A SHOTGUN LIPIDOMICS APPROACH TO STABILIZE THE REGIOSPECIFICITY OF MONOGLYCERIDES USING A FACILE LOW-TEMPERATURE DERIVATIZATION ENABLING THEIR DEFINITIVE IDENTIFICATION AND QUANTITATION

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Supporting Information

Contents

Supplemental Experimental Materials and Methods:

Reagents. All reagents were purchased commercially and utilized without further purification. MG isomers including 1-LG, 2-LG, 1-AG, 2-AG, 2-OG, and 2-PG were purchased from Cayman Chemical (Ann Arbor, MI). 1-OG and 1-stearoyl glycerol were obtained from Sigma-Aldrich (St. Louis, MO). 1-O-hexadecyl-*sn*-glycerol was obtained from Avanti Polar Lipids (Alabaster, AL). The *sn*-1 MG solutions were prepared in methanol, incubated for 30 min at room temperature, and evaporated under N_2 stream prior to resuspension in acetonitrile for storage in -80°C. The *sn*-2 MG solutions were prepared in acetonitrile containing 0.2 M acetic acid and stored in -80°C. The other MG species (predominately *sn*-1 isomers) were from Nu-Chek PREP, INC (Elysian, MN). Stable isotope labeled 7,7,8,8-d_4-16:0 FA was purchased from Cambridge Isotope Laboratory Inc. (Andover, MA). Acetic anhydride, butyric anhydride, hexanoic anhydride, acetic acid, formic acid, 4-(dimethylamino) pyridine (DMAP) and methyl *tert*-butyl ether (MTBE) were purchased from Sigma-Aldrich (St. Louis, MO). All other HPLC grade solvents were obtained from Burdick and Jackson (Honeywell International Inc., Muskegon, MI). Optima™ LC/MS grade water was purchased from Fisher Scientific (St. Peters, MO).

Synthesis of vinyl ether monoglycerides. Vinyl ether diglyceride 1-O-1'-(Z)-hexadecenyl-2-oleoyl-*sn*-glycerol (p16:0-18:1) was synthesized from commercially available plasmenylcholine 1-O-1'-(Z)-hexadecenyl-2-oleoyl-*sn*-glycero-3-phosphocholine by phospholipase C-catalyzed phosphocholine headgroup hydrolysis as described previously.[1] The resultant p16:0-18:1 vinyl ether diglyceride was then subjected to base hydrolysis to hydrolyze the *sn*-2 ester bond while the *sn*-1 vinyl ether bond remained intact.[2] Briefly, to the dried lipid residue of 16:0p-18:1 vinyl ether diglyceride, 200µL of Lithium methoxide (1M) in methanol was added, followed by bath sonication for 2 min. The solution was incubated at 4°C for 2 hr. The reaction was quenched by adding 2 mL of aqueous acetic acid solution (1.2%, v/v) followed by pH adjustment to 4-5 using acetic acid if needed. Two mL of hexane was then added. After vortexing and centrifugation, the lower aqueous layer was carefully removed without contamination from interface and saved on ice. To the remaining hexane layer, 2 mL of methanol was added. After vortexing and centrifugation, the lower methanol layer was removed and combined with the above aqueous layer. To the mixture, 2 mL of chloroform was added. After vortexing and centrifugation, the lower chloroform layer was removed, dried under N_2 and stored in -80°C until use within one week.

Extraction of monoglycerides from biological samples. Four-month old wild type (WT, C57BL/6J) male mice were purchased from Jackson Laboratories (Bar Harbor, ME). The animals were sacrificed by cervical dislocation and tissues (brain, heart, liver, and white adipose) quickly harvested according to a protocol approved by the Washington University Animal Studies committee. Heart tissue was cut in half and quickly rinsed with cold 10% aqueous phosphate buffered saline (pH 7.4) and blotted to remove blood before freezing. All the other tissues were immediately frozen in liquid nitrogen. Frozen tissues were then pulverized into fine wafers before extraction. During sample extraction, all the steps were carried out at 4°C or below to prevent isomerization or degradation of the MG molecules. Each frozen tissue sample (about 20 mg or above) was weighed into a screw top glass culture tube; and two mL of ice-cold MTBE was added immediately. The sample was incubated at -20°C for 20 min before adding MG internal standard (1-19:1 MG in MTBE) as indicated. After 5-min sonication in an ice water bath, the sample was shaken slowly at 4°C for 30 min. The solvent was removed and stored at -20°C while the tissue residue was repeatedly extracted. Combined extracts were pulse centrifuged and the supernatant was removed to a fresh tube as MTBE extract.

From the MTBE extract, MGs were next enriched in acetonitrile[3] and separated from other non-polar lipids by liquid/liquid partitioning using hexane *vs.* acetonitrile containing 0.2 M acetic acid to prevent isomerization. Briefly, acetonitrile-saturated hexane (*i.e.*, hexane$_A$) and hexane-saturated acetonitrile (*i.e.*, acetonitrile$_H$) were prepared by mixing equal volumes of hexane and acetonitrile in the presence of 0.2 M acetic acid followed by centrifugation for phase separation. To the dried MTBE extract on ice, 2 mL of ice-cold acetonitrile$_H$ were added, followed by ice bath sonication for 2 min. One mL of ice-cold hexane$_A$ was then added, followed by 2-min mixing. After 5-min centrifugation, the lower acetonitrile layer was removed and saved on ice. The remaining upper layer was extracted with 1 mL of ice-cold acetonitrile$_H$ once. The combined lower layers were dried under a N_2 stream.

Derivatization of monoglycerides. Derivatization was performed using acetic anhydride as derivatizing agent in the presence of 4-(dimethylamino) pyridine (DMAP) in chloroform at low temperature (-20°C) to prevent isomerization during the reaction. Briefly, to the dried MG acetonitrile residue, 20 µL of freshly prepared 0.2 M acetic anhy-

dride in chloroform was added, followed by vigorous vortexing and 2-min ice bath sonication for dissolution. Twenty μL of 0.2 M DMAP (catalyst) in chloroform was then added, followed by incubation at -20°C for 30 min.

Next, the reaction mixture was extracted with hexane/methanol/water to enrich derivatized MGs into hexane and remove the majority of excess reagents and polar lipids into methanol as previously described[1] with minor modifications. Briefly, methanol-saturated hexane (*i.e.*, $hexane_M$) and hexane-saturated methanol (*i.e.*, $methanol_H$) were prepared by mixing hexane, methanol and water at 1:1:0.1 (v/v/v) followed by centrifugation for phase separation. To the reaction solution, 1.5 mL of $methanol_H$ was added, followed by vortexing for 1 min. One and a half mL of $hexane_M$ was then added and the mixture was vortexed for 1 min. After a 5-min centrifugation, the upper hexane layer was removed and saved on ice. The remaining lower layer was extracted with 1.5 mL of $hexane_M$ once. The combined upper layers were dried under a N_2 stream. The residue was reconstituted in 1:1 (v/v) chloroform/methanol and stored in -20°C before mass spectrometric analysis within one week.

Mass spectrometric analysis. Mass spectrometric analysis of derivatized MG standards and lipid extracts were performed using a TSQ Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an automated nanospray apparatus (*i.e.*, Nanomate HD, Advion BioSciences, Ithaca, NY) for direct infusion as previously described.[4,5] The capillary temperature of the mass spectrometer was set at 150 °C. The spray voltage and gas pressure of the Nanomate were set at 1.2 kV and 0.3 psi, respectively. Samples were diluted in chloroform /methanol/isopropanol (1:2:4, v/v/v) in the presence of 10 mM ammonium acetate prior to mass spectrometric analysis. Tandem mass spectrometric analysis (*i.e.*, product ion scanning or precursor ion scanning) was preformed in the positive ion mode at collision gas pressure of 1 mTorr and indicated collision energies. Instrument control and data acquisition were performed using the Thermo Xcalibur version 2.1 software.

Table S1 Correction factors for quantitation of monoglyceride (MG) regioisomers using tandem mass spectra of precursor ion scanning (PIS) of *m/z* 159 and 1-19:1 MG as internal standard[###]

MG species	PG	OG	LG	AG	MG species of acyl chains of 0-3 double bonds
Normalized relative intensity (%) of *m/z* 159 in product ion spectra of *sn*-1 isomer[#]	47.3 ± 0.2	54.1 ± 0.2	49.0 ± 0.1	58.5 ± 0.1	50.5 ± 0.9
Normalized relative intensity (%) of *m/z* 159 in product ion spectra of *sn*-2 isomer[#]	16.9 ± 0.3	18.6 ± 0.2	20.9 ± 0.1	43.5 ± 0.3	18.8 ± 0.5
Correction factor for *sn*-1 isomer (isomeric composition (% of *sn*-2) = 0)	1	1	1	1.5[##] ± 0.04	1
Correction factor for isomer mixture (isomeric composition (% of *sn*-2) = x)	= 47.3/(47.3-(47.3-16.9)*x)	= 54.1/(54.1-(54.1-18.6)*x)	= 49.0/(49.0-(49.0-20.9)*x)	=1.5* 58.5/(58.5-(58.5-43.5)*x)	= 50.5/(50.5-(50.5-18.8)*x)
Correction factor for *sn*-2 isomer (isomeric composition (% of *sn*-2) = x =1)	2.8	2.9	2.3	2.1	2.7
Correction factor for 1-a16:0 MG	5.1				

[#]: At least four replicates were analyzed for each isomer. It is highly recommended that product ion spectra of authentic isomers are acquired by the mass spectrometer using the same tune and calibration files that will be used for MG analysis of biological samples, from which the normalized relative intensity (%) of *m/z* 159 for *sn*-1 or *sn*-2 isomer is determined as described in the text and used for the correction factor calculations as displayed above.

[##]: Obtained from the difference in relative peak intensity in the spectrum of PIS of *m/z* 159 of an equal molar mixture of 1-AG and 1-19:1 MG (internal standard) (Figure S10A).

[###]: Additional possibilities of ~5% error should be taken into consideration when these correction factors are used for quantitation of MG regioisomers due to the utilization of commercially available *sn*-2 MGs of 95%-98% purity in obtaining these correction factors.

