. For chromatographic separation, solvent A composed of acetonitrile:water (60:40 v/v) while solvent B contained isopropanol:acetonitrile:water (90:10:1 v/v/v); with both solvents containing 10 mM ammonium formate and 0.1% formic acid. TG concentrations in *E. solidaginis* larvae fat are expressed on nanomol percent (nmol %) and microgram per gram fresh weight (µg/g FW) basis.

2.6. Data processing

Xcalibur data acquisition and interpretation software

(<https://www.thermofisher.com/order/catalog/product/OPTON-30965#> , version 4.0,

Thermo Fisher Scientific, Ontario, Canada) was used for the C30-RPLC-HRAM-MS/MS

lipidomics data acquisition and processing. For the identification at a molecular level, the

relative positions of medium and long-chain fatty acids (FA) in TG molecular species

were assigned based on the general rules of the relative abundances of fragment ions in

their MS/MS spectra. The rule was established based on common findings that the loss

of fatty acid(s) at the *sn*-1 and *sn*-3 positions are observed at higher abundance than *sn*-

2 fatty acid loss²⁸⁻³⁰. However, it was found to be an exception for TG containing short

chain fatty acids. For example, in this study we observed that the loss of CH₃COONH₄

from the MS/MS spectra of MAcDG standards was at the lowest abundance although

acetic acid was esterified to the *sn*-3 position. For relative quantitation, integrated peak

areas and exact masses of TG molecular species were used to calculate the relative

abundance (nmol %) of TG molecular species in *E. solidaginis* larvae fat. Furthermore,

the absolute quantitation was conducted based on a limited number of available

standards to estimate the concentration of TG molecular species in *E. solidaginis* larvae

fat (µg/g FW). It was based on integrated peak areas of TG molecular species and

standards curves generated from authenticated standards (Triglycerides mixtures-

17811-1AMP) purchased from Supelco (Oakville Ontario, Canada) and MAcDG

standards obtained from Chemforce Laboratories (Edmonton, Canada). The standard

curves were generated using representative standards from each class due to the lack

of standards for all individual molecular species, and thus not completely accounted for

the matrix effects common in biological samples. However, it was found that the

corresponding factor of lipid ions depended mainly on their headgroup, thus only one

representative standard is required for each lipid class/subclass in absolute quantitation^{31,32}.

2.7. Statistical analysis

TG molecular species and subclass composition in *E. solidaginis* larvae fat were assessed using the descriptive statistics procedure in XLSTATS. One-way analysis of variance (ANOVA) was used to determine significant differences between the levels of TG molecular species and subclasses comprising the distributions of TG present in *E. solidaginis* larvae fat. Where differences were significant, the means were compared with Fisher's Least Significant Difference (LSD), $\alpha = 0.05$. XLSTAT (Premium version, Addinsoft, New York, USA) was used for statistical analysis.

3.0. Results and discussion

3.1. Analysis of MAcDG in complex lipid standard mixture using thin layer chromatography coupled to flame ionization detection (TLC-FID)

One objective of this study was to demonstrate thin layer chromatography coupled with flame ionization detection (TLC-FID) as a simple analytical method for routine, accurate identification and quantitation of MAcDG from other TG subclasses in complex biological samples. In the TLC-FID approach, we utilized a solvent system composed of benzene:chloroform:formic acid optimized for chromatographic separation of neutral lipid classes on polar silica chromarods^{20,33}. We observed that TG subclasses in the complex standard mixture separated into four major peaks based on retention times corresponding to OH-MAcDG (0.41 min), MAcDG (0.25 min), mcTG (0.22 min), and lcTG (0.18 min) subclasses (Fig. 1a). The retention times demonstrated the polarity of the different TG subclasses using this solvent system is as follows: OH-MAcDG < MAcDG < mcTG < lcTG

(Fig. 1a). The resolution of MAcDG and lcTG subclass are consistent with that reported by Marshall et al. (2014) for the analysis of *E. solidaginis* larvae fat²⁰. The concentration range observed to provide excellent resolution and quantitation using TLC-FID is 100 - 1000 µg/mL (0.1 to 1 mg/mL). Below 100 µg/mL the peak size and appearance is not consistent and above 1000 µg/mL we observed very large, broad shaped peaks that can interfere with the resolution of MAcDG from the different TG subclasses. Furthermore, the response is linear between 100 - 1000 µg/mL for the quantification of MAcDG as well as other TG subclasses. The amount of MAcDG in a sample can be quantified by TLC-FID from the average peak area of MAcDG (7 chromarods) present in each sample using the linear regression equation ($y = mx + c$) and calculated as follows¹⁷:

$$MAcDG (\mu g/g \text{ sample}) = \frac{(y - c)x V}{m \times W}$$

where y = average peak area of MAcDG, c and m = intercept and slope of the regression line, respectively, V = total volume of lipid solution (mL), and W = weight of sample used (g).

