Core E – Analysis of Neutral Lipids from Cells
June 4, 2010
Thomas J. Leiker and Robert M. Barkley

This protocol describes the extraction and direct measurement of cholesterol esters (CEs), triacylglycerols (TAGs), and monoetherdiacylglycerols (MeDAGs) that are found in cells. Diacyl glycerols (DAGs) and monoacyl glycerols (MAGs) are extracted and then determined after derivatization with 2,4-difluorophenyl isocyanate. The steps include addition of internal standards (IS) to the thawed sample, liquid/liquid extraction, and analysis of extracts by normal phase HPLC/MS/MS using multiple reaction monitoring (MRM) for CEs, full spectral scanning (Q1) for TAGs and MeDAGs, and, after derivatization, constant neutral loss scanning (NL) for both 1,2- and 1,3-DAGs and MAGs. Samples of approximately 10^6 cells are received in 1.0 mL of phosphate buffered saline solution contained in 10 mL glass culture tubes that are sealed with Teflon-lined screw caps and frozen on dry ice. Upon receipt, samples are stored as received at -70 ^{o}C until the neutral lipids are extracted.

Reagents required:

Dichloromethane (DCM)
Ethyl acetate
Isooctane
Methyl *tert*-butyl ether (MTBE)
Acetonitrile
Deionized water
Ammonium acetate
Dulbecco's Phosphate buffered solution (1X concentration) without calcium or magnesium (DPBS)
4-Dimethylamino pyridine (DMAP)
2,4-Difluorophenyl isocyanate (DFPI)
Appropriate Internal and Reference Standards - see table of Internal Standards and descriptions of quantitative procedures below.

I. Sample Preparation - Extraction and Derivatization

All glassware is solvent rinsed with DCM and 25:75 (v/v) ethyl acetate:isooctane prior to use.
The entire sample is extracted and prepared for analysis, with an aliquot then reserved for derivatization.

1. Just prior to extraction, samples are removed from the -70 ^{o}C freezer and thawed at room temperature.
2. 2.0 mL of DPBS is added to the thawed sample.
3. Table of Internal Standards - add to each sample

Neutral Lipid	Internal Standard (IS)	Avanti ID	Concentration IS Solution	Volume IS Added
CE	$^{13}C_{18}$18:1-CE (See note) 18:1-d_5-CE (Alternate)	 LM-4200	10 ng/μL 10 ng/μL	25 μL 25 μL
MeDAG	18:1e/16:0/17:1-MeDAG	110597	1 ng/μL	25 μL
TAG	14:0/16:1/14:0-d_5-TAG	110541	1 ng/μL	25 μL
DAG and MAG	1,3-20:0/20:0-d_5-DAG	110540	1 ng/μL	25 μL

(Note: This internal standard was synthesized according to the procedure described in PM Hutchins, RM Barkley, RC Murphy, J. Lipid Res. 2008, 49:804–813.)

4. To each sample is added 2 mL of freshly prepared 25:75 (v/v) ethyl acetate:isooctane.
5. The sample is sealed, shaken, and vortexed.
6. The sample is centrifuged at 3,000 RPM for 3 min at 20 ^{o}C.
7. The organic (upper) layer is transferred to a clean glass culture tube using a clean disposable glass pipette.
8. Steps 4 through 7 are repeated once and extracts are combined.
9. The extract is sealed with a Teflon-lined cap and placed in a -20 ^{o}C freezer until the extract is processed for the final stage of analysis, or immediately analyzed by LC/MS/MS.
10. Prior to analysis, the extract is reduced to dryness using a SpeedVac.
11. 50 μL of DCM is added to each extract.

12. A 25 µL aliquot is removed and placed in a 0.8 mL conical shaped crimp top LC vial. The remaining 25 µL of extract is set aside for derivatization of DAGs and MAGs (go to step 16).
13. The DCM in the LC vial is evaporated under a gentle stream of N_2 at room temperature.
14. The extracted is re-dissolved in 20 µL of 4:96 (v/v) MTBE:isooctane.
15. The vial is sealed with a crimp-top and and analyzed according to procedures in **II. A. B.** and **C.**, or stored at -20 ^{o}C until the extract is analyzed.
16. To the remaining extract from step 12 above is added 400 µL of DCM, 10 µL of a 10 µg/µL DFPI solution in DCM and 10 µL of a 10 µg/µL DMAP solution in DCM.
17. The culture tube is sealed with a Teflon-line cap and placed in a 60 ^{o}C heated block for 30 min.
18. The tube is removed from the heated block and allowed to remain at room temperature overnight.
19. The DCM is transferred to a 0.8 mL conical shaped crimp top LC vial.
20. The DCM in the LC vial is removed under a gentle stream of N_2 at room temperature.
21. The extracted is re-dissolved in 20 µL of 4:96 (v/v) MTBE:isooctane.
22. The vial is sealed with a crimp-top and the extract is analyzed immediately according to the procedure in **II. D** and **E.**

