

UHPLC-MS/MS method for determination of 1,2-and 1,3-diacylglycerol regioisomers using N,N-dimethylglycine derivatization

Piotr Zabielski

Medical University of Białystok

Agnieszka U. Błachnio-Zabielska

agnieszka.blachnio@umb.edu.pl

Medical University of Białystok

Article

Keywords: diacylglycerols mass spectrometry, derivatization, ultra-high performance liquid chromatography, regioisomers

Posted Date: October 1st, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11006204/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Diacylglycerols (DAGs) are bioactive lipids involved in metabolic pathways and cellular signaling, with their activity influenced by molecular species and regioisomeric configuration. 1,2-DAGs are potent activators of protein kinase C isoforms, whereas 1,3-DAGs show markedly lower activity. Accurate determination of these regioisomers is therefore important for investigating their distinct biological roles. However, DAG analysis remains challenging due to poor ionization efficiency, potential acyl migration, and limited chromatographic resolution. Here, we developed and validated a UHPLC-MS/MS method for selective determination of individual 1,2- and 1,3-DAG molecular species. DAGs were derivatized with N,N-dimethylglycine to enhance ionization under positive electrospray conditions. Separation was achieved on a reversed-phase Accucore C30 column, with detection by triple quadrupole mass spectrometry in selected reaction monitoring mode. Fatty-acyl-specific product ions improved molecular-species selectivity and discrimination of isobaric DAG species. The method showed excellent linearity over 1.56–200 ng ($R^2 = 0.9969\text{--}0.9997$), with limits of detection of 0.214–1.786 pg/injection. Recovery ranged from 94.60% to 105.85% at the low spike level and from 99.27% to 107.54% at the high spike level, while intra-day and inter-day precision was below 9%. The method provides sensitive, selective, and reproducible determination of 1,2- and 1,3-DAG molecular species, and may serve as a useful analytical platform for investigating DAG metabolism and signaling in metabolic disease.

1. Introduction

Diacylglycerols (DAGs) are a class of neutral lipids that serve as important intermediates in triacylglycerol and glycerophospholipid metabolism and also function as signaling molecules in numerous cellular pathways (1). Their signaling functions include activation of protein kinase C (PKC) isoforms and regulation of processes such as insulin signaling, cell growth, and differentiation (2). The biological activity of DAGs depends strongly on their molecular structure, including the chain length and degree of unsaturation of the esterified fatty acids (3). An additional level of structural complexity arises from regioisomerism, defined by the position of the acyl chains on the glycerol backbone (1,2- versus 1,3-DAG). These regioisomers can differ substantially in their biological properties and metabolic fate (4). For instance, 1,2-DAGs are potent activators of PKC, whereas 1,3-DAGs are substantially less effective in this regard (1). Consequently, analytical methods capable of accurately identifying and quantifying individual DAG molecular species, including their regioisomeric forms, are essential for elucidating their physiological and pathological functions.

Despite their biological importance, comprehensive analysis of DAGs by mass spectrometry remains challenging. DAGs are neutral lipids lacking a permanent ionizable group and therefore exhibit relatively low ionization efficiency in electrospray ionization (ESI). They are commonly detected as ammonium or metal adducts, which facilitates their MS detection (5, 6) but such adduct formation provides limited structural information for distinguishing closely related regioisomers. In particular, 1,2- and 1,3-DAGs have identical elemental compositions and highly similar physicochemical properties, making their chromatographic separation and identification challenging. Accurate analysis is further complicated by acyl migration, a spontaneous intramolecular rearrangement in which 1,2-DAGs are converted into the thermodynamically more stable 1,3-isomers. This process may occur during sample preparation, storage, or chromatographic analysis, particularly under elevated temperatures or acidic or basic conditions, potentially altering the native regioisomeric composition and compromising quantitative accuracy. Chromatographic separation is therefore an important component of the reliable analysis of DAG regioisomers. Direct chromatographic separation of DAG regioisomers has previously been achieved using normal-phase chromatographic approaches. Piyatheerawong et al. separated both regio- and enantiomeric DAG isomers by coupling a silica column with a cellulose-based chiral stationary phase and employing a normal-phase mobile phase composed of n-hexane and 2-propanol (300:7, v/v) (7). Detection was performed using an evaporative light scattering detector (ELSD). Although this approach provided high chromatographic selectivity, the use of a highly nonpolar n-hexane-based mobile phase is not well suited to direct coupling with ESI-MS because of its poor electrospray compatibility. Moreover, analysis of underivatized DAGs does not address their intrinsically low ionization efficiency or susceptibility to acyl migration. Thus, although normal-phase chromatography can provide effective separation of DAG regioisomers, there remains a need for LC-MS-compatible approaches that combine regioisomeric resolution with improved ionization efficiency and preservation of the native DAG composition. Chemical derivatization provides a complementary strategy for improving the mass spectrometric analysis of DAGs. In particular, charge-tagging approaches introduce an ionizable group into the DAG molecule, thereby substantially improving ionization efficiency and analytical sensitivity in ESI-MS. Derivatization of the free hydroxyl group may provide an additional advantage by reducing susceptibility to acyl migration, thereby helping to preserve the regioisomeric composition throughout the analytical workflow. Several derivatization strategies have been proposed to improve the mass spectrometric analysis of DAGs. Wang et al. described a one-step charge-derivatization approach for shotgun lipidomics that markedly improved ionization efficiency and enabled highly sensitive quantification of DAGs directly from crude lipid extracts (8). However, because shotgun lipidomics does not provide chromatographic separation, this approach cannot distinguish 1,2- and 1,3-DAG regioisomers. More recently, N,N-dimethylglycine has been used for selective derivatization of DAGs through esterification of the free hydroxyl group. This reaction introduces a tertiary amine that can be readily protonated under positive ESI conditions, substantially enhancing ESI-MS sensitivity. DMG derivatives also generate a characteristic neutral loss of 103 Da during tandem mass spectrometry, providing an additional basis for selective DAG detection. However, DMG-based neutral-loss detection alone does not provide sufficient structural information to distinguish all isobaric DAG molecular species. In particular, DAG species with different fatty-acyl compositions may generate identical precursor ions and the same characteristic neutral loss, despite representing structurally distinct molecular species. Consequently, chromatographic separation combined with MS/MS transitions providing fatty-acyl-specific structural information is required for selective determination of individual DAG molecular species. Therefore, the aim of the present study was to develop a robust UHPLC-MS/MS method for the simultaneous determination of 1,2- and 1,3-DAG molecular species. The analytical strategy combined DMG derivatization with reversed-phase chromatographic separation on a C30 stationary phase and fatty-acyl-specific SRM detection. Particular emphasis was placed on achieving effective chromatographic separation of 1,2- and 1,3-DAG regioisomers, enhancing analytical sensitivity, and enabling selective quantification of individual DAG molecular species, including isobaric compounds. The analytical performance of the method was evaluated in mouse liver samples using calibration linearity, limit of detection (LOD), overall method recovery, and intra-day and inter-day precision.