The output presented in this paper demonstrate the ability of the method for simple, rapid, and sensitive quantification of OH-MAcDG, MAcDG, mcTG and lcTG in biological samples. To the best of our knowledge, this is the first time hydroxylated MAcDG (OH-MAcDG) has been resolved from other lipid classes using TLC-FID. This presents opportunities for purification and further evaluation of the hydroxylated version of MAcDG, which is a more polar version of MAcDG, as well as determine possible functions and applications. It is well recognised that the bioactivities and physical properties of MAcDG is distinctly different from that of other TG subclasses. How hydroxylation further influences these properties is unknown. The application of TLC-FID to resolve and quantify polar and neutral lipid classes in complex biological samples ranging from animal (egg yolk, chicken fat, lamb fat, milk), marine (lobster, krill oil, red porgy wild, greater

weever, piper gurnard) plant (sesame seeds, canola gum, macadamia nuts, olive oil), and edible fungus (mushrooms) origins have been widely reported^{19,34-36}. These applications demonstrate the versatility of this analytical technique for routine, simple, efficient, accurate and sensitive analysis of neutral lipids. MAcDG can now be included as a subclass in routine neutral lipid analysis in these sources using TLC-FID. The proposed TLC-FID method provides a simple, comparatively inexpensive, sensitive and rapid method to separate, identify and quantitate TG subclasses including MAcDG and OH-MAcDG in biological samples. In particular, the unique biochemical composition of MAcDG in biological samples including *E. solidaginis* can confer novel uses and application in the food science field (considering insects are emerging sources of dietary proteins and health-promoting functional lipids), healthcare (treating sepsis, cancers and tumors), and biofuel industry (potential additive to improve performance of biofuels in cold climates^{26,37}. These uses suggest possible applications for MAcDG for which rapid, relatively inexpensive, accurate and sensitive analytical methods for quantification will be essential. Our findings demonstrate that TLC-FID could be used to accomplish this analysis across a range of biological samples.

3.2. Analysis of MAcDG in complex lipid standard mixture using C30 reversed phase liquid chromatography coupled to heated electrospray ionization- high resolution accurate mass tandem mass spectrometry (C30-RPLC-HESI-HRAM- MS/MS)

We further demonstrated that C30-reversed phase liquid chromatography coupled to high resolution accurate mass tandem mass spectrometry (C30-RPLC-HRAM-MS/MS) could be used complimentary to TLC-FID for the simultaneous analysis of the fatty acids and molecular species composition of intact MAcDG and other TG subclasses in biological samples. To this end, we used a mobile phase consisting of solvent A (acetonitrile: H₂O 60:40 v/v) and solvent B (isopropanol:acetonitrile:water 90:10:1 v/v/v) both containing

ammonium formate buffer as the solvent system applied with C30-RPLC coupled with accurate mass tandem mass spectrometry optimized for separation and quantification of intact neutral lipid species²⁶. We observed clear resolution of TG molecular species including mcTG, lcTG, MACDG, and OH-MACDG present in standard mixture based on polarity, fatty acid composition and fatty acid chain length ranging from TG(8:0)₃ to TG(18:1)₃, in positive ion mode (Fig. 1b). Resolution of non-acetylated TG species including mcTG: TG(8:0)₃ occurred at 16.47 min, TG(10:0)₃ at 20:00 min and TG(12:0)₃ at 22:39 min from lcTG: TG(14:0)₃ at 24:88 min, TG(16:1)₃ at 25:14 min, TG(16:0)₃ at 27.95 min and TG(18:1)₃ at 28:27 min standards was achieved. These non-acylated TG molecular species were well separated from the acetylated TG including OH-MACDG which occurred at 19:77 min and MACDG at 21:53 min (Fig. 1b). The presence of OH group in OH-MACDG appears to have increased the relative polarity of MACDG-OH thereby reducing its retention on the C30-RP column relative to MACDG and lcTG species in the standard mixture³⁸. As such MACDG-OH eluted before MACDG and lcTG species, as shown in Fig. 1b. Furthermore, separation of mcTG and lcTG molecular species present in the standard mixture was based on their fatty acid chain lengths²⁶. It is accepted that hydrophobic interactions between the stationary phase and hydrophobicity of fatty acyl chains (based on chain length, number and position of double bonds of fatty acids) provides the resolution of neutral lipid species present in the standard mixture³⁹. McTG molecular species have shorter fatty acyl chains compared to lcTG species which makes the former less hydrophobic compared to lcTG. As such, mcTG molecular species were less retained under C30-RPLC conditions compared to the more hydrophobic lcTG species⁴⁰.