II. Analysis by HPLC/MS and HPLC/MS/MS

Liquid Chromatography

Chromatographic separations, one for CEs, TAGs and MeDAGs, and another for derivatized neutral lipids, were performed using the following conditions with a Shimadzu HPLC consisting of a Sil-HTc controller and auto-sampler, and three LC10AD pumps:

Normal Phase Separation

Phenomenex silica column (Luna): 150 mm X 0.2 mm id, 5 µm particle size

Solvent system

Pump A-100% isooctane

Pump B-50:50 MTBE:isooctane

Gradient

Total flow - 0.2 mL/min

Time (min)	%B
0.0	Start
8.0	5.0
25.0	30.0
29.0	90.0
35.0	90.0
40.0	5.0
50.0	Stop

Auto Sampler

Injection volume –	1.0 µL
Rinse volume –	500 µL
Needle stroke –	52 mm
Rinse speed –	35 µL/s
Sampling speed –	15.0 µL
Cooler enabled –	Yes
Cooler temperature –	15 ^{o}C

Post Column Modifier

Pump C Flow rate – 0.03 mL/min

Modifier – 10 mM ammonium acetate in 5:95 (v/v) water:acetonitrile.

Mass Spectrometry

The total effluent from the HPLC was admitted to the mass spectrometer. Electrospray ionization mass spectral analyses were conducted using an Applied Biosystems 4000 QTrap LC/MS/MS system equipped with a Turbo Ion Spray source. The conditions for each class of neutral lipid are given in the descriptions and tables below. Data for standard curves are acquired and curve linearity established for each batch of samples.

A. Quantitation of Cholesterol Esters

Cholesterol esters are quantified from multiple reaction monitoring (MRM) data and application of standard curves that relate the responses for known amounts of reference standards 18:1-, 18:2- and 20:4-CE to that for a single internal standard. The curves are prepared from solutions of reference

standards at 0, 0.29, 0.98, 2.9, and 9.8 µg/5mL that also contain the same amount of IS that is used for extracts of cells. For species for which there is no standard curve, the standard curve used for quantitation is that for the closest reference standard based on degree of unsaturation and composition of the fatty acid side chain. Some species in the table will be present at levels below the limit of quantitation.

MRM Conditions

Polarity –	Positive
Scan mode –	MRM
Ion source –	Turbo Spray
Resolution Q1 –	Unit
Resolution Q3 –	Unit
Intensity Threshold –	0.00 cps
Settling Time –	0.000 ms
MR pause –	5.0070 ms
MCA –	No
Step Size –	0.00 amu
Cycle Time –	2.63 s
Transitions –	See table below

Mass Spectrometer Parameters

CUR –	10.0
IS –	5500.0
TEM –	300.0
GS1 –	40.0
GS2 –	20.0
ihe –	ON
CAD –	7.0
DP –	60.0
EP –	9.0
CE –	25.0
CXP –	6.0

Cholesterol Ester MRM Transitions

LM ID	Cholesterol Ester (Acyl Carbon Number: Double bonds)	Molecular Mass	Q1 $[NH_4]^+$	Q3	Dwell Time (ms)
LMST01020021	14:1	594.6	612.6	369.3	100
LMST01020004	14:0	596.6	614.6	369.3	100
LMST01020022	15:1	608.6	626.6	369.3	100
LMST01020027	15:0	610.6	628.6	369.3	100
LMST01020024	16:2	620.6	638.6	369.3	100
LMST01020006	16:1	622.6	640.6	369.3	100
LMST01020005	16:0	624.6	642.6	369.3	100
LMST01020023	17:1	636.6	654.6	369.3	100
LMST01020026	17:0	638.6	656.6	369.3	100
LMST01020009	18:3	646.6	664.6	369.3	100
LMST01020008	18:2	648.6	666.6	369.3	100
LMST01020003	18:1	650.6	668.6	369.3	100
LMST01020007	18:0	652.7	670.7	369.3	100
LMST01020030	18:1-d_5 IS (Alternate)	655.7	673.7	369.3	100
	$^{13}C_{18}$-18:1 IS	668.7	686.7	369.3	100
LMST01020014	20:4	672.6	690.6	369.3	100
LMST01020013	20:3	674.6	692.6	369.3	100
LMST01020012	20:2	676.7	694.7	369.3	100
LMST01020011	20:1	678.7	696.7	369.3	100
LMST01020010	20:0	680.7	698.7	369.3	100
LMST01020019	22:6	696.6	714.6	369.3	100
LMST01020031	22:5	698.6	716.6	369.3	100
LMST01020018	22:4	700.6	718.6	369.3	100
LMST01020017	22:2	704.7	722.7	369.3	100
LMST01020025	22:1	706.7	724.7	369.3	100
LMST01020016	22:0	708.7	726.7	369.3	100