2. Materials and Methods

2.1 Materials

All deuterated internal standards including the 1,2-DAG species (1,2–15:0/18:1-d7-DAG, 1,2–17:0/14:1-d5-DAG, 1,2–17:0/18:1-d5-DAG) and 1,3-DAG species (1,3–14:0/14:0-d5-DAG, 1,3–16:1/16:1-d5-DAG, 1,3–17:0/17:0-d5-DAG, 1,3–18:1/18:1-d5-DAG and 1,3–18:0/18:0-d5-DAG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Unlabeled DAG standards used for preparation of calibration curves, representing both 1,2- and 1,3-DAG regioisomers, were purchased from Avanti Polar Lipids, Inc. or Cayman Chemical (Ann Arbor, MI, USA). Individual DAG stock solutions were prepared in dichloromethane/methanol (1:1, v/v) at a concentration of 0.5 mg/mL. The derivatization reagents including DMG hydrochloride, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All solvents used for lipid extraction and sample preparation were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All solvents used for LC separation were LC/MS grade.

2.2. Animal Experiments

Male C57BL/6J mice were obtained from the Center of Experimental Medicine, Medical University of Białystok (Białystok, Poland). The animals were housed under controlled environmental conditions (23°C, 12-h light/12-h dark cycle) with free access to food and water. Mice were fed ad libitum for 12 weeks with either a control low-fat diet (10% kcal from fat; Research Diets Inc., D12450J) or a high-fat diet (HFD; 60% kcal from fat; Research Diets Inc., D12492). After 12 weeks, the mice were euthanized by intraperitoneal administration of pentobarbital at a dose of 250 mg/kg body weight, followed by cervical dislocation to ensure death. The livers were rapidly excised, perfused with ice-cold phosphate-buffered saline (PBS), and immediately freeze-clamped in liquid nitrogen. The samples were stored at – 80°C until lipid extraction and analysis. The experimental protocol was approved by the Local Ethical Committee for Animal Experiments in Olsztyn, Poland (approval no. 23/2025). All procedures were performed in accordance with applicable guidelines and regulations governing the use of animals in research.