The high resolving power of the Q-Exactive Orbitrap mass spectrometer allowed for accurate and unambiguous resolution and structural identification of TG molecular species including MACDG and OH-MACDG present in the standard mixture in positive ion mode

(Fig. 2). The C30-RPLC-HRAM-MS/MS spectra of MAcDG and OH-MAcDG molecular species present in the standard mixture are shown in Fig. 2a-d. Ammonium formate buffer was used in the C30-RPLC method for column optimization. Therefore, all intact TG molecular species present in the standard mixture formed ammonium adducts $[TG + NH_4]^+$ under the C30-RPLC-HRAMS-MS/MS conditions used in this study^{26,41}. For example, the MS/MS spectrum of MAcDG 16:0/18:1/2:0 $[M+NH_4]^+$ at m/z 654.57 is shown in Fig. 2a. The diagnostic *sn*-3 acetyl moiety at m/z 577.52 corresponds to the neutral loss of $[CH_3COO^-NH_4^+]$ or 77 Da from m/z 654.57 $[MAcDG\ 16:0/18:1/2:0 + NH_4]^+$ molecular ion⁴². The composition and positions of fatty acids (FA) in TG molecular species including MAcDG and OH-MAcDG present in the standard mixture were identified based on the neutral loss of the fatty acid fragments as ammonium adducts, and presence of the fatty acid ketene ions $[FA + H-H_2O]^+$ in the MS/MS spectra^{26,43}. As such for MAcDG 16:0/18:1/2:0 (Fig. 2a), the fragment at m/z 355.28 correspond to the neutral loss of 18:1 + NH_3 (*sn*-2 FA) or 299 Da, while the fragment at m/z 381.30 correspond to the neutral loss of 16:0 + NH_3 (*sn*-1 FA) or 273 Da from m/z 654.57 $[MAcDG\ 16:0/18:1/2:0 + NH_4]^+$ ion⁴⁴. Distinction between *sn*-2 and *sn*-1 fatty acids is based on the relative abundance of these two FA fragments, with relative abundance of *sn*-2 FA fragment $[18:1 + NH_3]$ lower than that of *sn*-1 FA fragment $[16:0 + NH_3]$, which is a trend typical of *sn*-1 and *sn*-2 FAs in TG⁴⁵. Furthermore, fragment ions at m/z 239.24 and 265.25 in Fig. 2a were assigned to fatty acid ketene ions $[16:0 + H-H_2O]^+$ and $[18:1 + H-H_2O]^+$ corresponding to *sn*-1 FA and *sn*-2 FA, respectively. The distinction between *sn*-2 and *sn*-1 positions on TG molecular species including MAcDG and OH-MAcDG could also be identified by the relative abundance of these fatty acid ketene ions²⁶. The relative abundance of $[18:1 + H-H_2O]^+$ ketene ion at m/z 265.26 corresponding to *sn*-2 FA was higher than that of $[16:0 + H-H_2O]^+$ ion at m/z 239.24 for *sn*-1 FA of MAcDG 16:0/18:1/2:0, which is a trend typical of *sn*-1 and *sn*-2 FA ketene ions in TG species (Fig. 2a). Thus, the trend based on relative

abundance of *sn-1* and *sn-2* [FA + H-H₂O]⁺ ketene ions is opposite to that observed for fragments formed by neutral loss of FAs from [MAcDG 16:0/18:1/2:0 + NH₄]⁺ ion, and is consistent with the literature on determining TG molecular species^{43,44}.