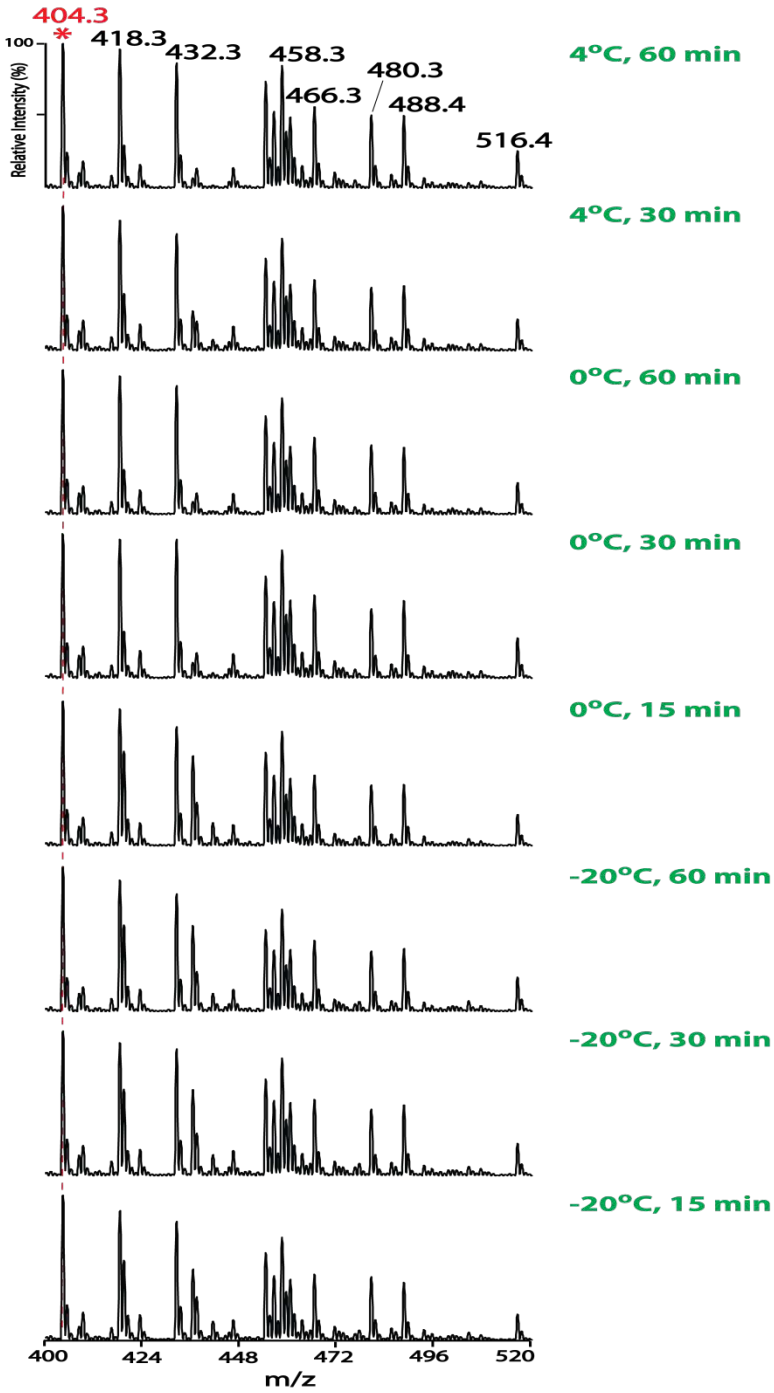


Figure S1. Effects of reaction temperature and time on acetylation of the mixture of various monoglyceride (MG) species. The mixture comprised multiple MG species of various acyl chain lengths (14-22 carbons) and degrees of unsaturation (0-6). The dried lipid residue was derivatized by addition of 20 µL of freshly prepared 0.2 M acetic anhydride in chloroform and 20 µL of 0.2 M DMAP (catalyst) in chloroform, followed by incubation at the indicated reaction temperatures and times. The derivatized MGs were then enriched in hexane fraction via liquid/liquid partitioning using hexane/methanol/water as described in the Experimental Section. Full MS spectra of the hexane fractions were acquired.

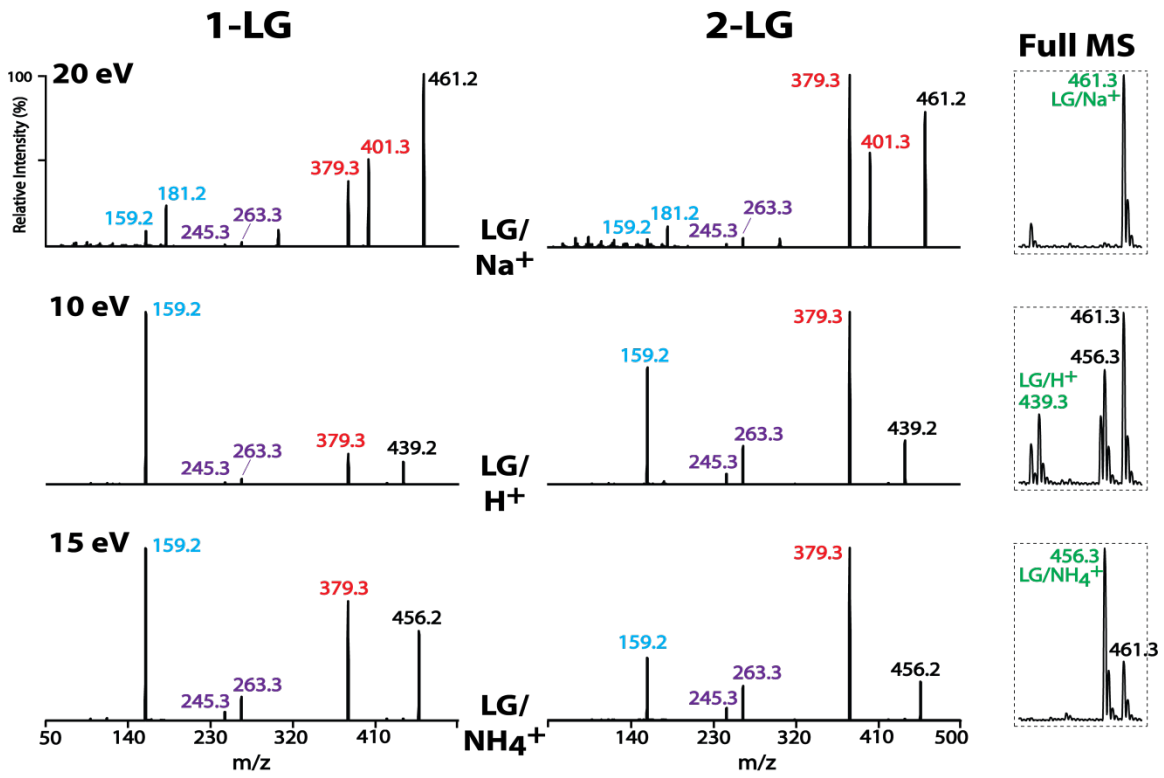


Figure S2. Full MS and tandem MS analysis of different adduct ions of acetic acid (AcA)-derivatized linoleoylglycerol (LG) regioisomers. AcA-derivatized 1-LG and 2-LG were prepared as described in Figure S1. Their Na^+, H^+ and NH_4^+ adduct ions were analyzed by full MS scan and product ion scan at optimized collision energies as indicated. Ionization efficiency was greatest with sodiated and ammoniated diacetyl LGs but relatively weak with protonated diacetyl LGs as displayed in the full MS spectra. Sodiated diacetyl LGs demonstrate considerably less differentiated MS/MS spectra for regioisomers, where the fragment ions (at *m/z* 401 and *m/z* 379) resulting from neutral loss of acetic acid and sodium acetate predominated while the counterpart fragment ions (at m/z 181 and m/z 159) from neutral loss of linoleic acid and linoleic acid sodium salt were present in very low abundance.