B. Quantitation of Triacylglycerols

Triacylglycerols are quantified by integration of molecular ions (as ammonium ion adducts) from full scan mass spectra and application of standard curves that relate the responses of known amounts of reference standards to that for a single internal standard. The curves are prepared from solutions of reference standards at 0, 5, 10, 25, 50, 100, 200, and 400 ng/5mL that also contain the same amount of IS that is used for extracts of cells. The reference standards used are 49:2- (Avanti ID 110528), 50:1- (Avanti ID 110521), 50:0- (Avanti ID 110520) and 54:3- (Avanti LM3216) TAGs. For species for which there is no standard curve, the standard curve used for quantitation is that for the closest reference standard based on degree of unsaturation and composition of the fatty acid side chains. Final calculations of amounts of analyte per sample require isotope corrections and application of deisotope factors. Some species in the table will be present at levels below the limit of quantitation.

Q1 Scan Conditions

Polarity –	Positive
Scan mode –	Profile
Ion source –	Turbo Spray
Resolution Q1 –	Unit
Intensity threshold –	0.00 cps
Settling Time –	0.00 ms
MR pause –	5.0070 ms
MCA –	No
Center/Width –	No
Step size –	0.1 amu
Scan range –	500-1200 amu
Scan time –	4 s

Mass Spectrometer Parameters

Cur –	14.0
IS –	5500.0
TEM –	300.0
GS1 –	1800
GS2 –	20.0
ihe –	ON
DP –	88.0
EP –	10.0

Triacylglycerol Ions

TAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4]^+$	TAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4]^+$
40:2	$C_{43}H_{78}O_6$	690.6	708.6	44:0	$C_{47}H_{90}O_6$	750.7	768.7
40:1	$C_{43}H_{80}O_6$	692.6	710.6	IS	$C_{47}{}^2H_5H_{83}O_6$	753.7	771.7
40:0	$C_{43}H_{82}O_6$	694.6	712.6	45:3	$C_{48}H_{86}O_6$	756.6	774.7
41:5	$C_{44}H_{74}O_6$	698.6	716.6	45:2	$C_{48}H_{88}O_6$	760.7	778.7
41:4	$C_{44}H_{76}O_6$	700.6	718.6	45:1	$C_{48}H_{90}O_6$	762.7	780.7
41:3	$C_{44}H_{78}O_6$	702.6	720.6	45:0	$C_{48}H_{92}O_6$	764.7	782.7
41:2	$C_{44}H_{80}O_6$	704.6	722.6	46:5	$C_{49}H_{84}O_6$	768.6	786.7
41:1	$C_{44}H_{82}O_6$	706.6	724.6	46:4	$C_{49}H_{86}O_6$	770.6	788.7
41:0	$C_{44}H_{84}O_6$	708.6	726.7	46:3	$C_{49}H_{88}O_6$	772.7	790.7
42:5	$C_{45}H_{76}O_6$	712.6	730.6	46:2	$C_{49}H_{90}O_6$	774.7	792.7
42:4	$C_{45}H_{78}O_6$	714.6	732.6	46:1	$C_{49}H_{92}O_6$	776.7	794.7
42:3	$C_{45}H_{80}O_6$	716.6	734.6	46:0	$C_{49}H_{94}O_6$	778.7	796.7
42:2	$C_{45}H_{82}O_6$	718.6	736.7	47:5	$C_{50}H_{86}O_6$	782.6	800.7
42:1	$C_{45}H_{84}O_6$	720.6	735.7	47:4	$C_{50}H_{88}O_6$	784.7	802.7
42:0	$C_{45}H_{86}O_6$	722.6	740.7	47:3	$C_{50}H_{90}O_6$	786.7	804.7
43:2	$C_{46}H_{84}O_6$	732.6	750.7	47:2	$C_{50}H_{92}O_6$	788.7	806.7
43:1	$C_{46}H_{86}O_6$	734.6	752.7	47:1	$C_{50}H_{94}O_6$	790.7	808.7
43:0	$C_{46}H_{88}O_6$	736.7	754.7	47:0	$C_{50}H_{96}O_6$	792.7	810.7
44:5	$C_{47}H_{80}O_6$	740.6	758.6	48:6	$C_{51}H_{86}O_6$	794.6	812.7
44:4	$C_{47}H_{82}O_6$	742.6	760.6	48:5	$C_{51}H_{88}O_6$	796.7	814.7
44:3	$C_{47}H_{84}O_6$	744.6	762.7	48:4	$C_{51}H_{90}O_6$	798.7	816.7
44:2	$C_{47}H_{86}O_6$	746.6	764.7	48:3	$C_{51}H_{92}O_6$	800.7	818.7
44:1	$C_{47}H_{88}O_6$	748.7	766.7	48:2	$C_{51}H_{94}O_6$	802.7	820.7

TAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4]^+$
48:1	$C_{51}H_{96}O_6$	804.7	822.8
48:0	$C_{51}H_{98}O_6$	806.7	824.8
49:5	$C_{52}H_{90}O_6$	810.7	828.7
49:4	$C_{52}H_{92}O_6$	812.7	830.7
49:3	$C_{52}H_{94}O_6$	814.7	832.7
49:2	$C_{52}H_{96}O_6$	816.7	834.7
49:1	$C_{52}H_{98}O_6$	818.7	836.8
49:0	$C_{52}H_{100}O_6$	820.8	838.8
50:5	$C_{53}H_{92}O_6$	824.7	842.7
50:4	$C_{53}H_{94}O_6$	826.7	844.7
50:3	$C_{53}H_{96}O_6$	828.7	846.8
50:2	$C_{53}H_{98}O_6$	830.7	848.8
50:1	$C_{53}H_{100}O_6$	832.8	850.8
50:0	$C_{53}H_{102}O_6$	834.8	852.8
51:5	$C_{54}H_{94}O_6$	838.7	856.7
51:4	$C_{54}H_{96}O_6$	840.7	858.8
51:3	$C_{54}H_{98}O_6$	842.7	860.8
51:2	$C_{54}H_{100}O_6$	844.8	864.8
51:1	$C_{54}H_{102}O_6$	846.8	864.8
51:0	$C_{54}H_{104}O_6$	848.8	866.8
52:5	$C_{55}H_{96}O_6$	852.7	870.8
52:4	$C_{55}H_{98}O_6$	854.7	872.8
52:3	$C_{55}H_{100}O_6$	856.8	874.8
52:2	$C_{55}H_{102}O_6$	858.8	876.8
52:1	$C_{55}H_{104}O_6$	860.8	878.8
52:0	$C_{55}H_{106}O_6$	862.8	880.8
53:5	$C_{56}H_{98}O_6$	866.7	884.8
53:4	$C_{56}H_{100}O_6$	868.8	886.8
53:3	$C_{56}H_{102}O_6$	870.8	888.8
53:2	$C_{56}H_{104}O_6$	872.8	890.8
53:1	$C_{56}H_{106}O_6$	874.8	892.8
53:0	$C_{56}H_{108}O_6$	876.8	894.8
54:6	$C_{57}H_{98}O_6$	878.7	896.8
54:5	$C_{57}H_{100}O_6$	880.8	898.8
54:4	$C_{57}H_{102}O_6$	882.8	900.8
54:3	$C_{57}H_{104}O_6$	884.8	902.8
54:2	$C_{57}H_{106}O_6$	886.8	904.8
54:1	$C_{57}H_{108}O_6$	888.8	906.8
54:0	$C_{57}H_{110}O_6$	890.8	908.8
55:6	$C_{58}H_{100}O_6$	892.8	910.8
55:5	$C_{58}H_{102}O_6$	894.8	912.8
55:4	$C_{58}H_{104}O_6$	896.8	914.8
55:3	$C_{58}H_{106}O_6$	898.8	916.8
55:2	$C_{58}H_{108}O_6$	900.8	918.8
55:1	$C_{58}H_{110}O_6$	902.8	920.9
55:0	$C_{58}H_{112}O_6$	904.9	922.9
56:6	$C_{59}H_{102}O_6$	906.8	924.8
56:5	$C_{59}H_{104}O_6$	908.8	926.8
56:4	$C_{59}H_{106}O_6$	910.8	928.8
56:3	$C_{59}H_{108}O_6$	912.8	930.8
56:2	$C_{59}H_{110}O_6$	914.8	932.9
56:1	$C_{59}H_{112}O_6$	916.8	934.9
56:0	$C_{59}H_{114}O_6$	918.9	936.9
57:6	$C_{60}H_{104}O_6$	920.8	938.8
57:5	$C_{60}H_{106}O_6$	922.8	940.8
57:4	$C_{60}H_{108}O_6$	924.8	942.8
57:3	$C_{60}H_{110}O_6$	926.8	944.9
57:2	$C_{60}H_{112}O_6$	928.9	946.9
57:1	$C_{60}H_{114}O_6$	930.9	948.9
57:0	$C_{60}H_{116}O_6$	932.9	950.9
58:6	$C_{61}H_{106}O_6$	934.8	952.8
58:5	$C_{61}H_{108}O_6$	936.8	954.8
58:4	$C_{61}H_{110}O_6$	938.8	956.9
58:3	$C_{61}H_{112}O_6$	940.9	958.9
58:2	$C_{61}H_{114}O_6$	942.9	960.9
58:1	$C_{61}H_{116}O_6$	944.9	962.9
58:0	$C_{61}H_{118}O_6$	946.9	964.9
60:12	$C_{63}H_{98}O_6$	950.7	968.8
59:2	$C_{62}H_{116}O_6$	956.9	974.9
59:1	$C_{62}H_{118}O_6$	958.9	976.9
59:0	$C_{62}H_{120}O_6$	960.9	978.9
60:4	$C_{63}H_{114}O_6$	966.9	984.9
60:3	$C_{63}H_{116}O_6$	968.9	986.9
60:2	$C_{63}H_{118}O_6$	970.9	988.9
60:1	$C_{63}H_{120}O_6$	972.9	990.9
60:0	$C_{63}H_{122}O_6$	974.9	992.9