The proposed C30-RPLC-MS/MS method also allowed for resolution and identification of OH-MAcDG molecular species in the complex standard mixture. Structurally, while OH-MAcDG and MAcDG are distinguished from non-acetylated TG species including scTG, mcTG and lcTG by the presence of *sn-3* acetyl groups, OH-MAcDG differs from MAcDG by having one hydroxyl group on the *sn-2* fatty acid chain. These structural differences were seen in the mass spectra of MAcDG and OH-MAcDG molecular species present in the standard mixture under C30-RPLC-MS/MS conditions (Fig. 2a-b). For example, the MS/MS spectra of OH-MAcDG 16:0/18:0(OH)/2:0 *m/z* 672.58 is shown in Fig. 2b. The neutral loss of [CH₃COO·NH₄]⁺ or 77 Da from [TG + NH₄]⁺ ions is representative of MAcDG molecular species⁴². In contrast OH-MAcDG molecular species are distinguished from MAcDG by the neutral loss of [CH₃COO·NH₄⁺ + H₂O] or 95 Da from [TG + NH₄]⁺ ions⁴⁶. Accordingly, the fragment at *m/z* 577.52 corresponding to neutral loss of [CH₃COO·NH₄⁺ + H₂O] or 95 Da from *m/z* 672.58 [OH-MAcDG 16:0/18:0(OH)/2:0 + NH₄]⁺ ion is diagnostic of the *sn-3* acetate moiety in OH-MAcDG 16:0/18:0(OH)/2:0 (Fig. 2b). Furthermore, relative abundances of fatty acid fragments arising from neutral loss from [TG + NH₄]⁺ ions and fatty acid ketene ions were used to assign *sn-1* and *sn-2* fatty acid composition of OH-MAcDG species. Consistent with conventions in the literature for assigning the *sn1* and *sn2* fatty acids based on relative abundance⁴⁵, fragments at *m/z* 355.28 (18:0 *sn-2*) and *m/z* 381.30 (16:0 *sn-1*) correspond to neutral loss of [18:0 + NH₃] and [16:0 + NH₃] from [OH-MAcDG 16:0/18:0(OH)/2:0 + NH₄]⁺ ion, respectively (Fig. 2b). As previously alluded to, the relative abundance of fragment corresponding to *sn-2* FA is lower compared to *sn-1* FA fragment⁴⁵. In a similar fashion, the fragments at *m/z* 239.24 and *m/z* 265.25 were diagnostic of fatty acid ketene ions [16:0 + H-H₂O]⁺ and [18:0 + H-H₂O]⁺

respectively, with the relative abundance of the *sn*-2 FA ketene ion higher than the *sn*-1 FA ketene ion (Fig. 2b). In summary, neutral loss of 77 Da and 95 Da from [TG + NH₄]⁺ ions was used to distinguish between MAcDG and OH-MAcDG molecular species present in the standard mixture.

In contrast, non-acetylated TG including scTG, mcTG and lcTG present in the standard mixture did not form fragments corresponding to neutral loss of 77 Da and 95 Da from [TG + NH₄]⁺ ions under C30-RPLC-HRAM-MS/MS conditions, which provided a basis for their identification relative to acylated TG (MAcDG and OH-MAcDG). For example, the MS/MS spectra of mcTG 8:0/8:0/8:0 *m/z* 488.39 is shown in Fig. 2c. The neutral loss of 161 Da is representative of C8:0 FA loss from *m/z* 488.39 [mcTG 8:0/18:0/8:0 + NH₄]⁺ ion, which corresponds to the fragment at *m/z* 327.25 (Fig. 2c), while the fragment at *m/z* 127.11 corresponds to the fatty acid ketene ion [8:0 + H-H₂O]⁺ at *sn*-1, *sn*-2 and *sn*-3 positions of mcTG 8:0/8:0/8:0²⁶. The same approach was applicable for structural elucidation of lcTG molecular species present in the standard mixture. Accordingly, the *m/z* 551.50 fragment in Fig. 2d corresponds to the neutral loss of FA 16:0 (273 Da) from *m/z* 824.68 [lcTG 16:0/16:0/16:0 + NH₄]⁺ ion, while fatty acid ketene ion [16:0 + H-H₂O]⁺ was at *m/z* 127.11^{26,28}. Application of C30-RPLC-MS/MS to identify and quantify neutral lipid molecular species in biological samples have been reported in the literature. Narvaez et al. (2016) applied a RPLC-MS/MS method in positive ion mode to resolve and analyze neutral lipids including TG in rat liver and rat plasma lipid extracts²². A similar application has been used for analysis of TG composition of Calu-3 cells, rat blood and human skin tissues⁴⁷. However, none of these techniques have included the analysis of MAcDG and OH-MacDG. We have developed the method utilizing a C30-RPLC-HRAM-MS/MS technique to analyze neutral lipids including TG and was successful in detecting MAcDG in wild cervid meats for the first time (moose and caribou)⁴⁸. Taken together, the proposed C30-RPLC-HRAM-MS/MS method allows for accurate detection and quantification of TG

molecular species including OH-MAcDG and MAcDG with good resolution, sensitivity, and throughput during routine lipidomics.