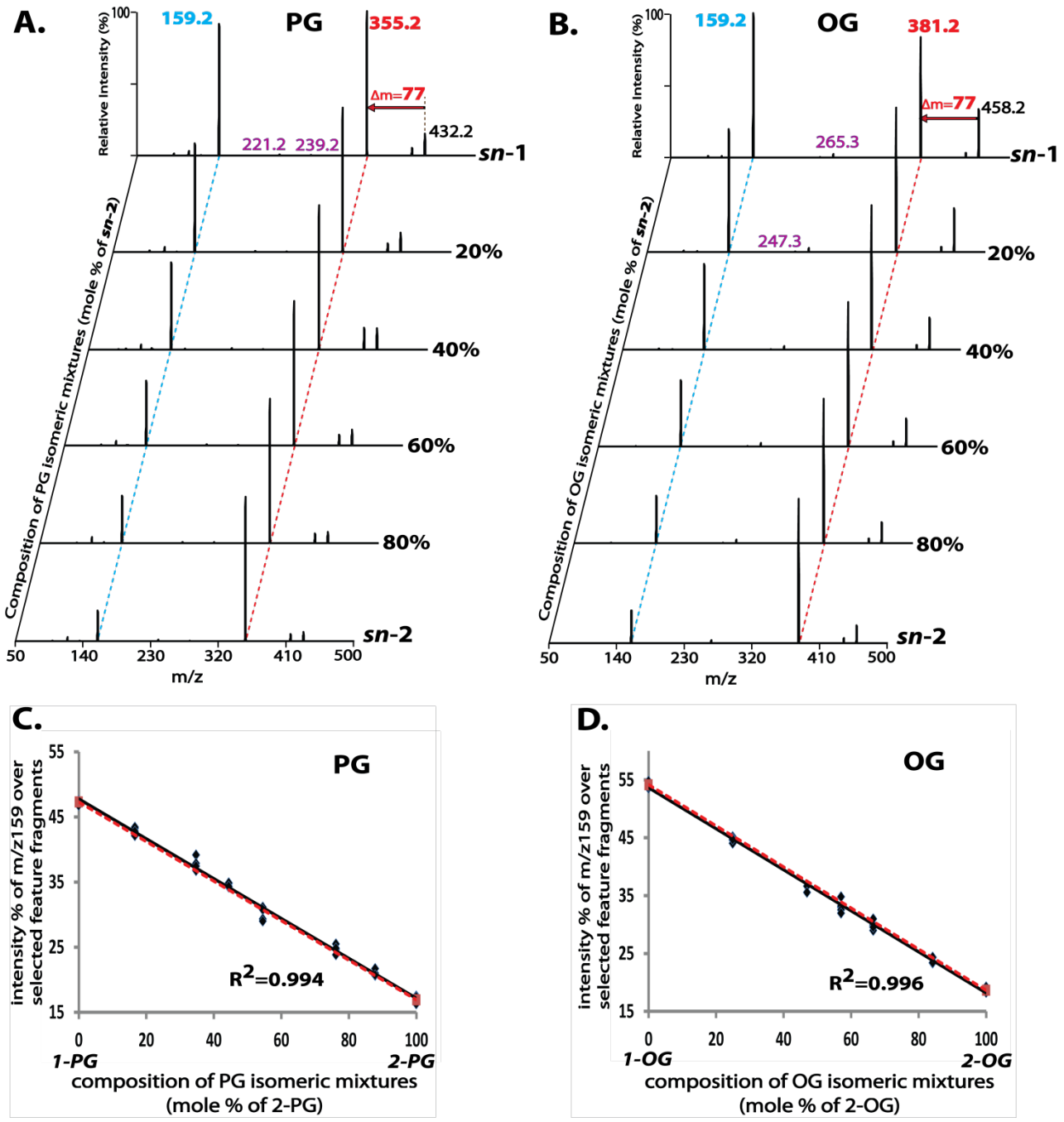
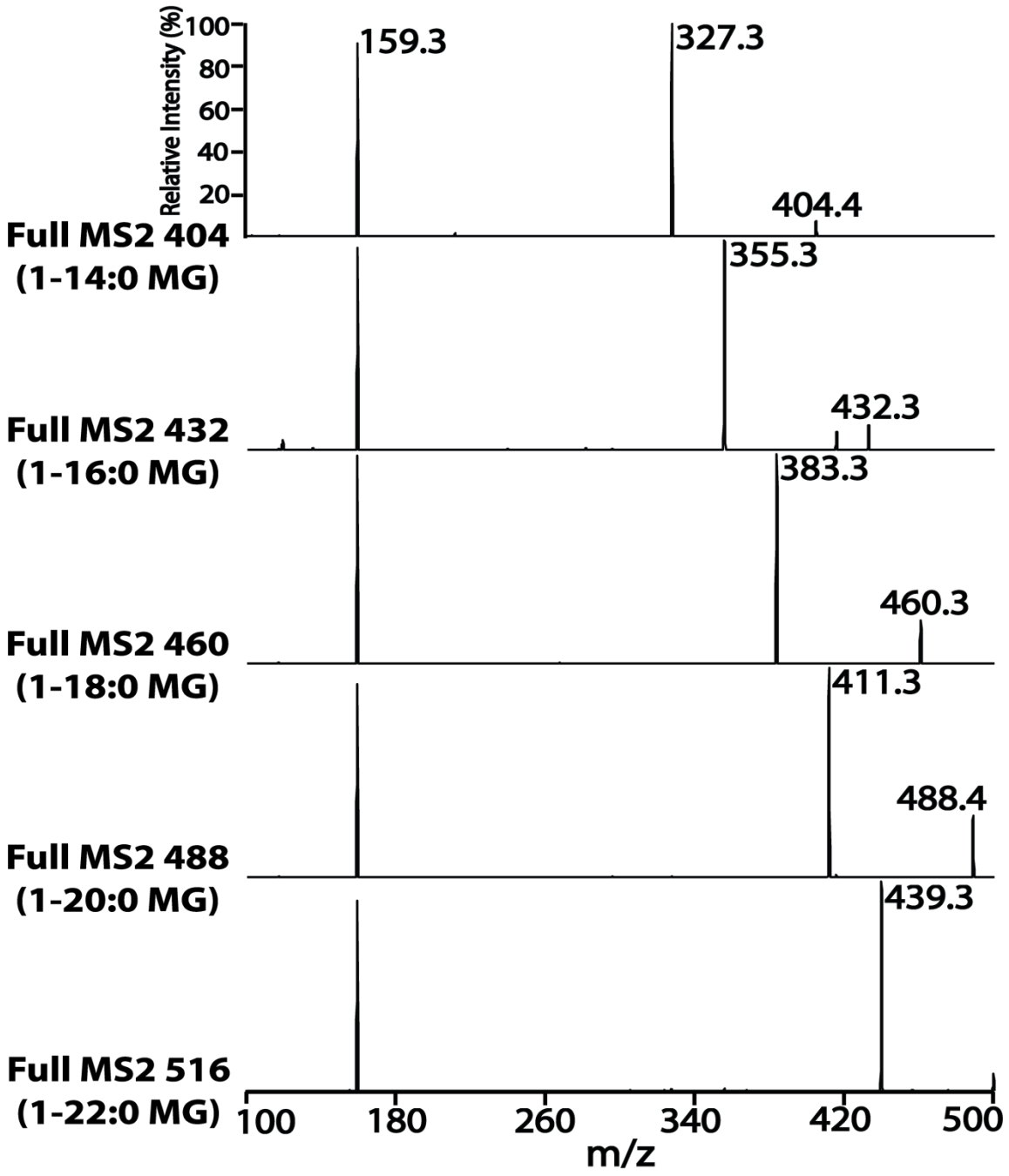
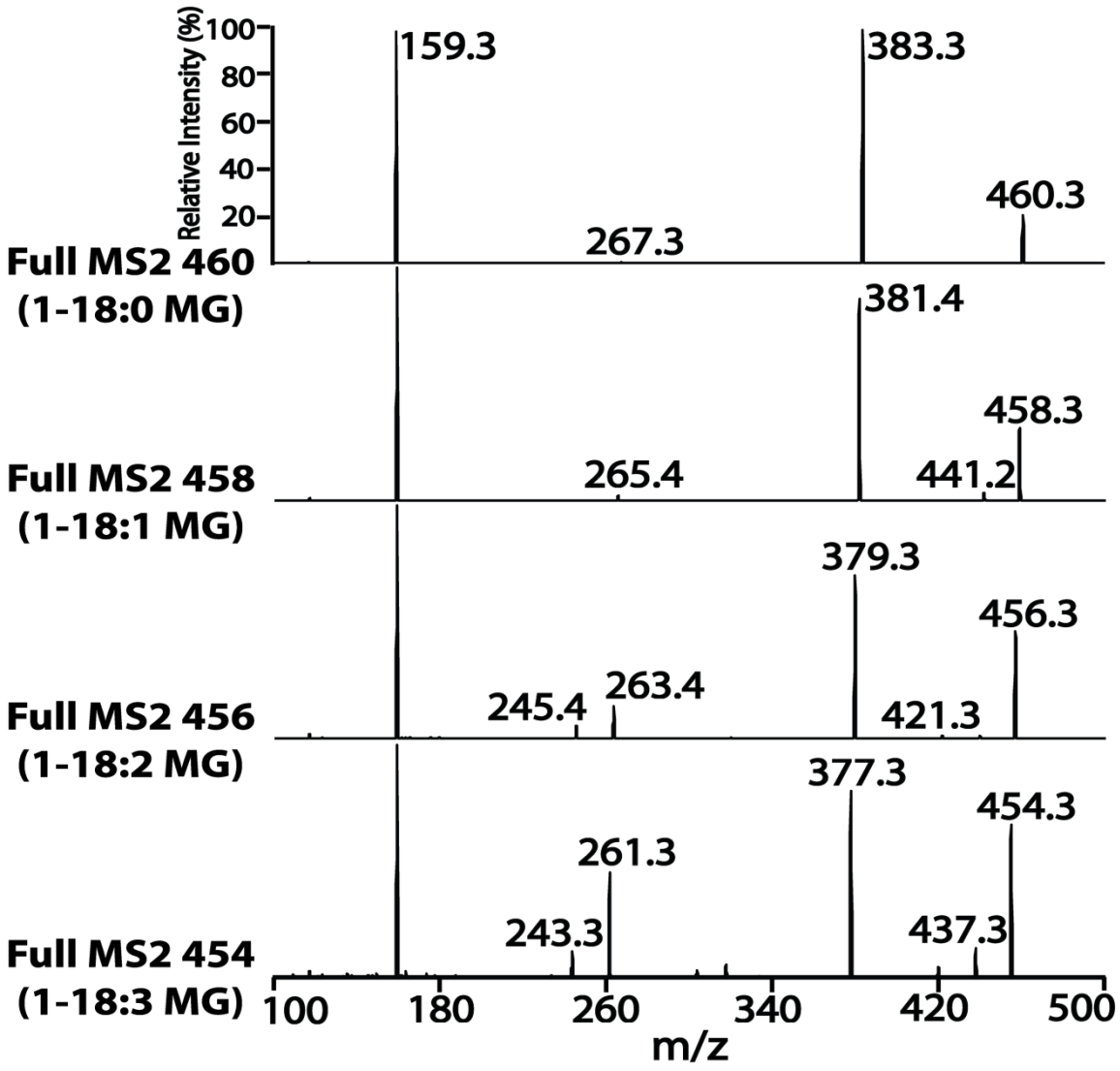


Figure S3. Tandem mass spectra of mixtures of monoglyceride (MG) regioisomers at defined compositions and the linear relationship between their tandem mass spectral features and isomeric compositions. Product ion spectra of mixtures of diacetyl MG regioisomers at defined compositions were acquired in the presence of 10 mM ammonium acetate at a collision energy of 15 eV, and displayed in **(A)** for PG and in **(B)** for OG. The two featured fragment ions (*m/z* 159 and neutral loss of 77 amu) are indicated by the dashed blue and red lines, respectively. The linearity between the tandem mass spectral features (represented by the normalized intensity of the *m/z* 159 fragment as described in the text) and the molar compositions of isomeric mixtures is demonstrated in **(C)** for PG and in **(D)** for OG. Four replicates were analyzed for each mixture. Two types of linearity are displayed: established from a series of data points of isomeric mixtures at defined compositions (black solid line), and from the two data points of authentic *sn*-1 and *sn*-2 isomers (red dashed line).