2.3. Extraction, Cleanup and Derivatization of Diacylglycerols

Liver tissue samples (20 mg) were pulverized under liquid nitrogen and homogenized in 200 µL of ice-cold phosphate-buffered saline (PBS, pH 7.4). An aliquot of each homogenate was transferred to a glass tube and spiked with 50 µL of a mixture of deuterated internal standards (40 ng of each), representing both 1,2- and 1,3- DAG regioisomers: 1,2–15:0/18:1-d7-DAG, 1,2–17:0/14:1-d5-DAG, 1,2–17:0/18:1-d5-DAG, 1,3–14:0/14:0-d5-DAG, 1,3–16:1/16:1-d5-DAG, 1,3–17:0/17:0-d5-DAG, 1,3–18:1/18:1-d5-DAG and 1,3–18:0/18:0-d5-DAG. The internal standards were selected to cover a broad range of acyl chain lengths and degrees of unsaturation representative of the analyzed DAG species. Samples were vortex-mixed before lipid extraction. Total lipids were extracted using a modified Bligh-Dyer procedure. Briefly, 600 µL of ice-cold methanol/chloroform (2:1, v/v) containing 0.01% (w/v) butylated hydroxytoluene (BHT) was added to each sample. Samples were vortexed for 2 min, followed by the addition of 200 µL chloroform and 200 µL water and then vortexed for an additional 1 min and centrifuged at 1,000 × g for 5 min to achieve phase separation. The lower organic phase was carefully collected using a glass Pasteur pipette while avoiding contamination of the protein interphase. The remaining aqueous phase was re-extracted with 200 µL chloroform, followed by vortex mixing and centrifugation under the same conditions. The second lower organic phase was collected and combined with the first extract. The pooled organic phase was evaporated to dryness under a gentle stream of nitrogen. To reduce phospholipid content and improve sample cleanliness before derivatization, a cold acetone precipitation step was performed. The dried lipid extract was reconstituted in 150 µL chloroform and sonicated for 2 min to ensure complete dissolution. Subsequently, 3.5 mL of acetone pre-cooled to – 20°C was added and the samples were thoroughly vortexed. Then the samples were incubated at – 20°C for 1 h and then centrifuged at 5,000 × g for 15 min at 4°C. The supernatant was carefully transferred to a clean glass tube without disturbing the pellet and evaporated to dryness under nitrogen. For DMG derivatization, the dried lipid extracts were treated with 8 µL of a solution containing DMG (0.125 M) and DMAP (0.5 M) prepared in anhydrous chloroform, followed by the addition of 4 µL EDC (0.25 M) in anhydrous chloroform. Samples were vortexed for 20 s, gently flushed with nitrogen, tightly capped, and incubated at 45°C for 60 min (9). During incubation, samples were briefly mixed every 15 min. The derivatization reaction was quenched by adding 10 µL methanol to each sample. Subsequently, 180 µL water containing 0.1% formic acid, 300 µL chloroform, and 200 µL methanol were added. Samples were vortexed thoroughly and centrifuged at 10,000 × g for 5 min at 4°C. The upper phase was discarded and the lower organic phase, containing the derivatized DAGs, was collected and evaporated to dryness under nitrogen. The derivatized extracts were reconstituted in 100 µL of a solvent mixture consisting of 45% mobile phase A and 55% mobile phase B (v/v). Samples were centrifuged at 5,000 × g for 5 min, and the resulting supernatants were transferred to autosampler vials for UHPLC–MS/MS analysis.

2.4. UHPLC/MS/MS conditions

Chromatographic separation was performed using a Thermo Scientific Vanquish UHPLC system coupled to a Thermo Scientific TSQ Altis Plus triple quadrupole mass spectrometer. DMG-derivatized DAG were separated on a Thermo Scientific Accucore C30 column (100 × 2.1 mm, 2.6 µm particle size) maintained at 60°C. The mobile phases were as follows: mobile phase A, water/methanol (4:6, v/v) containing 2 mM ammonium acetate; and mobile phase B, 2-propanol/acetonitrile (9:1, v/v) containing 0.1% formic acid. The flow rate was 0.3 mL/min. The gradient program was as follows: 55% B from 0 to 1 min; 60% B at 4 min, maintained until 6 min; 70% B from 6 to 7 min, maintained until 8 min; 75% B from 8 to 10 min; 95% B from 10 to 12.5 min, maintained until 13.5 min; and 55% B from 13.5 to 14 min, maintained until 15 min. The injection volume was 1 µL, and the autosampler temperature was maintained at 4°C. The mass spectrometer was operated in positive electrospray ionization (ESI+) mode. DMG-derivatized DAGs were detected using selected reaction monitoring (SRM). The method was validated using eight DAG molecular species, analyzed as both 1,2- and 1,3-regioisomers: C14:0/14:0, C18:2/18:2, C16:0/16:0, C18:1/18:1, C18:0/18:2, C16:0/18:0, C16:0/18:1, and C18:0/18:0. Separate eight-point calibration curves ranging from 1.56 to 200 ng were prepared for each regioisomer using analyte-to-internal-standard peak-area ratios. Collision-induced dissociation was performed using argon as the collision gas at a pressure of 1.5 mTorr. The resolution for both Q1 and Q3 was set to 0.7 FWHM. The source parameters were as follows: sheath gas: 50 arbitrary units (arb), auxiliary gas: 10 arb, sweep gas: 1 arb, ion transfer tube temperature: 325°C, vaporizer temperature: 350°C, spray voltage: 4100 V. To improve the selectivity of DAG molecular-species identification, particularly for isobaric species, SRM transitions were selected based on fatty-acyl-derived product ions rather than solely on the characteristic 103-Da neutral loss of the DMG moiety. The selected product ions corresponded to characteristic fatty-acyl-derived

fragments $[RCO - H_2O]^+$) and provided structural information on the acyl composition of the DAG molecular species. For example, C18:1/C18:1-DAG produced a characteristic product ion at m/z 265.133, whereas C18:0/C18:2-DAG generated product ions at m/z 267.133 and 263.133, corresponding to the respective fatty-acyl-derived fragments. Monitoring fatty-acyl-specific product ions therefore provided additional selectivity compared with monitoring the characteristic DMG-derived neutral loss alone. The optimized SRM transitions, collision energies, and retention times are summarized in Table 1

Table 1
Table 1. SRM transitions, collision energies, and retention times of DMG-derivatized 1,2- and 1,3-DAG molecular species.