3.3. Application of C30-RPLC-HESI-HRAM-MS/MS for separation and identification of MAcDG molecular species in larvae of *E. solidaginis*

TG subclass and molecular species composition in *E. solidaginis* is of interest due to its role as a model system in cold stress tolerance. In this model system, MAcDG have been demonstrated to provide cryoprotection during exposure to low temperature stress²⁰. This work has also led to subsequent studies recently on the potential of producing specialized oil seed crops with superior levels of MAcDG for applications in the production of biofuels specific for cold temperatures or northern climates^{15,49}. For example, Liu et al. (2015) reported that EaDAcT genetically modified camelina and soybean accumulated MAcDG at up to 70 mol% of the total seed oil produced by the resultant crops. A similar strategy of genetic modification increased MAcDG content to 85 mol% in field-grown transgenic camelina¹⁵. Furthermore, recent interests in insects as a potential source of proteins and functional ingredients for food and animal feed to improve population health and wellbeing has gained recognition, for which the larvae of *E. solidaginis* could be a promising food source^{50,51}. TG in *E. solidaginis* larvae fat have been reported to contain about 36 mol% MAcDG compared to long chain TG²⁰. However, no report was done on the molecular species composition of MAcDG in *E. solidaginis*. MAcDG is associated with several health benefits in human and these include treating sepsis¹⁰, tumor growth and cancers¹¹, rheumatoid arthritis¹² and asthma¹³.

We applied the proposed C30-RPLC-HRAM-MS/MS method to separate MAcDG lipid species present in the fat of *E. solidaginis* larvae from other TG molecular species. We observed MAcDG eluted between 18.9 to 20.64 min followed by the other TG subclasses between 21.44 and 27.63 under C30-RPLC conditions (Fig. 3a-b). Four main classes of

412 TG molecular species were identified in the larvae of *E. solidaginis* including MAcDG,
 413 scTG, mcTG and lcTG (Fig. 3b). A clear separation of these TD subclasses was observed
 414 based on hydrophobicity, chain lengths and molecular weight as follows: MAcDG < scTG <
 415 mcTG < lcTG (Fig. 3b). No OH-MAcDG specie was observed in *E. solidaginis* fat, which is
 416 line with the literature ²⁰. The C30-RPLC separated the more polar MAcDG species from
 417 non-acetylated TG species (scTG, mcTG and lcTG), which allowed for facile mass
 418 spectrometric identification and quantification (Fig. 3b). High resolution accurate mass
 419 tandem mass spectrometry (HRAM-MS/MS) was used to assign the molecular species
 420 composition of MAcDG, scTG, mcTG and lcTG species in *E. solidaginis* larvae fat as
 421 shown in Fig. 4a-e & Fig. S-1 – S-3. For example, the MS/MS spectra of MAcDG
 422 18:1/16:1/2:0 [M+H]⁺ at *m/z* 652.56 is shown in Fig. 4a. The fragment at *m/z* 575.51
 423 corresponds to the neutral loss of 77 Da [CH₃COO·NH₄⁺] from *m/z* 652.56 [MAcDG
 424 18:1/16:1/2:0 + NH₄]⁺ ion. This neutral loss 77 Da is representative of the *sn*-3 acetyl
 425 moiety [CH₃COO·NH₄⁺] characteristic of all MAcDG species. The two fatty acid fragments
 426 were at *m/z* 381.31 (16:1 *sn*-2) and *m/z* 353.27 (18:1 *sn*-1) corresponding to neutral loss
 427 of [16:1 + NH₃] and [18:1 + NH₃] from [MAcDG 18:1/16:1/2:0 + NH₄]⁺ ion, respectively (Fig.
 428 4a). Furthermore, corroboration of the fatty acid composition of MAcDG 18:1/16:1/2:0 was
 429 also based on the diagnostic fatty acid ketene ions at *m/z* 237.22 (*sn*-2 FA) and *m/z* 265.26
 430 (*sn*-1 FA) which correspond to [16:1 + H-H₂O]⁺ and [18:1 + H-H₂O]⁺ ions, respectively (Fig.
 431 4a). Assignment of *sn*-1 FA and *sn*-2 FA of MAcDG 18:1/16:1/2:0 was made based on the
 432 relative abundance of the neutral loss FA fragments and fatty acid ketene ions as
 433 elaborated previously in the discussion. A similar approach was applied to elucidate the
 434 structures of other MAcDG species detected in *E. solidaginis* larvae fat (Fig. 4b & Fig. S-
 435 1 – S-3). Significantly, the proposed C30-RPLC-HRAM-MS/MS method provided facile
 436 resolution of MAcDG and TG molecular species present as isomers in *E. solidaginis* larvae
 437 fat. For example, the MS/MS spectra of MAcDG species present in *E. solidaginis* larvae