A.


Relative Intensity (%)
100
80
60
40
20
159.3
327.3
404.4
Full MS2 404
(1-14:0 MG)
355.3
Full MS2 432
(1-16:0 MG)
432.3
383.3
Full MS2 460
(1-18:0 MG)
460.3
411.3
Full MS2 488
(1-20:0 MG)
488.4
439.3
Full MS2 516
(1-22:0 MG)
100
180
260
340
420
500
m/z

B.


Relative Intensity (%)
100
80
60
40
20
159.3
383.3
460.3
267.3
Full MS2 460
(1-18:0 MG)
381.4
458.3
Full MS2 458
(1-18:1 MG)
265.4
441.2
379.3
456.3
Full MS2 456
(1-18:2 MG)
245.4
263.4
421.3
377.3
454.3
261.3
Full MS2 454
(1-18:3 MG)
243.3
437.3
100
180
260
340
420
500
m/z

C.

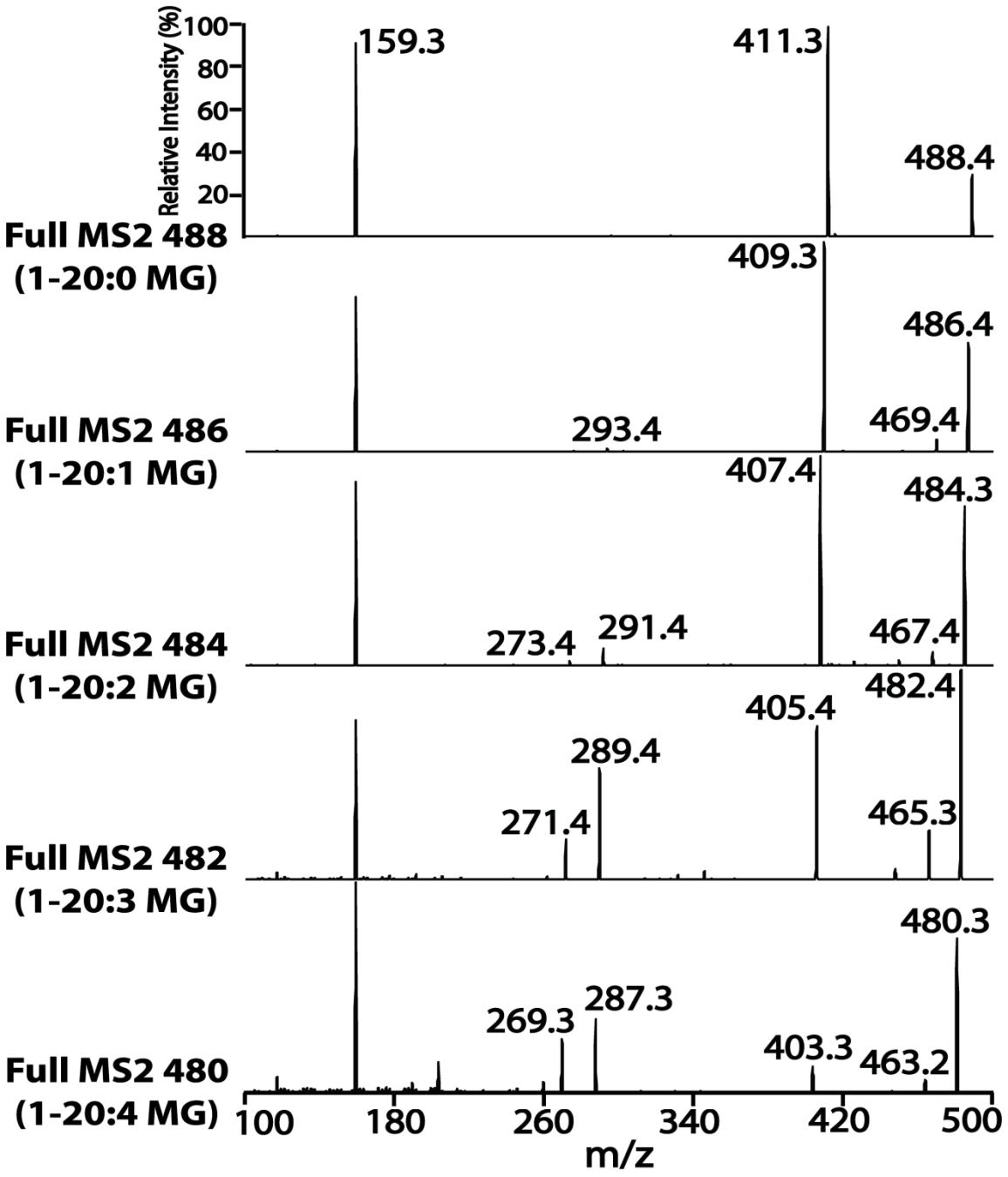
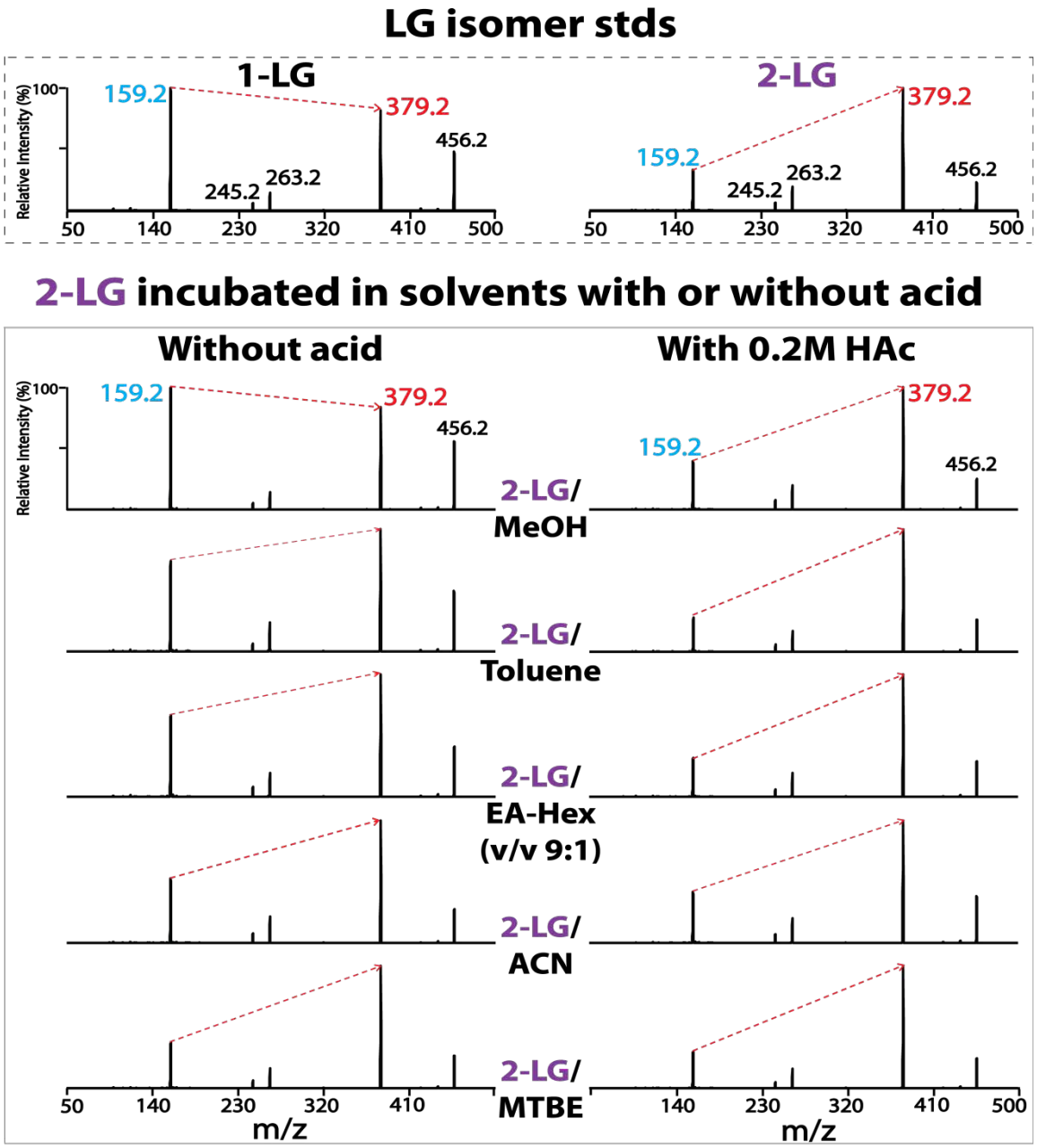
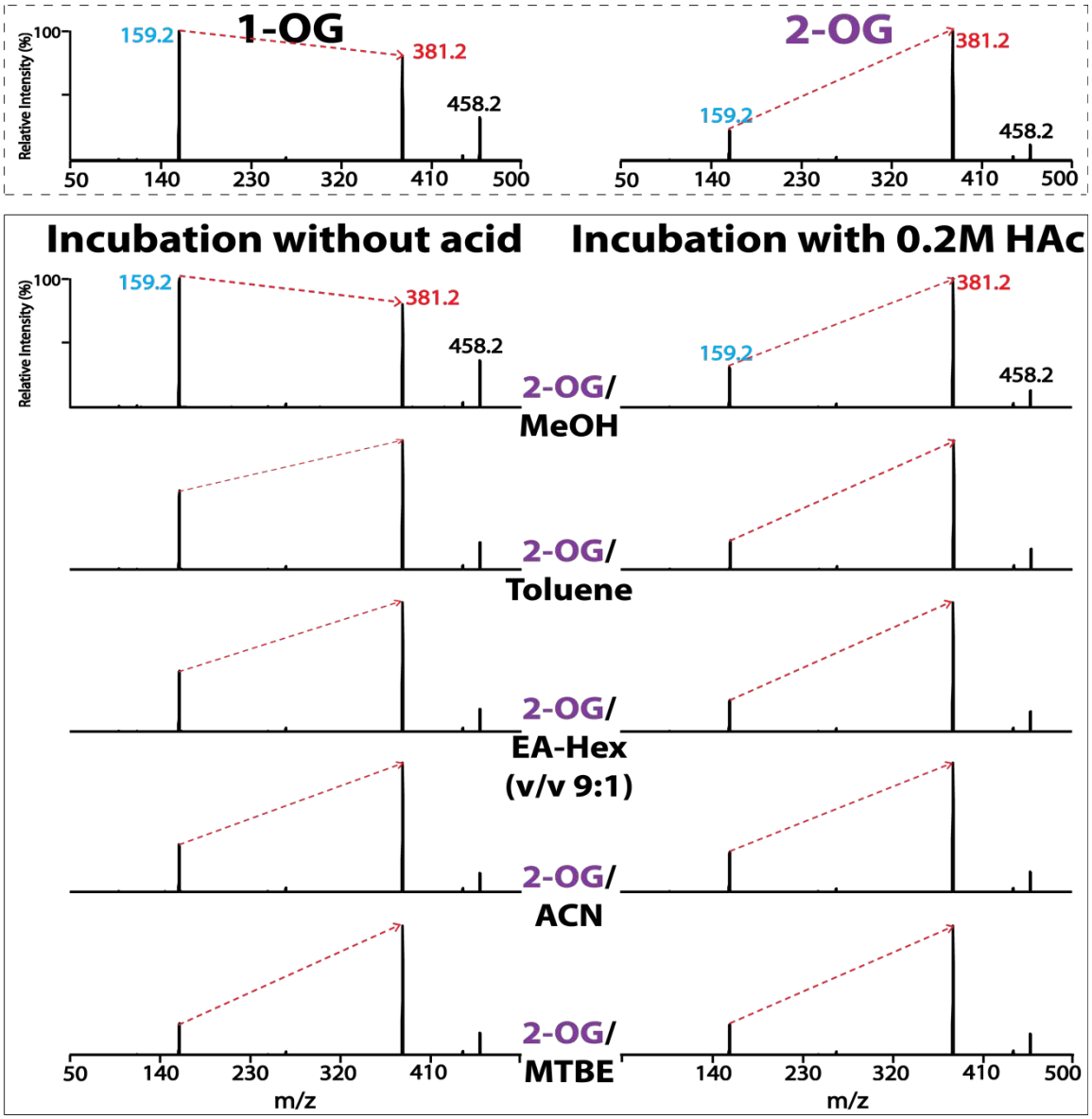