DAG species	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	Retention time (min)
1,2-C14:0/14:0	598.50	211.133	27.5	2.57
1,3-C14:0/14:0	598.50	211.133	27.5	2.87
1,2-C18:2/18:2	702.45	263.133	30	3.59
1,3-C18:2/18:2	702.45	263.133	30	4.03
1,2-C16:0/16:0	654.55	239.133	28.5	4.69
1,3-C16:0/16:0	654.55	239.133	28.5	5.23
1,2-C18:1/18:1	706.55	265.133	30.5	5.22
1,3-C18:1/18:1	706.55	265.133	30.5	5.82
1,2-C18:0/18:2	706.55	267.133	30	5.51
1,3-C18:0/18:2	706.55	267.133	30	6.11
1,2-C16:0/18:0	682.55	239.133	30	6.23
1,3-C16:0/18:0	682.55	239.133	30	6.98
1,2-C16:0/18:1	680.55	265.133	30	4.97
1,3-C16:0/18:1	680.55	265.133	30	5.52
1,2-C18:0/18:0	710.55	267.133	30	8.02
1,3-C18:0/18:0	710.55	267.133	30	8.51

2.5. Method validation

2.5.1. Linearity.

Calibration curves were prepared at eight concentration levels ranging from 1.56 to 200 ng for each 1,2- and 1,3-DAG regioisomer. Calibration was performed by plotting the analyte-to-internal standard peak area ratio against the corresponding amount of analyte using linear regression. Method linearity was assessed based on the coefficient of determination (R^2). The results are summarized in Table 2.

Table 2
Validation parameters of the UHPLC-MS/MS method for the quantification of 1,2- and 1,3-DAG molecular species in mouse liver.

DAG	R ²	LOD (pg/injection)	Recovery (low) (%)	Recovery (high) (%)	Intra-day precision RSD (%)		Inter-day precision mean RSD ± SD (%)	
					Control	HFD	Control	HFD
1,2 14:0/14:0	0.9977	0.214	98.23 ± 7.84	101.12 ± 2.38	5.83 ± 0.50	5.13 ± 1.78	7.62 ± 0.94	8.97 ± 1.27
1,2 16:0/18:0	0.9980	0.371	100.70 ± 5.40	100.37 ± 5.73	5.56 ± 1.16	4.42 ± 0.52	3.63 ± 0.99	4.66 ± 1.15
1,2 16:0/16:0	0.9972	0.309	94.60 ± 7.58	101.65 ± 1.35	3.53 ± 0.40	4.77 ± 0.45	1.78 ± 1.57	5.96 ± 3.85
1,2 16:0/18:1	0.9981	0.836	102.31 ± 3.85	103.99 ± 4.90	4.60 ± 0.40	3.46 ± 0.32	2.47 ± 1.20	2.68 ± 0.53
1,2 18:0/18:0	0.9986	0.294	99.93 ± 6.98	100.84 ± 3.23	6.07 ± 0.78	5.59 ± 0.92	8.67 ± 2.52	8.60 ± 0.86
1,2 18:0/18:2	0.9989	1.430	96.33 ± 6.78	107.54 ± 5.94	3.08 ± 0.73	4.96 ± 0.25	3.62 ± 2.79	7.22 ± 2.70
1,2 18:1/18:1	0.9980	0.469	103.85 ± 2.91	101.34 ± 6.45	4.57 ± 0.58	3.92 ± 0.67	1.84 ± 0.95	2.74 ± 1.45
1,2 18:2/18:2	0.9982	0.287	101.18 ± 9.09	101.10 ± 4.30	5.49 ± 0.89	4.09 ± 0.46	3.39 ± 2.23	4.17 ± 1.33
1,3 14:0/14:0	0.9997	0.375	98.49 ± 5.11	99.27 ± 3.08	6.69 ± 0.96	5.85 ± 1.12	7.57 ± 2.16	8.22 ± 0.87
1,3 16:0/18:0	0.9969	0.345	97.44 ± 5.80	103.25 ± 6.14	5.95 ± 0.82	4.84 ± 1.19	4.05 ± 1.24	6.97 ± 0.65
1,3 16:0/16:0	0.9986	0.586	98.56 ± 4.70	100.31 ± 4.49	5.07 ± 0.57	5.00 ± 0.76	4.00 ± 3.34	6.69 ± 2.22
1,3 16:0/18:1	0.9992	0.740	96.17 ± 6.50	103.62 ± 7.09	4.88 ± 0.49	3.60 ± 0.61	3.82 ± 1.28	3.95 ± 1.71
1,3 18:0/18:0	0.9994	0.872	102.20 ± 5.07	101.30 ± 5.57	4.56 ± 1.33	7.04 ± 0.67	8.53 ± 2.72	7.37 ± 1.02
1,3 18:0/18:2	0.9981	1.612	105.85 ± 6.57	103.54 ± 3.72	5.80 ± 0.48	5.48 ± 0.92	6.01 ± 1.10	7.79 ± 0.65
1,3 18:1/18:1	0.9973	1.130	102.39 ± 4.24	102.61 ± 4.57	4.74 ± 0.68	3.93 ± 0.49	2.63 ± 1.17	3.77 ± 1.41
1,3 18:2/18:2	0.9983	1.786	104.30 ± 1.91	99.56 ± 4.20	6.65 ± 0.79	5.66 ± 0.76	6.54 ± 0.99	7.34 ± 1.84
Values are presented as mean ± SD. Recovery was assessed at low and high spike levels using liver samples from control and HFD-fed mice. Intra-day and inter-day precision were evaluated separately for control and HFD groups								

2.5.2. Limits of detection

The limit of detection (LOD) was determined based on signal-to-noise (S/N) measurements. The LOD was defined as the lowest amount of analyte producing an S/N ratio of ≥ 3. LOD values for the individual DAG molecular species are presented in Table 2.