438 were distinguished from scTG, mcTG and lcTG species by the neutral loss of $[\text{CH}_3\text{COO}^-$
 439 $\text{NH}_4^+]$ or 77 Da from $[\text{TG} + \text{NH}_4]^+$ ion, which is diagnostic of the *sn*-3 acetyl moiety of all
 440 MAcDG species (Fig. 4a-e). Using the approach based on the relative abundances of
 441 neutral loss of the fatty acid fragments and fatty acid ketene ions explained above, as well
 442 as in the literature ⁵², the two isomers at m/z 874.79 $[\text{TG } 52:3 + \text{NH}_4]^+$ reported in Fig. 4d-
 443 e were assigned as lcTG 18:1/18:1/16:1 and lcTG 18:2/18:1/16:0 molecular species
 444 respectively.

445 Furthermore, it was possible to resolve isomers of MAcDG from that of other TG
 446 subclasses present in *E. solidaginis* larvae using the proposed method (Fig. 5a-b). For
 447 example, MAcDG 18:0/18:1/2:0 $[\text{M}+\text{H}]^+$ m/z 682.59 eluted at 20.64 min under C30-RPLC
 448 conditions (Fig. 5b) is an isomer of scTG 16:0/18:0/4:0 $[\text{M}+\text{H}]^+$ m/z 682.59 which eluted at
 449 21.44 min (Fig. 5a). These isomers were easily resolved based on their MS/MS
 450 fragmentation patterns (Fig. 5a-b). For scTG 16:0/18:1/4:0, the three fatty acid
 451 composition were assigned as follows: the fragments at m/z 577.53 is diagnostic of 4:0
 452 fatty acid at *sn*-3, m/z 409.34 for 16:0 at *sn*-1 and m/z 383.32 for 18:1 at the *sn*-2 positions
 453 of the glycerol moiety. This correspond to the neutral loss of $[4:0 + \text{NH}_3]$, $[16:0 + \text{NH}_3]$ and
 454 $[18:1 + \text{NH}_3]$ from m/z 682.59 $[\text{scTG } 16:0/18:2/2:0 + \text{NH}_4]^+$ ion, respectively (Fig. 5a).
 455 Assignment of the *sn*-1, *sn*-2 and *sn*-3 FAs of scTG 16:0/18:0/4:0 was based on the
 456 relative abundance of these fragments with *sn*-1 FA > *sn*-2 FA > *sn*-3 FA, a trend typical
 457 of TG species ^{26,28}. Furthermore, diagnostic fatty acid ketene ions at m/z 239.24 and m/z
 458 265.26 correspond to $[16:0 + \text{H}-\text{H}_2\text{O}]^+$ and $[18:1 + \text{H}-\text{H}_2\text{O}]^+$, which is representative of *sn*-
 459 1 FA and *sn*-2 FA respectively (Fig. 5a). In contrast, the structure of MAcDG 18:0/18:1/2:0
 460 $[\text{M}+\text{H}]^+$ m/z 682.59 in Fig. 5b was distinguished from scTG 16:0/18:1/4:0 by the fragment
 461 at m/z 605.56 which correspond to neutral loss of 77 Da from m/z 682.59 $[\text{MAcDG}$
 462 $18:0/18:1/2:0 + \text{NH}_4]^+$ ion. Assignment of the *sn*-1 and *sn*-2 FAs in MAcDG 18:0/18:1/2:0
 463 was based on the relative abundance of the fragments associated with the neutral loss of

each fatty acid and the fatty acid ketene ions (Fig. 5b). A similar approach was used to assign all TG and MAcDG molecular species including isomers present in *E. solidaginis* (Fig. 4-5 & Fig. S1-S2). This work shows the advantage of C30-RPLC-HESI-HRAM-MS/MS as a superior platform for resolving isomers of MAcDG from other TG subclasses in biological samples. We believe MAcDG maybe observed to be more common in biological samples if their analysis is facilitated during routine lipidomics.