Figure S4. Product ion spectra of various diacetyl monoglyceride (MG) species. MG species containing acyl chains with differential chain length **(A)** and degree of unsaturation **(B and C)** were derivatized and analyzed as described in Figure S3. The tandem MS spectra of MG species containing acyl chains with 3 double bonds (*e.g.*, 18:3 MG and 20:3 MG) demonstrate fragmentation features similar to those of MG species containing acyl chains with 0-2 double bonds but markedly different from that of MG species of higher degree of unsaturation (*e.g.*, 20:4 MG). The intensity of the fragment ion resulting from neutral loss of 77 amu (*e.g.*, *m/z* 377 for 18:3 MG and *m/z* 405 for 20:3 MG) is nearly equal to that of the fragment ion from loss of the acyl chain *(i.e.*, m/z 159) in their sn-1 isomers (B and C).

A.


LG isomer stds
1-LG
2-LG
Relative Intensity (%)
100
159.2
379.2
456.2
245.2
263.2
50
140
230
320
410
500
2-LG incubated in solvents with or without acid
Without acid
With 0.2M HAc
2-LG/
MeOH
2-LG/
Toluene
2-LG/
EA-Hex
(v/v 9:1)
2-LG/
ACN
2-LG/
MTBE
m/z

B.


1-OG
2-OG
Relative Intensity (%)
100
159.2
381.2
458.2
50
140
230
320
410
500
Incubation without acid
Incubation with 0.2M HAc
2-OG/
MeOH
2-OG/
Toluene
2-OG/
EA-Hex
(v/v 9:1)
2-OG/
ACN
2-OG/
MTBE
m/z

C.

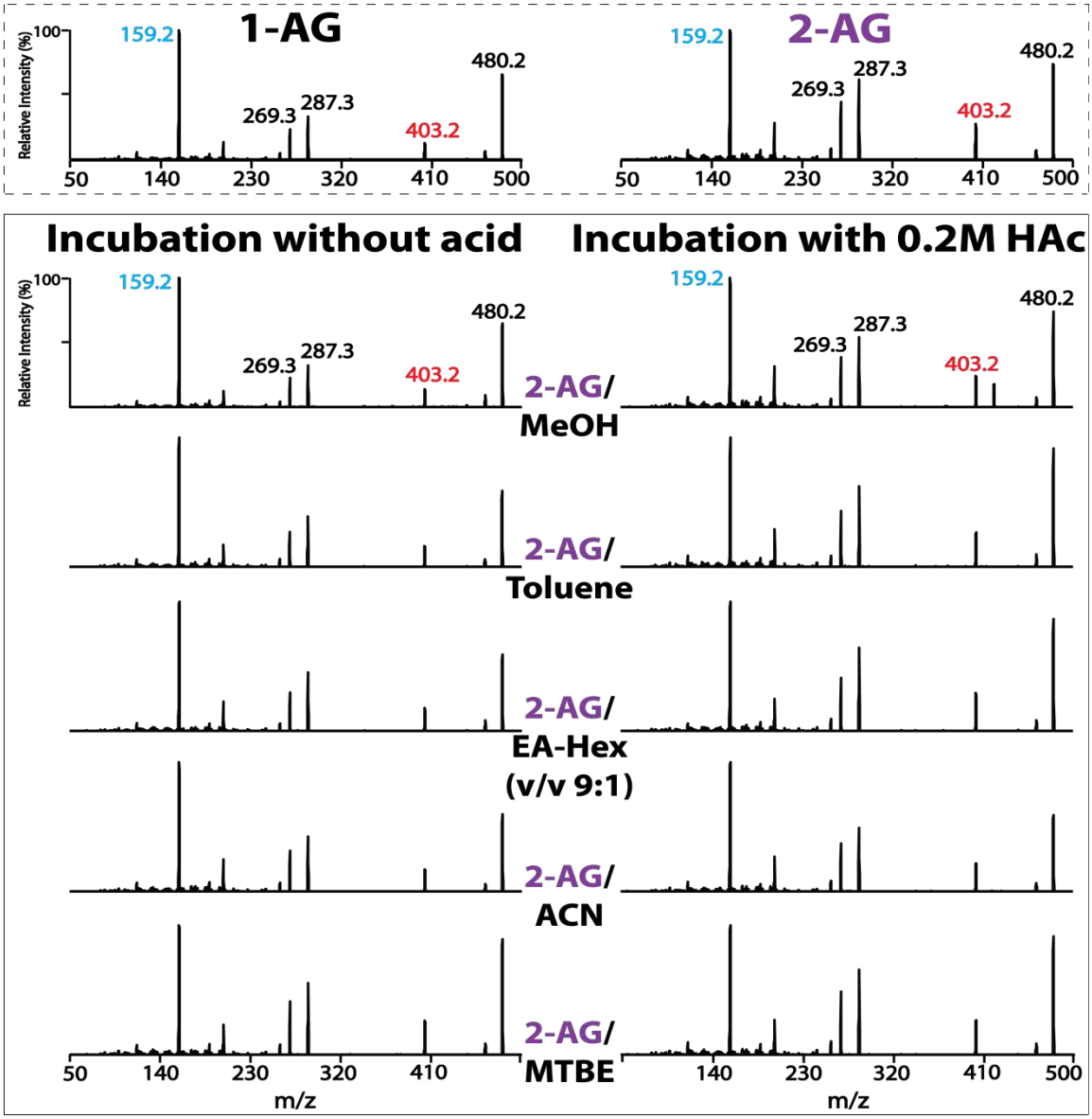


Figure S5. Product ion spectra of *sn*-2 monoglyceride (MG) species after incubation and evaporation in various solvents in the presence or absence of 0.2 M acetic acid prior to derivatization. The incubation with 2 mL of solvent was performed at 4° C for 1 hr. Solvent evaporation was performed at room temperature using a N_2 stream. The dried residues were derivatized by acetic anhydride and extracted as described in the Experimental Section. Each top panel shows the product ion spectra of authentic *sn*-1 and *sn*-2 isomers for each examined MG species. Each bottom panel shows the product ion spectra of *sn*-2 isomers after incubation in solvents for 2-LG **(A)**, 2-OG **(B)**, or 2-AG **(C)**.