2.5.3. Overall method recovery

Overall method recovery was evaluated using biological matrices obtained from six individual mouse liver samples, including three samples from control diet-fed mice and three samples from HFD-fed mice. For each liver sample, three 200-μL aliquots of homogenate were prepared. All aliquots were initially spiked with 50 μL of the deuterated internal-standard mixture (40 ng of each internal standard). One aliquot remained unspiked with the target analytes and served as the endogenous control. The second and third aliquots were additionally spiked with 12.5 μL and 100 μL, respectively, of the mixed analyte stock solution used for calibration curves preparation, corresponding to the low and high spike levels, respectively. All samples were subsequently subjected to the same extraction, cleanup, DMG derivatization, and UHPLC-MS/MS procedures. The concentrations of the target DAG molecular species were determined using the corresponding calibration curves. Overall method recovery was calculated by comparing the measured increase in analyte concentration in the spiked samples with the theoretical amount of analyte added during the spiking procedure.

$$\text{Recovery (\%)} = \left[\frac{C_{\text{spiked}} - C_{\text{unspiked}}}{C_{\text{added}}} \right] \times 100$$

where C spiked is the measured analyte amount in the spiked sample, C unspiked is the measured endogenous analyte amount in the corresponding unspiked sample, and C added is the theoretical amount of analyte added by spiking. Recovery was evaluated at both the low and high spike levels and expressed as the mean ± SD.

2.5.4. Intra-day and inter-day precision

Method precision was assessed as intra-day and inter-day precision using six individual mouse liver samples, including three samples from control diet-fed mice and three samples from HFD-fed mice. For intra-day precision, two independent 20-mg aliquots from each liver sample were processed separately through the complete analytical workflow, including lipid extraction, sample cleanup, DMG derivatization, and UHPLC-MS/MS analysis, on the same day. The RSD (%) was calculated from the two replicate measurements obtained for each liver sample. The resulting RSD values were then summarized as mean RSD $\pm$ SD for the three animals within each dietary group. For inter-day precision, independent aliquots of the same liver samples were processed and analyzed on three separate days using the same analytical procedure. The RSD (%) was calculated for each individual liver sample from the three measurements obtained on the respective days. Results were expressed as mean RSD $\pm$ SD for the three animals within each dietary group.

3. Results and Discussion

3.1. Optimization of Chromatographic Separation of 1,2- and 1,3-DAG Regioisomers

3.2. Selective Detection of Isobaric DAG Species Using Fatty Acyl-Specific Product Ions

An additional analytical challenge in the mass spectrometric analysis of DAGs is the discrimination of isobaric molecular species. For example, C18:1/C18:1-DAG and C18:0/C18:2-DAG have the same molecular formula ($C_{39}H_{72}O_5$) and therefore generate the same precursor ion after DMG derivatization. Consequently, monitoring only the characteristic 103-Da neutral loss of the DMG moiety does not provide sufficient structural information to distinguish these molecular species. To overcome this limitation, we developed an SRM strategy based on fatty-acyl-derived product ions. Instead of monitoring solely the characteristic neutral loss of the DMG moiety, product ions derived from the fatty-acyl chains were monitored to provide additional structural information. For example, C18:1/C18:1-DAG generated a characteristic product ion at m/z 265.133, corresponding to the C18:1-derived fragment, whereas C18:0/C18:2-DAG generated product ions at m/z 267.133 and m/z 263.133, corresponding to the C18:0- and C18:2-derived fragments, respectively. Thus, monitoring fatty-acyl-specific product ions provided additional molecular-species selectivity beyond that obtained from the DMG neutral-loss transition alone. Several DAG molecular species exhibited closely spaced retention times, resulting in partially overlapping chromatographic peaks (Fig. 2). In such cases, the use of fatty-acyl-specific SRM transitions provided an additional level of selectivity and facilitated reliable detection and integration of individual molecular species. Taken together, the combination of C30-based reversed-phase chromatography, DMG derivatization, and fatty-acyl-specific SRM detection enabled selective determination of individual DAG molecular species, including isobaric compounds that could not be reliably distinguished using DMG neutral-loss detection alone.

3.3. Method validation

3.3.1. Linearity

Excellent linearity was obtained over the investigated calibration range of 1.56–200 ng for all analyzed DAG molecular species. The coefficients of determination (R^2) ranged from 0.9969 to 0.9997 for the eight 1,2- and eight 1,3-DAG molecular species (Table 2). Representative calibration curves for 1,2- and 1,3-DAG molecular species are shown in Fig. 3.

The high R^2 values demonstrate a strong linear relationship between the analyte-to-internal-standard peak-area ratio and the corresponding analyte amount over the investigated concentration range.

3.3.2. Limit of detection

The LOD values ranged from 0.214 to 1.786 pg/injection across the investigated DAG molecular species (Table 2), demonstrating the high sensitivity of the developed UHPLC-MS/MS method. Differences in LOD among individual molecular species may reflect differences in ionization efficiency, chromatographic behavior, and MS/MS response associated with their specific fatty-acyl composition.