C. Quantitation of Monoetherdiacylglycerols

Monodiacylglycerides are quantified by integration of molecular ions (as ammonium ion adducts) from full scan mass spectra and application of standard curves that relate the responses of known amounts of reference standards to that for a single internal standard. Since reference and internal standards are unavailable for MeDAGs, quantitation is performed using the TAG standards (see **B** above), choosing the closest reference standard based on degree of unsaturation and composition of fatty acid side chains. Final calculations of amounts of analyte per sample require isotope corrections and application of deisotope factors. Some species in the table will be present at levels below the limit of quantitation.

Q1 Scan Conditions

Polarity –	Positive
Scan mode –	Profile
Ion source –	Turbo Spray
Resolution Q1 –	Unit
Intensity threshold –	0.00 cps
Settling Time –	0.00 ms
MR pause –	5.0070 ms
MCA –	No
Center/Width –	No
Step size –	0.1 amu
Scan range –	500-1200 amu
Scan time –	4 s

Mass Spectrometer Parameters

Cur –	14.0
IS –	5500.0
TEM –	300.0
GS1 –	1800
GS2 –	20.0
ihe –	ON
DP –	88.0
EP –	10.0

Monodiacylglycerols Ions

MeDAG (CN:DB)	**Formula**	**Molecular Mass**	**Mass** $[NH_4]^+$
40:2	$C_{43}H_{80}O_5$	676.6	694.6
40:1	$C_{43}H_{82}O_5$	678.6	696.6
40:0	$C_{43}H_{84}O_5$	680.6	698.7
41:5	$C_{44}H_{76}O_5$	684.6	702.6
41:4	$C_{44}H_{78}O_5$	686.6	704.6
41:3	$C_{44}H_{80}O_5$	688.6	706.6
41:2	$C_{44}H_{82}O_5$	690.6	708.7
41:1	$C_{44}H_{84}O_5$	692.6	710.7
41:0	$C_{44}H_{86}O_5$	694.7	712.7
42:5	$C_{45}H_{78}O_5$	698.6	716.6
42:4	$C_{45}H_{80}O_5$	700.6	718.6
42:3	$C_{45}H_{82}O_5$	702.6	720.6
42:2	$C_{45}H_{84}O_5$	704.6	722.7
42:1	$C_{45}H_{82}O_5$	706.7	724.7
42:0	$C_{45}H_{88}O_5$	708.7	726.7
43:3	$C_{46}H_{84}O_5$	716.6	734.7
43:2	$C_{46}H_{86}O_5$	718.6	736.7
43:1	$C_{46}H_{88}O_5$	720.7	738.7
43:0	$C_{46}H_{90}O_5$	722.7	740.7
44:5	$C_{47}H_{82}O_5$	726.6	744.7
44:4	$C_{47}H_{84}O_5$	728.6	746.7
44:3	$C_{47}H_{86}O_5$	730.7	748.7
44:2	$C_{47}H_{88}O_5$	732.7	750.7
44:1	$C_{47}H_{90}O_5$	734.7	752.7
44:0	$C_{47}H_{92}O_5$	736.7	754.7
45:5	$C_{48}H_{84}O_5$	740.6	758.6
45:4	$C_{48}H_{86}O_5$	742.6	760.7
45:3	$C_{48}H_{88}O_5$	744.7	762.6
45:2	$C_{48}H_{90}O_5$	746.7	764.7
45:1	$C_{48}H_{92}O_5$	748.7	766.7
45:0	$C_{48}H_{94}O_5$	750.7	768.7
46:5	$C_{49}H_{86}O_5$	754.7	772.7
46:4	$C_{49}H_{88}O_5$	756.7	774.7
46:3	$C_{49}H_{90}O_5$	758.7	776.7
46:2	$C_{49}H_{92}O_5$	760.7	778.7
46:1	$C_{49}H_{94}O_5$	762.7	780.7
46:0	$C_{49}H_{96}O_5$	764.7	782.8
47:5	$C_{50}H_{88}O_5$	768.7	786.7
47:4	$C_{50}H_{90}O_5$	770.7	788.7
47:3	$C_{50}H_{92}O_5$	772.7	790.7
47:2	$C_{50}H_{94}O_5$	774.7	792.8
47:1	$C_{50}H_{96}O_5$	776.7	794.8
47:0	$C_{50}H_{98}O_5$	778.7	796.8
48:6	$C_{51}H_{88}O_5$	780.7	798.7
48:5	$C_{51}H_{90}O_5$	782.7	800.7
48:4	$C_{51}H_{92}O_5$	784.7	802.7

MeDAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4]^+$
48:2	$C_{51}H_{94}O_5$	786.7	804.8
48:3	$C_{51}H_{96}O_5$	788.7	806.8
48:1	$C_{51}H_{98}O_5$	790.7	808.8
48:0	$C_{51}H_{100}O_5$	792.8	810.8
49:5	$C_{52}H_{92}O_5$	796.7	814.7
49:4	$C_{52}H_{94}O_5$	798.7	816.7
49:3	$C_{52}H_{96}O_5$	800.7	818.8
49:2	$C_{52}H_{98}O_5$	802.7	820.8
49:1	$C_{52}H_{100}O_5$	804.8	822.8
49:0	$C_{52}H_{102}O_5$	806.8	824.8
50:5	$C_{53}H_{94}O_5$	810.7	828.7
50:4	$C_{53}H_{96}O_5$	812.7	830.8
50:3	$C_{53}H_{98}O_5$	814.7	832.8
50:2	$C_{53}H_{100}O_5$	816.8	834.8
50:1	$C_{53}H_{102}O_5$	818.8	836.8
50:0	$C_{53}H_{104}O_5$	820.8	838.8
51:5	$C_{54}H_{96}O_5$	824.7	842.8
51:4	$C_{54}H_{98}O_5$	826.7	844.8
51:3	$C_{54}H_{100}O_5$	828.8	846.8
51:2	$C_{54}H_{102}O_5$	830.8	848.8
51:1	$C_{54}H_{104}O_5$	832.8	850.8
51:0	$C_{54}H_{106}O_5$	834.8	852.8
52:5	$C_{55}H_{98}O_5$	838.7	856.8
52:4	$C_{55}H_{100}O_5$	840.8	858.8
52:3	$C_{55}H_{102}O_5$	842.8	860.8
52:2	$C_{55}H_{104}O_5$	844.8	862.8
52:1	$C_{55}H_{106}O_5$	846.8	864.8
52:0	$C_{55}H_{108}O_5$	848.8	866.8
53:5	$C_{56}H_{100}O_5$	852.8	870.8
53:4	$C_{56}H_{102}O_5$	854.8	872.8
53:3	$C_{56}H_{104}O_5$	856.8	874.8
53:2	$C_{56}H_{106}O_5$	858.8	876.8
53:1	$C_{56}H_{108}O_5$	860.8	878.9
53:0	$C_{56}H_{110}O_5$	862.8	880.9
54:6	$C_{57}H_{100}O_5$	864.8	882.8
54:5	$C_{57}H_{102}O_5$	866.8	884.8
54:4	$C_{57}H_{104}O_5$	868.8	886.8
54:3	$C_{57}H_{106}O_5$	870.8	888.8
54:2	$C_{57}H_{108}O_5$	872.8	890.9
54:1	$C_{57}H_{110}O_5$	874.9	892.9
54:0	$C_{57}H_{112}O_5$	876.9	894.9
55:6	$C_{58}H_{102}O_5$	878.8	896.8
55:5	$C_{58}H_{104}O_5$	880.8	898.8
55:4	$C_{58}H_{106}O_5$	882.8	900.8
55:3	$C_{58}H_{108}O_5$	884.8	902.8
55:2	$C_{58}H_{110}O_5$	886.8	904.8
55:1	$C_{58}H_{112}O_5$	888.9	906.9
55:0	$C_{58}H_{114}O_5$	890.9	908.9
56:6	$C_{59}H_{104}O_5$	892.8	910.8
56:5	$C_{59}H_{106}O_5$	894.8	912.8