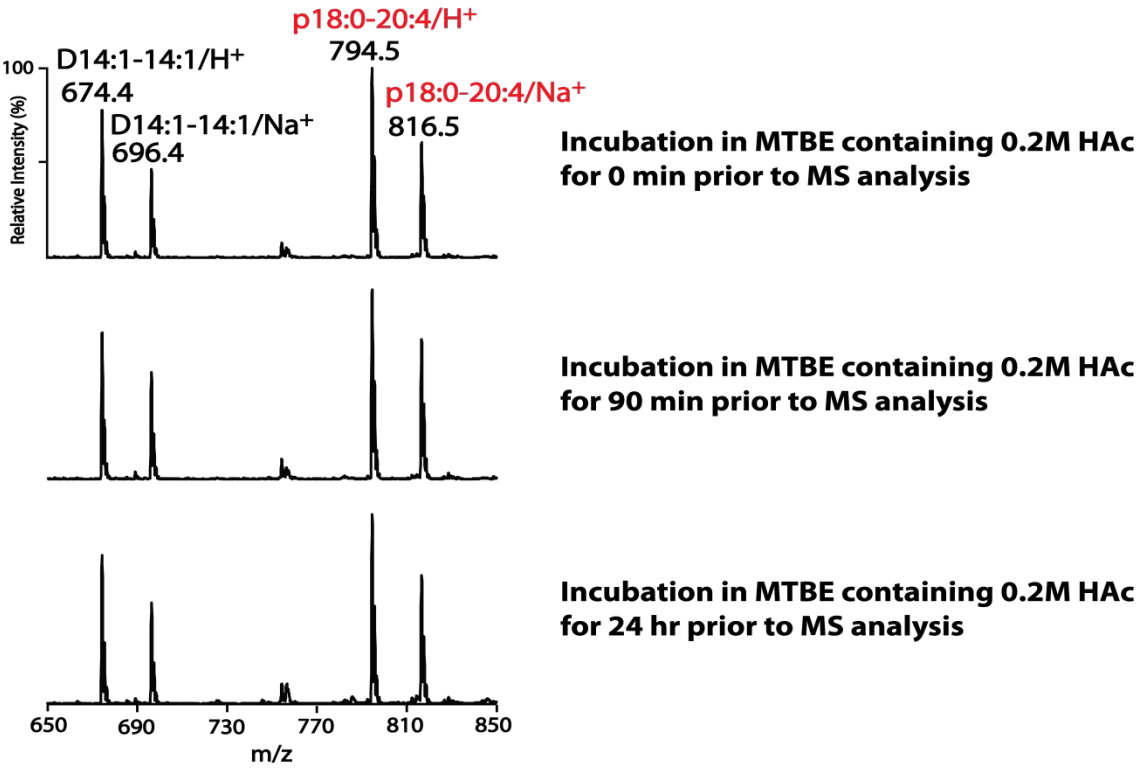
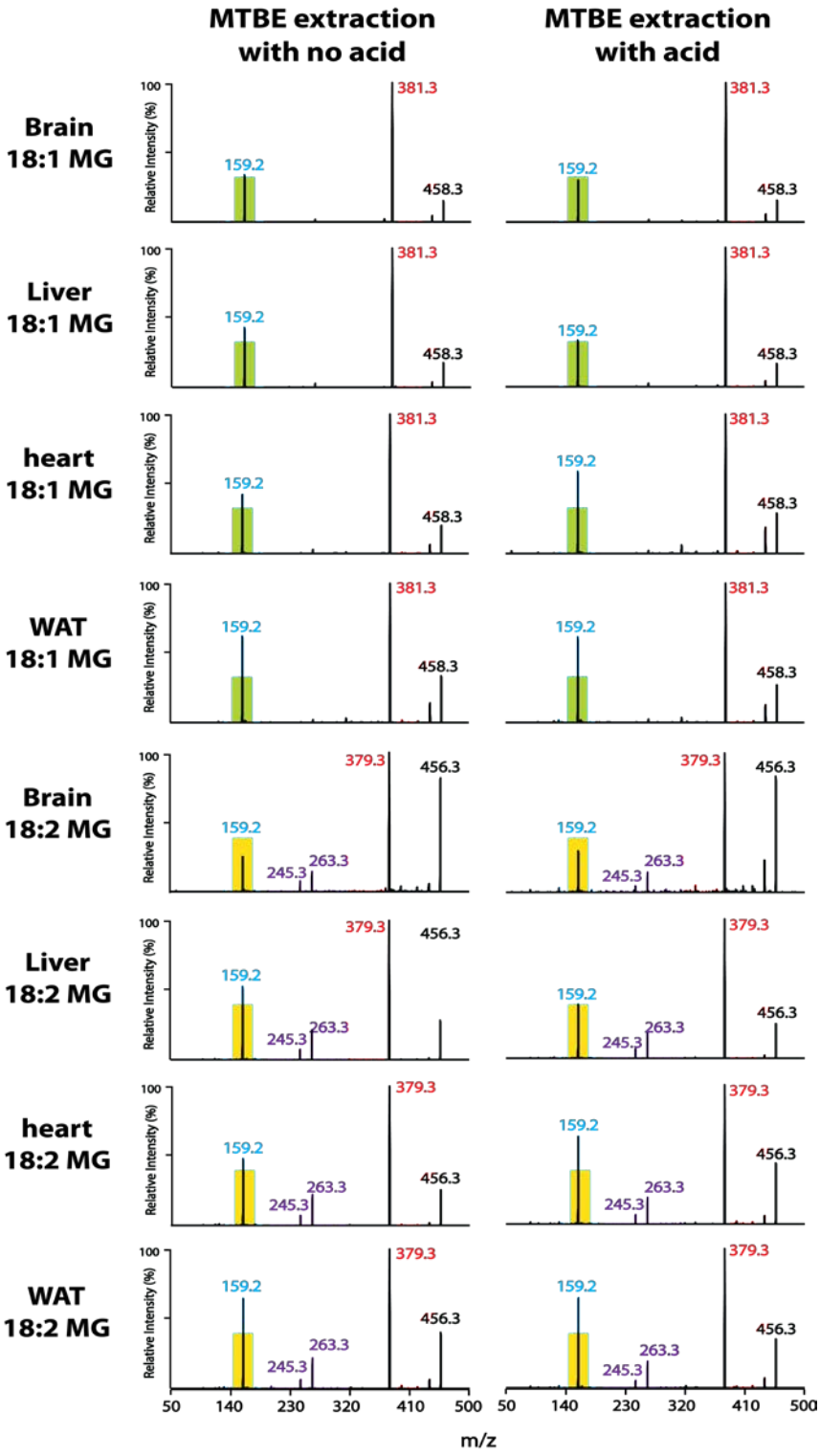


Figure S6. Stability of the vinyl ether linkage to the presence of 0.2 M acetic acid (HAc). A mixture of a choline plasmalogen species (p18:0-20:4) and a phosphatidylcholine species (D14:1-14:1) were prepared in methyl *tert*-butyl ether (MTBE) containing 0.2 M HAc, and incubated at 4°C for the specified times. The plasmalogen species contain a vinyl ether linkage that is acid labile with respect to acid concentration- and exposure time. The phosphatidylcholine species contains ester bonds that are stable under acidic conditions and thus serves as an internal control to examine the acid-mediated hydrolysis of the plasmalogen species. Full mass spectrum of the solution was directly acquired after dilution. The results demonstrate unchanged peak intensity ratios between the plasmalogen and phosphatidylcholine species, indicating the lack of acid-mediated vinyl ether linkage cleavage under these conditions (*i.e.,* incubation in MTBE in the presence of 0.2 M HAc at 4°C for up to 24 hr). This precludes the potential loss of endogenous vinyl ether species during sample preparation in the presence of 0.2 M acetic acid.


MTBE extraction with no acid
MTBE extraction with acid
Brain 18:1 MG
Liver 18:1 MG
heart 18:1 MG
WAT 18:1 MG
Brain 18:2 MG
Liver 18:2 MG
heart 18:2 MG
WAT 18:2 MG
Relative Intensity (%)
100
381.3
458.3
159.2
379.3
456.3
245.3
263.3
50
140
230
320
410
500
m/z