The quantitation and distribution of MAcDG and other TG subclasses present in *E. solidaginis* larvae fat is shown in Fig. 6a-b. The fat of *E. solidaginis* is enriched with MAcDG accounting for 38% of the TG composition, while lcTG accounted for 48% on mass percent basis (Fig. 6c-d). These findings are consistent with those presented by Marshall et al. (2014) who demonstrated that the level of lcTG and MAcDG in *E. solidaginis* was 36% and 29% respectively, and varied with seasons under cold temperature stress following analysis using TLC-FID²⁰ A thorough discussion of the use of TLC-FID for analyzing MAcDG and long chain TG is presented in our previous publication²⁰. We also demonstrated for the first time that the fat of *E. solidaginis* consisted of 12% and 5% scTG and mcTG respectively (Fig. 6c-d). We further identified a total of 34 TG molecular species in *E. solidaginis*, which consisted of 12 MAcDG species, 2 mcTG species, 2 scTG species and 17 lcTG species (Fig. 6e). The MAcDG molecular species composition was predominated by 18:1/18:1/2:0, while 18:1/18:1/4:0, 18:1/18:1/12:0 and 18:1/18:1/18:1 respectively predominated scTG, mcTG, and lcTG compositions in *E. solidaginis* (Fig. 6e). Furthermore, the TG profile of *E. solidaginis* was enriched in PUFA TG consisting of 38 and 40 carbons, as well as two double bonds. Approximately, 48% of the TG species consisted of 2 double bonds compared to 71% for MAcDG (Fig. 6f-g). Furthermore, we observed that MADAG in *E. solidaginis* was composed of shorter carbon chains compared to TG (C34-C40 vs C34-C56) with C38 predominating in both. However, MADAG had higher levels of C38 carbon chains compared to other TG subclasses (69% vs 58%) as is

shown in Fig. 6f-g. The output from this method shows for the first time the molecular species composition of MAcDG compared with other TG subclasses in *E. solidaginis*. This is important considering *E. solidaginis* can survive at cold temperatures as low as -80 °C and is a well recognised model system for studying cold temperature stress in different biological systems. MAcDG has been previously reported to confer cryoprotective properties to *E. solidaginis* during temperature stress survival. Information on the molecular species composition of MAcDG will allow further work by researchers in the scientific community to improve our understanding of the mechanisms associated with MAcDG metabolism during cold temperature stress acclimation. Furthermore, insect larvae are an emerging source of high-quality dietary lipids. This information could be useful in the evaluation of *E. solidaginis* as a source of dietary MAcDG or functional lipids. In summary, the proposed C30-RPLC-HRAM-MS/MS method appears to be a suitable approach for the analysis of MAcDG and other TG subclasses, molecular species, isomers and fatty acid composition in *E. solidaginis* fat, that could also be applied for routine lipidomics analysis of other biological samples.

3.4. Advantages of a multimodal approach using high resolution C30-RPLC-HESI-HRAM-MS/MS for routine MAcDG analysis.

The multimodal approach proposed in this paper is advantageous compared to other approaches in the literature in that TLC-FID facilitate quick, cost effect simple resolution of MAcDG from other neutral lipids including subclasses of TG, (mcTG, scTG, lcTG), as well as polar lipids using silica rods and a non-polar solvent system. The flame ionization detection (FID) facilitates rapid , cost effective quantitation of total MAcDG in the sample. We used this approach to demonstrate for the first time the resolution of hydroxylated MAcDG from other TG subclasses. Applying C30-RPLC-HESI-HRAM-MS/MS allow determination of the molecular species composition including the fatty acids composition

of MAcDG in biological samples. Most lipidomics analysis currently use C18 RPLC columns to resolve TG and other neutral lipids during routine lipidomics. We use C30 RPLC instead, due to the superior resolving power of C30 stationary phase in separating TG and other lipid isomers. When combined with accurate mass tandem mass spectrometry this is a powerful platform to resolve MAcDG molecular species, fatty acids and isomers including positional isomers as demonstrated in this paper. This is very important because MAcDG is not typically analyzed as a component of routine or untargeted lipidomics. Generally, a targeted approach is employed that often involved pre concentration steps.