56:4	$C_{59}H_{108}O_5$	896.8	914.9
56:3	$C_{59}H_{110}O_5$	898.8	916.9
56:2	$C_{59}H_{112}O_5$	900.9	918.9
56:1	$C_{59}H_{114}O_5$	902.9	920.9
56:0	$C_{59}H_{116}O_5$	904.9	922.9
57:6	$C_{60}H_{106}O_5$	906.8	924.8
57:5	$C_{60}H_{108}O_5$	908.8	926.9
57:4	$C_{60}H_{110}O_5$	910.8	928.9
57:3	$C_{60}H_{112}O_5$	912.9	930.9
57:2	$C_{60}H_{114}O_5$	914.9	932.9
57:1	$C_{60}H_{116}O_5$	916.9	934.9
57:0	$C_{60}H_{118}O_5$	918.9	936.9
58:6	$C_{61}H_{108}O_5$	920.8	938.9
58:5	$C_{61}H_{110}O_5$	922.8	940.9
58:4	$C_{61}H_{112}O_5$	924.9	942.9
58:3	$C_{61}H_{114}O_5$	926.9	944.9
58:2	$C_{61}H_{116}O_5$	928.9	946.9
58:1	$C_{61}H_{118}O_5$	930.9	948.9
58:0	$C_{61}H_{120}O_5$	932.7	950.9
60:12	$C_{63}H_{100}O_5$	936.8	954.8
59:2	$C_{62}H_{118}O_5$	942.9	960.9
59:1	$C_{62}H_{120}O$	944.9	962.9
59:0	$C_{62}H_{122}O_5$	946.9	964.9
60:4	$C_{63}H_{116}O_5$	952.9	970.9
60:3	$C_{63}H_{118}O_5$	954.9	972.9
60:2	$C_{63}H_{120}O_5$	956.9	974.9
60:1	$C_{63}H_{122}O_5$	958.9	976.9
60:0	$C_{63}H_{124}O_5$	960.9	978.9

D. Quantitation of Diacylglycerols

Diacyglycerols are quantified by integration of molecular ions (as ammonium ion adducts) from constant neutral loss mass spectra and application of standard curves that relate the responses of known amounts of reference standards to that for a single internal standard for both the 1,2- and 1,3-DAG species. The curves for quantitation of 1,2- and 1,3-DAGs was prepared from solutions of 1,2-16:0/18:0-DAG at 0, 1.2, 6.2, 12.4, 31, 62, 124, and 248 pg/75µL and 1,3-18:0/18:0 DAG at 0, 1.1, 5.6, 11.2, 28, 56, 112, and 224 pg/75µL that also contain the same amount of IS that is used for extracts of cells. Final calculations of amounts of analyte per sample require isotope corrections and application of deisotope factors. Some species in the table will be present at levels below the limit of quantitation.