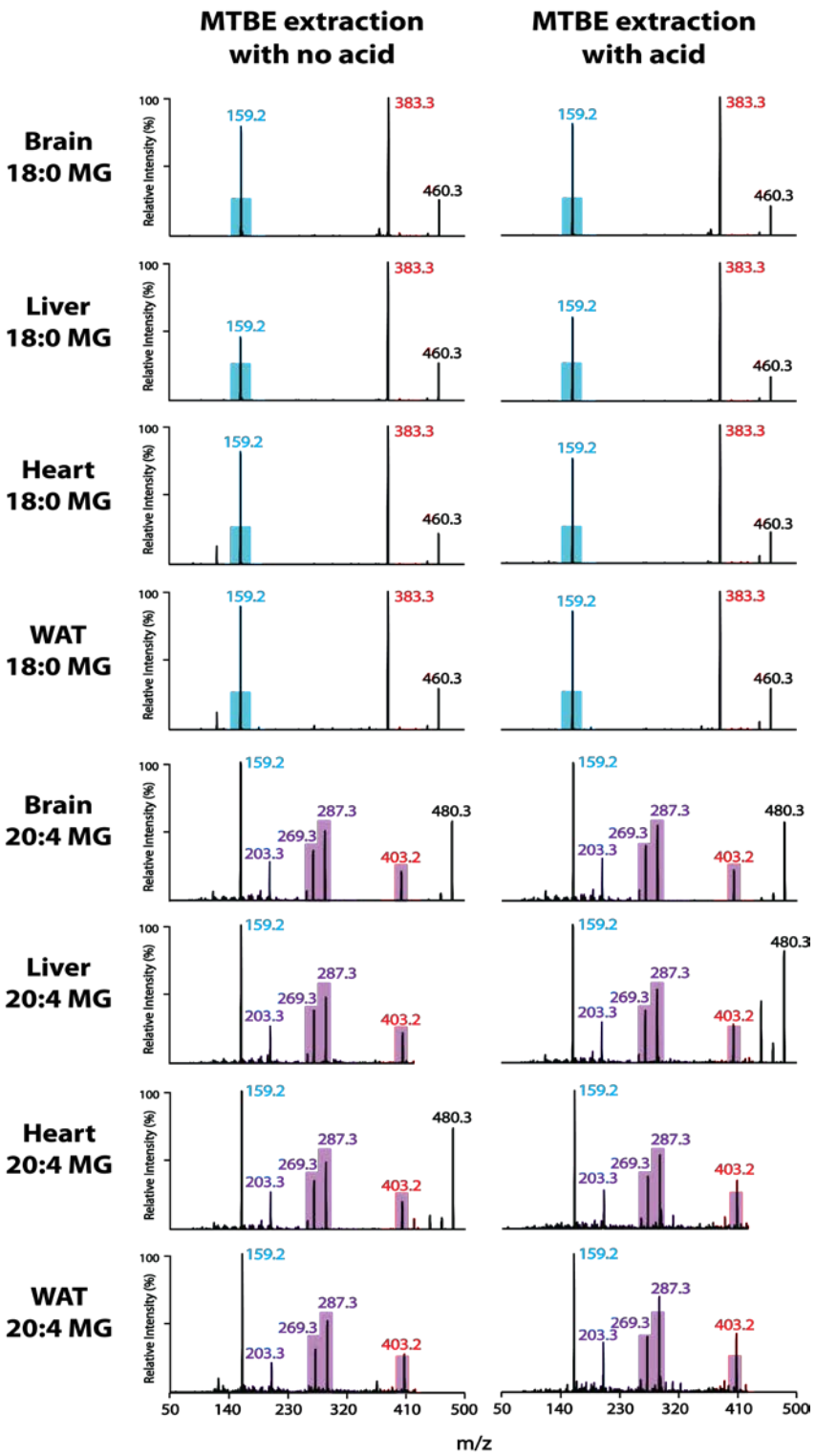


Figure S7. Minimal isomerization of monoglyceride (MG) species extracted from various tissues by a single-phase methyl *tert*-butyl ether (MTBE) extraction in the presence or absence of 0.2 M acetic acid. Lipid extracts of mouse brain, liver, heart and white adipose tissue (WAT) were prepared by a single-phase MTBE extraction with or without acid followed by acetonitrile enrichment as described in Figure 4 and in the text. Product ion spectra of representative MG species were acquired in the presence of 10 mM ammonium acetate at a collision energy of 15 eV. The filled color bars represent the same as described in Figure 5.

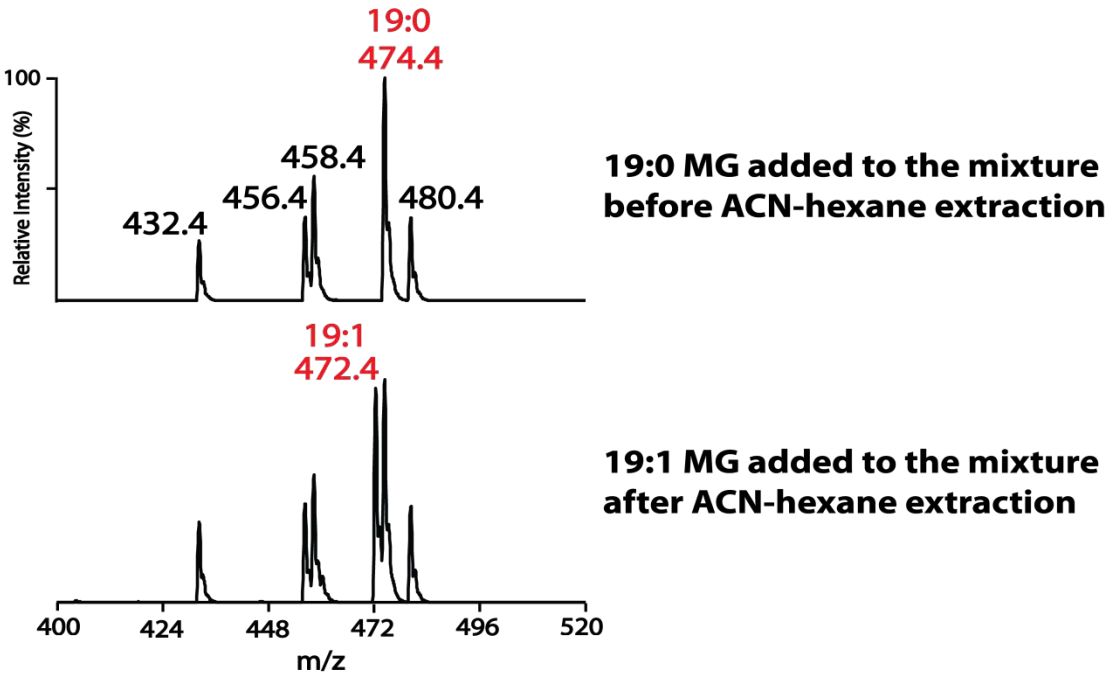


Figure S8. Complete recovery of monoglyceride (MG) species in the acetonitrile (ACN) fraction from ACN/hexane partitioning. The mixture of 2-PG, 2-LG, 2-OG, 2-AG and 1-19:0 MG was extracted by ACN/hexane liquid/liquid partitioning as described in the Experimental Section. The ACN fraction after diacetylation derivatization was analyzed by precursor ion scanning (PIS) of *m/z* 159 (top panel). In another experiment, an equal amount of 1-19:1 MG to that of 1-19:0 MG in the mixture was added to the ACN fraction of the mixture, followed by derivatization and analysis using PIS of *m/z* 159 (bottom panel). The nearly equal intensity of 1-19:0 MG and 1-19:1 MG (which was present in the mixture prior to ACN-hexane extraction, or added to the mixture after ACN-hexane extraction, respectively) indicates the nearly complete recovery of MG species into the ACN fraction by ACN-hexane liquid/liquid partitioning.

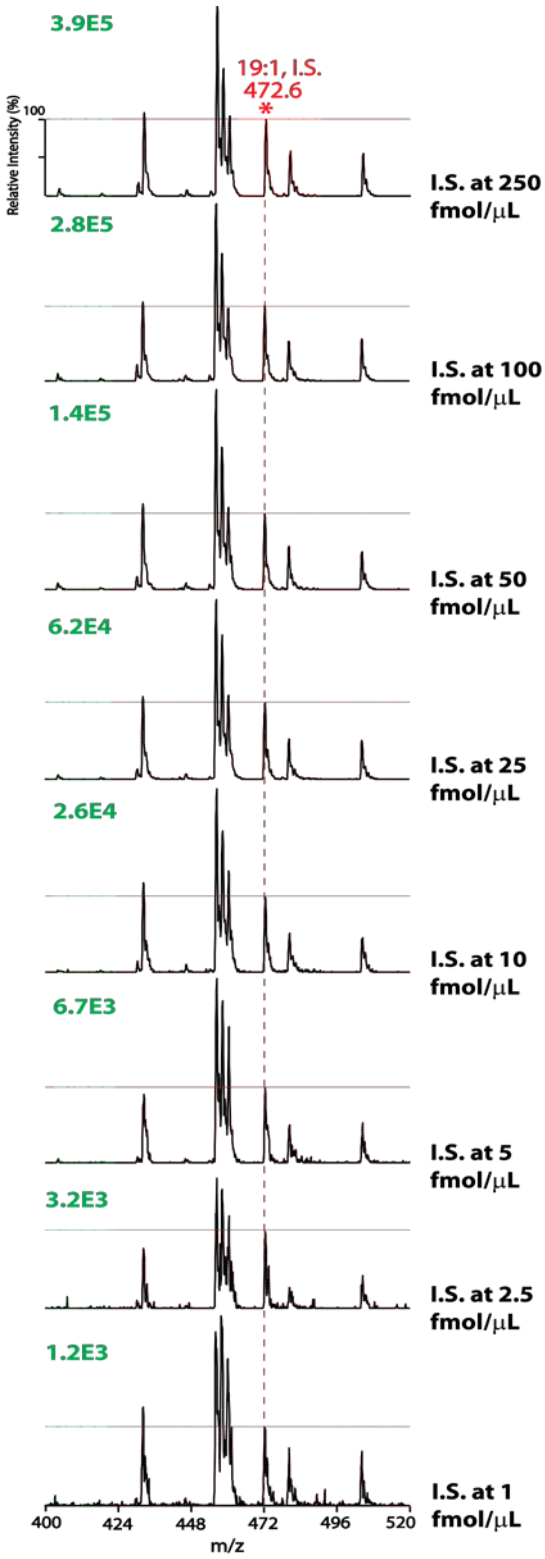


Figure S9. Sensitivity of MG profiling by precursor ion scanning (PIS) of *m/z* 159. A liver extract was prepared as described in Figure 5 and analyzed after a 20-5000 fold dilution in the presence of 10 mM ammonium acetate by PIS of *m/z*159 at a collision energy of 15 eV. The corresponding concentration of 1-19:1 MG internal standard (I.S.) and the absolute ion counts of spectral base peak for each diluted infusion solution are presented.