4.0. Conclusion

In this work, we present the utilization of TLC-FID and C30-RPLC-HESI-HRAM-MS/MS as a multimodal approach for the analysis of MAcDG, OH-MAcDG, scTG, mcTG and lcTG during routine lipidomics analysis. TLC-FID separated TG lipids into subclasses based on polarity, while C30 RPLC separated TG lipids based on chain length, polarity and fatty acid composition. We successfully applied the C30-RPLC-HESI-HRAM-MS/MS method to resolve MAcDG, scTG, mcTG and lcTG lipid subclasses, and molecular species and isomers in *E. solidaginis* as an example of the suitability of the method for routine lipidomics analysis of biological samples. Furthermore, C30-RPLC-HESI-HRAM-MS/MS provided excellent intra-class resolution of TG subclasses and isomers with different fatty acid compositions. We believe this method will allow for MAcDG to be included in routine lipidomics of biological samples and will have broad interests and applications in the scientific community.

Author contribution

R.H.T. conceptualized the research and designed the experiment; R.H.T. supervised the research; T.H.P., C.F.M and H.S. performed lab analyses; T.H.P. and C.F.M performed formal data analysis. C.F.M prepared manuscript draft. R.H.T., C.F.M. and T.H.P. reviewed the manuscript. All authors contributed to editing the final version of manuscript.

Competing interests

The authors declare no competing interests.

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5.0. References

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Fig. 1 a) TLC-FID separation of TG subclasses and **b)** C30-RPLC-HESI-HRAM-MS/MS chromatograms in positive ion mode of the standard lipid mixture of monoacetyldiacylglycerols (MAcDG), hydroxylated monoacetyldiacylglycerols (OH-MAcDG) medium and long chain triglycerides (mcTG and lcTG).

Fig. 2 C30-RPLC-HESI-HRAM-MS/MS spectra showing fragmentation of precursor ions isolated at 1 Da selection window in positive ion mode for the lipid standards representing: **a)** MAcDG 16:0/18:1/2:0 (36:1) at m/z 654.57, **b)** OH-MAcDG 16:0/18:0(OH)/2:0 (OH-36:0) at m/z 672.58, **c)** mcTG 8:0/8:0/8:0 (24:0) at m/z 488.39 and, **d)** lcTG 16:0/16:0/16:0 at m/z 824.68. All TG subclasses are shown as ammonium adducts ($[M+NH_4]^+$).

Fig. 3 a) C30-RPLC-HESI-HRAM-MS/MS chromatogram in positive ion mode of complex lipid extracted from the larvae of *Eurosta solidaginis* species. **b)** Zoomed in neutral lipids region of the C30-RPLC-MS chromatogram showing some major MAcDG, scTG, mcTG and lcTG contribution of the lipid profile.

Fig. 4 C30-RPLC-HESI-HRAM-MS/MS spectra showing fragmentation of precursor ions isolated at 1 Da selection window in positive ion mode for the major MAcDG species in *Eurosta solidaginis* species larvae corresponding to: **a)** MAcDG 18:1/16:1/2:0 (36:2) at m/z 652.56, **b)** MAcDG 16:0/18:1/2:0 (36:1) at m/z 654.55. **c)** mcTG 18:1/16:1/12:0 (38:3) m/z 678.56. Resolution of lcTG 52:3 isomers at m/z 874.79 representing: **d)** 18:1/18:1/16:1 and **e)** 18:2/18:1/16:0.

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729 **Fig. 5** C30-RPLC-HESI-HRAM-MS/MS spectra showing fragmentation of precursor ions
 730 isolated at 1 Da selection window in positive ion mode for isomers of scTG and MAdDG in *Eurosta*
 731 *solidaginis* corresponding to: **a)** scTG 16:0/18:1/4:0 (38:1) at m/z 682.59 and **b)** MAdDG
 732 18:0/18:1/2:0 (38:1) at m/z 682.59.

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Fig. 6 Quantitation and distribution of C and other TG subclasses present in the larvae of *E. solidaginis*. **a)** Standard curve used to calculate the amount of MAcDG in *E. solidaginis*, **b)** Representative structure of MAcDG, **c)** Percent composition, **d)** quantity, and **e)** molecular species distribution of MAcDG and other TG subclasses present in the larvae of *E. solidaginis*. **f)** Chain length as the number of carbons, **g)** and number of double bonds in the sum of the FA moieties in MAcDG present in *E. solidaginis*. Values represent means $\pm$ standard error (N = 7). Fisher LSD was used to separate the means at $\alpha = 0.05$ following analysis using one-way ANOVA.

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