3.3.3. Overall method recovery

Overall method recovery was evaluated using six individual mouse liver samples representing both control and HFD-fed animals. Recovery was assessed at two spike levels to evaluate the performance of the complete analytical workflow, including extraction, sample cleanup, derivatization, and instrumental analysis. At the low spike level, recovery values ranged from 94.60% to 105.85%, whereas recovery at the high spike level ranged from 99.27% to 107.54% across the investigated DAG molecular species (Table 2). The relatively narrow recovery ranges indicate consistent performance of the overall analytical procedure. Importantly, the use of individual liver samples rather than a pooled biological matrix allowed the recovery assessment to account for inter-individual differences in endogenous lipid composition. Overall, the recovery results demonstrate that the extraction, acetone precipitation, DMG derivatization, and subsequent UHPLC-MS/MS analysis provide satisfactory and reproducible recovery of the investigated DAG molecular species from mouse liver samples.

3.3.4. Intra-Day and Inter-Day Precision

The developed method demonstrated good intra-day and inter-day precision for all investigated DAG molecular species (Table 2). Intra-day precision, expressed as RSD, ranged from 3.08% to 7.04% across the investigated molecular species. Inter-day precision ranged from 1.78% to 8.97%. These results indicate good repeatability of the complete analytical procedure within a single day and satisfactory reproducibility across different analytical days. For intra-day precision, two independently processed aliquots from each individual liver sample were analyzed on the same day. This approach incorporated variability

associated with sample preparation, lipid extraction, sample cleanup, DMG derivatization, and instrumental analysis into the precision assessment. For inter-day precision, independent aliquots of the same liver samples were processed and analyzed on three separate days using the same analytical procedure, thereby allowing assessment of day-to-day variability across the complete analytical workflow. Precision was evaluated separately for control and HFD-fed animals. The low RSD values observed in both dietary groups indicate consistent analytical performance across the investigated biological matrices.

4. Conclusions

The present study describes the development and validation of a UHPLC-MS/MS method for the selective determination of individual 1,2- and 1,3-DAG molecular species in biological samples. The analytical workflow combines DMG derivatization, reversed-phase chromatographic separation on a C30 stationary phase, and fatty-acyl-specific SRM detection. DMG derivatization enhances DAG ionization under positive ESI conditions while simultaneously blocking the free hydroxyl group and thereby reducing the potential for acyl migration during the analytical workflow. The use of C30-based reversed-phase chromatography enabled effective separation of the investigated 1,2- and 1,3-DAG regioisomers within a 15-min analytical run. Furthermore, fatty-acyl-specific product ions provided additional molecular-species selectivity and enabled differentiation of isobaric DAG species that could not be reliably distinguished using the characteristic DMG neutral-loss transition alone. The developed method demonstrated excellent linearity over the investigated concentration range, low limits of detection, satisfactory overall method recovery, and good intra-day and inter-day precision. Importantly, method performance was evaluated using individual mouse liver samples representing both control and HFD-fed animals, providing evidence of the applicability of the method to complex biological matrices. Overall, the developed analytical platform provides a sensitive, selective, and reproducible approach for the determination of individual 1,2- and 1,3-DAG molecular species. The method may therefore be useful for investigating regioisomer-specific alterations in DAG metabolism and signaling in physiological and pathological conditions, including metabolic disease.

Declarations

Conflict of interest

The authors declare no conflict of interest.

Funding

The study was supported by Medical University of Bialystok (grant no: B.SUB.26.501)

Author Contribution

P. Z. - investigation, data curation, writing—review and editing, A. U. B-Z – conceptualization, methodology, investigation, resources, writing - original draft, writing—review and editing, supervision, funding acquisition;

Data Availability

The raw data generated during the current study are available at the following link: <https://doi.org/10.5281/zenodo.22085726>

References

1. Eichmann, T. O. & Lass, A. DAG tales: the multiple faces of diacylglycerol—stereochemistry, metabolism, and signaling. *Cell. Mol. Life Sci.* **72**, 3931–3952 (2015).
2. Liu, J. et al. DGKs in lipid signaling and disease intervention: structural basis, pathological mechanisms, and emerging therapeutic strategies. *Cell. Mol. Biol. Lett.* **30**, 129 (2025).
3. Heinonen, S., Lautala, S., Koivuniemi, A. & Bunker, A. Insights into the behavior of unsaturated diacylglycerols in mixed lipid bilayers in relation to protein kinase C activation-A molecular dynamics simulation study. *Biochim. Biophys. Acta Biomembr.* **1864**, 183961 (2022).
4. Hu, T., Chen, J., Tan, C. P. & Wang, Y. Differential digestive fate of palm oil and palm-based diacylglycerol oil: linking physicochemical properties, oxidative stability and pancreatic lipase specificity. *Food Chem.* **518**, 149547 (2026).
5. Blachnio-Zabielska, A. U., Zabielski, P. & Jensen, M. D. Intramyocellular diacylglycerol concentrations and [U-13C]palmitate isotopic enrichment measured by LC/MS/MS. *J. Lipid Res.* **54**, 1705–1711 (2013).
6. McAnoy, A. M., Wu, C. C. & Murphy, R. C. Direct qualitative analysis of triacylglycerols by electrospray mass spectrometry using a linear ion trap. *J. Am. Soc. Mass. Spectrom.* **16**, 1498–1509 (2005).
7. Piyatheerawong, W., Iwasaki, Y. & Yamane, T. Direct separation of regio- and enantiomeric isomers of diacylglycerols by a tandem column high-performance liquid chromatography. *J. Chromatogr. A.* **1068**, 243–248 (2005).
8. Wang, M., Hayakawa, J., Yang, K. & Han, X. Characterization and quantification of diacylglycerol species in biological extracts after one-step derivatization: a shotgun lipidomics approach. *Anal. Chem.* **86**, 2146–2155 (2014).