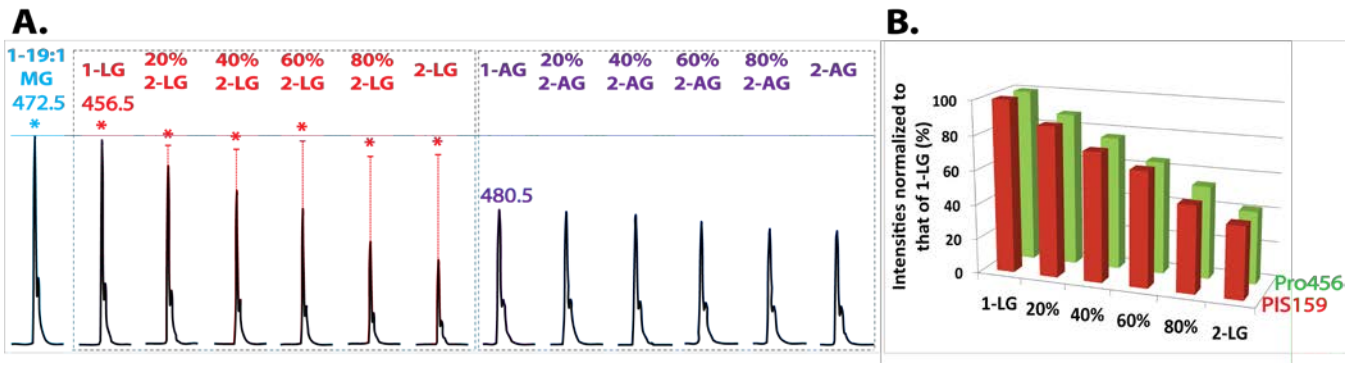
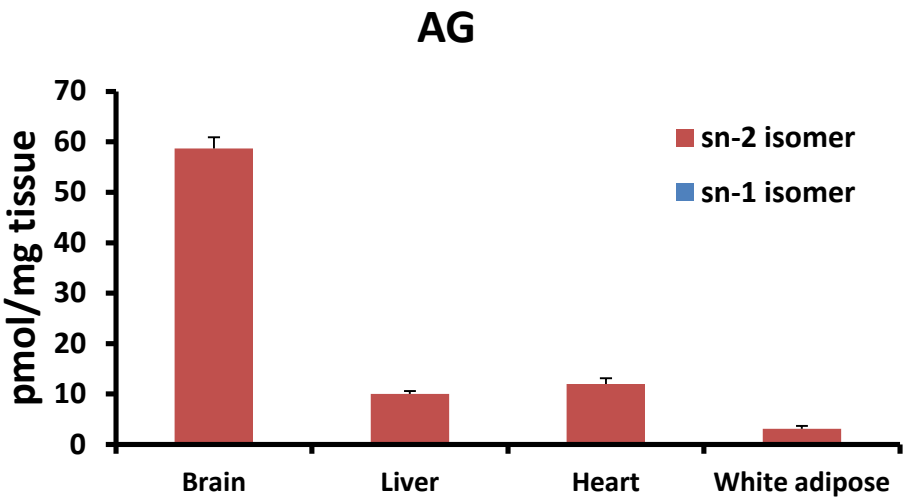
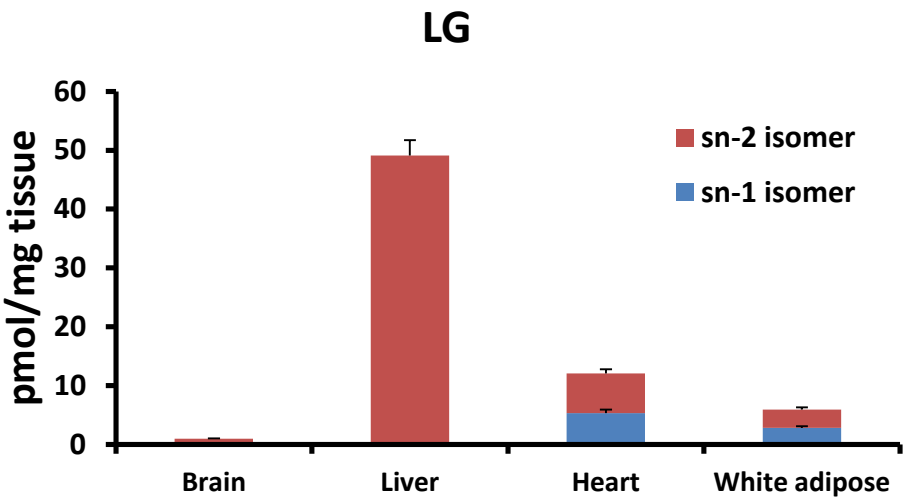
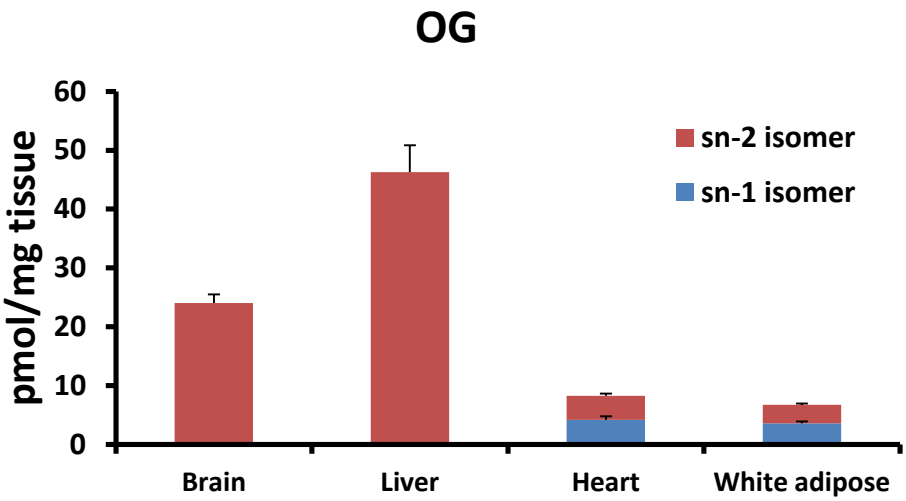


Figure S10. Determination of correction factors for quantitation of monoglyceride (MG) regioisomers using 1-19:1 MG internal standard (I.S.) and analysis of precursor ion scanning (PIS) of *m/z* 159. MG solutions used in Figure 3 including *sn*-1 and *sn*-2 isomers and their mixtures at defined compositions, after mixing with 1-19:1 MG I.S. at an equal molar concentration, were analyzed by PIS of *m/z* 159 for LG **(A, left panel)** and for AG **(A, right panel)**. All spectra were normalized to the 1-19:1 MG I.S. peak at *m/z* 472 (represented by the horizontal blue line). The relative intensity of the LG peak at *m/z* 456 in each spectrum of PIS of *m/z* 159 was then further normalized to that of 1-LG **(B, red bars)**. The y-axis data in Figure 3C obtained from spectra of the product ion scans of MG species were also normalized to that of 1-LG **(B, green bars)**. Data from spectra of PIS of *m/z* 159 (B, red bars) closely correlate with data from the product ion spectra (B, green bars). The latter are thus used to determine the correction factors for quantitation of MG regioisomers using spectral data of PIS of *m/z* 159 and 1-19:1 MG as I.S. (Table S1). Note that 1-LG needs no correction with 1-19:1 MG as I.S. "*" indicates the corrected intensities by using the correction factors for LG in Table S1.


AG
pmol/mg tissue
70
60
50
40
30
20
10
0
sn-2 isomer
sn-1 isomer
Brain
Liver
Heart
White adipose


LG
pmol/mg tissue
60
50
40
30
20
10
0
sn-2 isomer
sn-1 isomer
Brain
Liver
Heart
White adipose


OG
pmol/mg tissue
60
50
40
30
20
10
0
sn-2 isomer
sn-1 isomer
Brain
Liver
Heart
White adipose

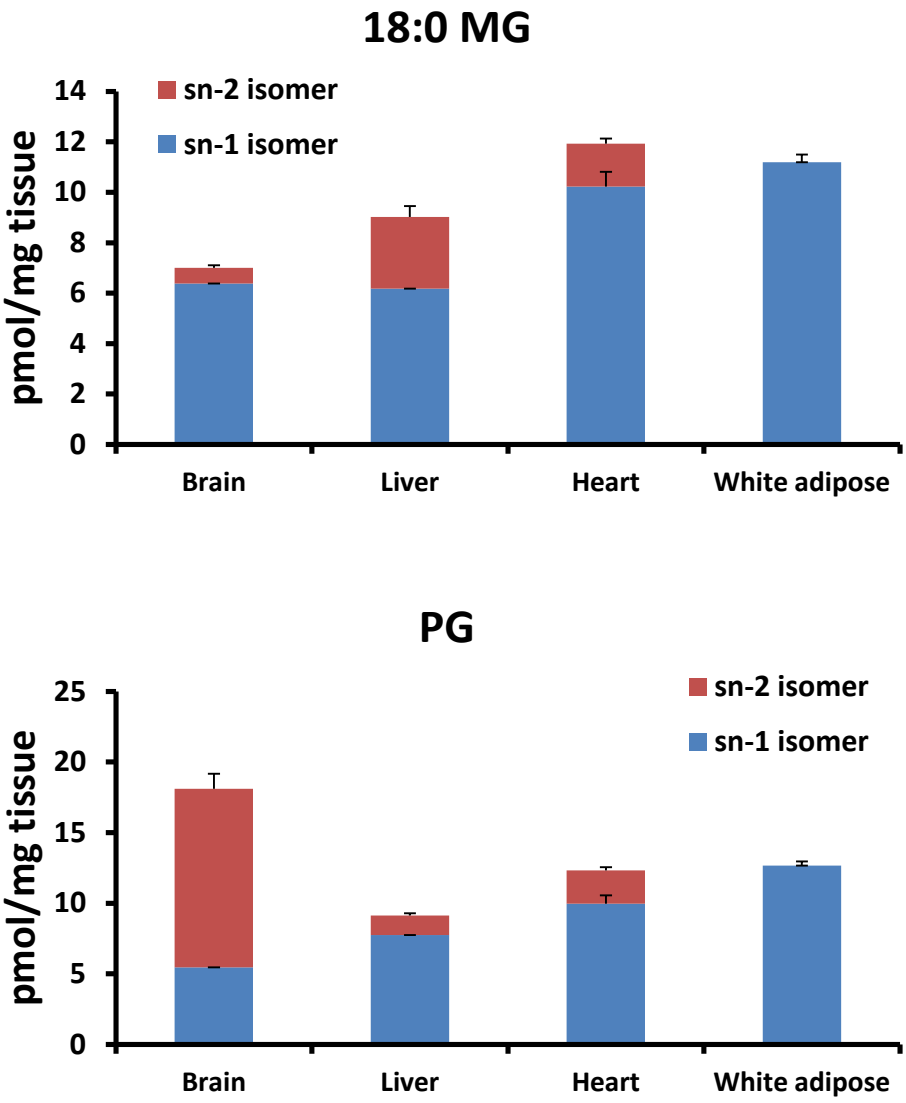


Figure S11. Quantitation of monoglyceride (MG) species including regioisomers in various tissues. The normalized intensity of the *m/z* 159 fragment ion was first calculated for each identified MG species from its product ion spectrum as shown in Figure 5B and described in the text. This normalized intensity was then used to quantify the isomeric composition of the MG species using the established linear relationship shown in Figure 3 and Figure S3. The determined isomeric composition was used to calculate the correction factor using Table S1. Next, the correction factor was used to quantify the total content of the MG species by using the spectra data of precursor ion scanning of *m/z* 159 as shown in Figure 5A with 1-19:1 MG as internal standard. Finally, from the total content and isomeric composition of the MG species, the content of each individual isomer of the MG species present in the extract was determined.

Reference

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