9. Liu, Y. D., Liu, H. J. & Gong, G. W. Monitoring diacylglycerols in biofluids by non-isotopically paired charge derivatization combined with LC-MS/MS. *Front. Chem.* **10**, 1062118 (2022).
10. Okumura, K. et al. 1,2-diacylglycerol content and its fatty acid composition in thoracic aorta of diabetic rats. *Diabetes* **40**, 820–824 (1991).
11. McKenna, C. F. et al. Intramuscular diacylglycerol accumulates with acute hyperinsulinemia in insulin-resistant phenotypes. *Am. J. Physiol. Endocrinol. Metab.* **327**, E183–E193 (2024).
12. Lyu, K. et al. and G. I. Shulman. 2020. A Membrane-Bound Diacylglycerol Species Induces PKC ϵ -Mediated Hepatic Insulin Resistance. *Cell Metab* **32**: 654–664e655 .
13. Majumder, D. et al. Comparative Analysis of Translational Innovations in Sn-2 and Sn-1,3-Specific Lipase-Mediated Regioselective Catalysis for Enhanced Diacylglycerol Production. *J. Agric. Food Chem.* **74**, 15–39 (2026).
14. Authors' Contributions P. Z. - investigation, data curation, writing—review and editing, A. U. B-Z – conceptualization, methodology, investigation, resources, writing - original draft, writing—review and editing, supervision, funding acquisition.

Figures

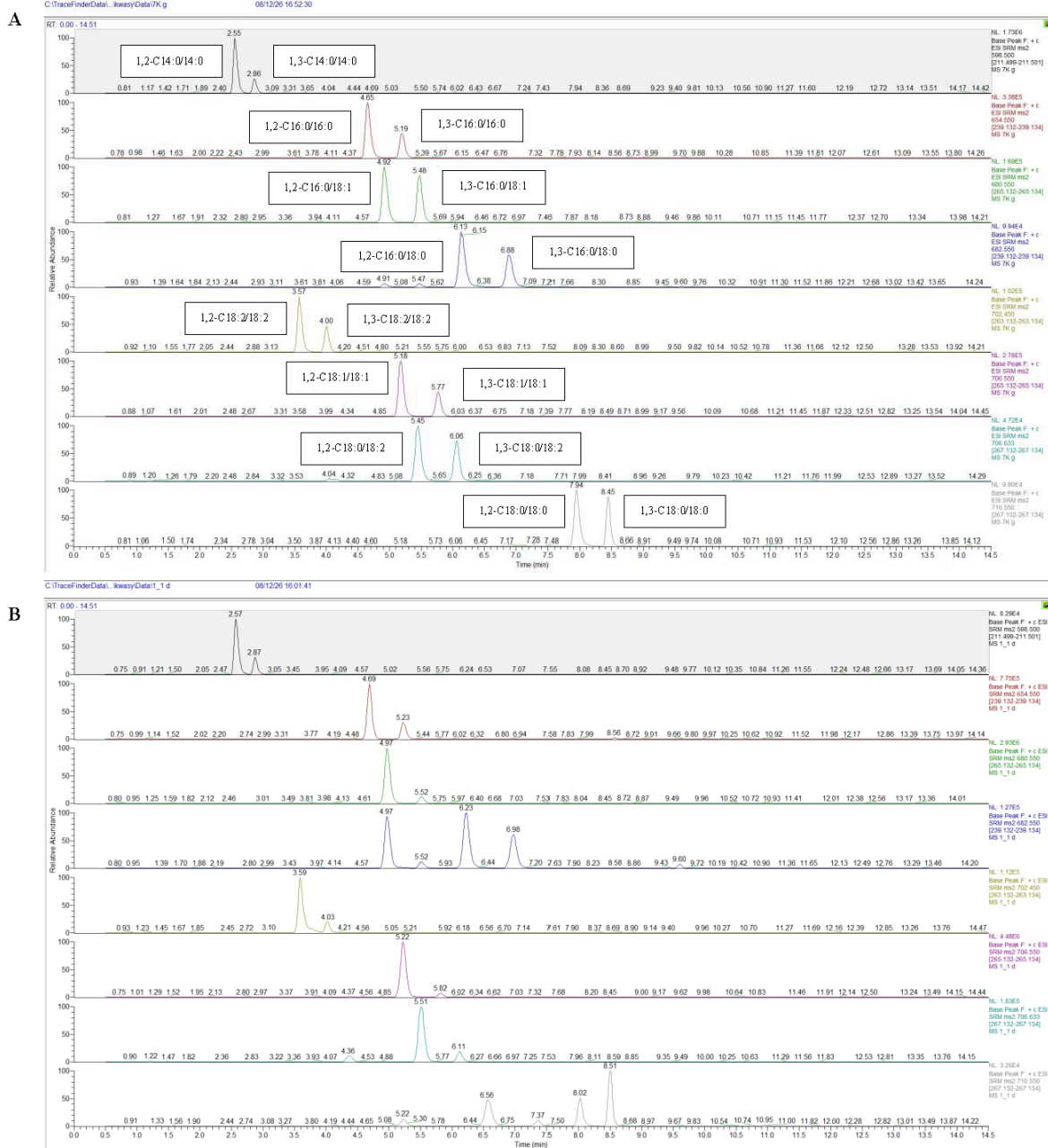


Figure 1

Figure 1

Chromatographic separation of 1,2- and 1,3-DAG regioisomers under optimized UHPLC-MS/MS conditions. Representative chromatographic profiles of DMG-derivatized 1,2- and 1,3-DAG molecular species in a calibration standard (A) and mouse liver extract (B), obtained using the Thermo Scientific Accucore C30 column. The optimized chromatographic conditions enabled separation of the investigated DAG regioisomers within a 15-min analytical run.

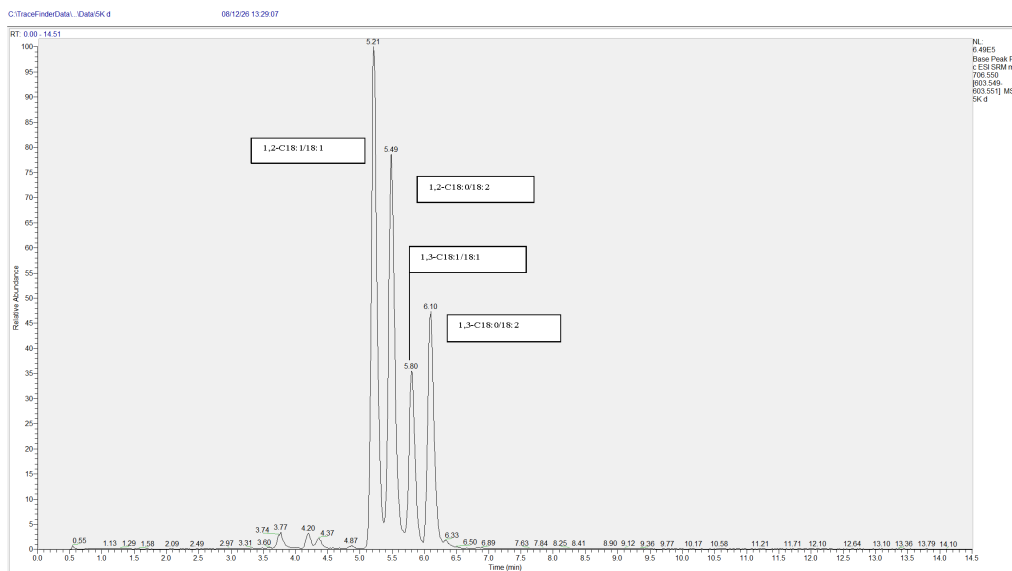


Figure 2

Figure 2

Limited chromatographic resolution of isobaric DAG molecular species. Representative chromatographic profiles of C18:1/C18:1-DAG and C18:0/C18:2-DAG, which have the same elemental composition and identical precursor ion masses following DMG derivatization. Detection based solely on the characteristic 103-Da neutral loss of the DMG moiety does not provide sufficient selectivity for reliable discrimination of these molecular species.

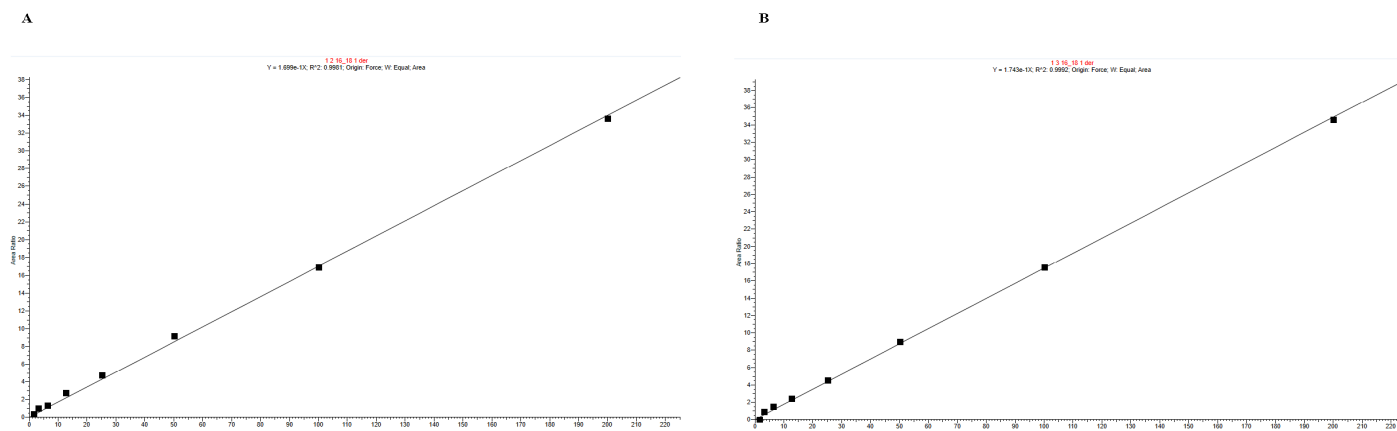


Figure 3

Figure 3

Representative calibration curves for DMG-derivatized DAG molecular species. (A) Representative calibration curve for 1,2-C16:0/18:1-DAG. (B) Representative calibration curve for 1,3-C16:0/18:1-DAG. Calibration curves were generated by plotting the analyte-to-internal-standard peak-area ratio against the corresponding analyte amount over the range of 1.56–200 ng.