Constant Neutral Loss Conditions

Polarity –	Positive
Scan mode –	Profile
Ion source –	Turbo Spray
Neutral loss –	190.1 Da
Resolution Q1 –	Unit
Resolution of Q3	Unit
Intensity threshold –	0.00 cps
Settling Time –	5.000 ms
MR pause –	5.0070 ms
MCA –	No
Center/Width –	No
Step size –	0.1 amu
Scan range –	500-1200 amu
Scan time –	5 s

Mass Spectrometer Parameters

Cur –	10.0
IS –	5500.0
TEM –	300.0
GS1 –	40.0
GS2 –	20.0
ihe –	ON
CAD –	7.0
DP –	85.0
EP –	9.0
CE –	52.0
CXP –	6.0

Diacylglycerol Ions

DAG (CN:DB)	**Formula**	**Molecular Mass**	**Mass** $[NH_4\text{-DFPI}]^+$	**DAG** (CN:DB)	**Formula**	**Molecular Mass**	**Mass** $[NH_4\text{-DFPI}]^+$
30:2	$C_{33}H_{60}O_5$	536.4	709.5	36:2	$C_{39}H_{72}O_5$	620.5	793.6
30:1	$C_{33}H_{62}O_5$	538.5	711.5	36:1	$C_{39}H_{74}O_5$	622.6	795.6
30:0	$C_{33}H_{64}O_5$	540.5	713.5	36:0	$C_{39}H_{76}O_5$	624.6	797.6
32:5	$C_{35}H_{58}O_5$	558.5	731.5	37:5	$C_{40}H_{68}O_5$	628.5	801.5
32:4	$C_{35}H_{60}O_5$	560.5	733.5	37:4	$C_{40}H_{70}O_5$	630.5	803.5
32:3	$C_{35}H_{62}O_5$	562.5	735.5	37:3	$C_{40}H_{72}O_5$	632.5	805.5
32:2	$C_{35}H_{64}O_5$	564.5	737.5	37:2	$C_{40}H_{74}O_5$	634.5	807.5
32:1	$C_{35}H_{66}O_5$	566.5	739.5	37:1	$C_{40}H_{76}O_5$	636.5	809.5
32:0	$C_{35}H_{68}O_5$	568.5	741.5	37:0	$C_{40}H_{78}O_5$	638.5	811.5
33:1	$C_{36}H_{68}O_5$	580.5	753.5	38:6	$C_{41}H_{68}O_5$	640.5	813.6
33:0	$C_{36}H_{70}O_5$	582.5	755.5	38:5	$C_{41}H_{70}O_5$	642.5	815.6
34:4	$C_{37}H_{64}O_5$	588.5	761.5	38:4	$C_{41}H_{72}O_5$	644.5	817.5
34:3	$C_{37}H_{66}O_5$	590.5	763.5	38:3	$C_{41}H_{74}O_5$	646.6	819.6
34:2	$C_{37}H_{68}O_5$	592.5	765.5	38:2	$C_{41}H_{76}O_5$	648.6	821.6
34:1	$C_{37}H_{70}O_5$	594.5	767.5	38:1	$C_{41}H_{78}O_5$	650.6	823.6
34:0	$C_{37}H_{72}O_5$	596.5	769.5	38:0	$C_{41}H_{80}O_5$	652.6	825.6
35:4	$C_{38}H_{64}O_5$	600.5	773.5	40:7	$C_{43}H_{70}O_5$	666.5	839.6
35:3	$C_{38}H_{66}O_5$	602.5	775.5	40:6	$C_{43}H_{72}O_5$	668.5	841.6
35:2	$C_{38}H_{68}O_5$	604.5	777.5	40:5	$C_{43}H_{74}O_5$	670.6	843.6
35:1	$C_{38}H_{70}O_5$	606.5	779.5	40:4	$C_{43}H_{76}O_5$	672.6	845.6
35:0	$C_{38}H_{72}O_5$	608.5	781.5	40:3	$C_{43}H_{78}O_5$	674.6	847.6
36:5	$C_{39}H_{66}O_5$	614.5	787.5	40:2	$C_{43}H_{80}O_5$	676.6	849.6
36:4	$C_{39}H_{68}O_5$	616.5	789.5	40:1	$C_{43}H_{82}O_5$	678.6	851.6
36:3	$C_{39}H_{70}O_5$	618.5	791.6	40:0	$C_{43}H_{84}O_5$	680.6	853.6
				IS	$C_{43}{}^2H_5H_{79}O_5$	685.6	858.6

E. Quantitation of Monoacylglycerols

Monoacylglycerols are quantified using the same internal and reference standards as for DAGs (see **D** above). Final calculations of amounts of analyte per sample require isotope corrections and application of deisotope factors. Some species in the table will be present at levels below the limit of quantitation.

Constant Neutral Loss Conditions

Polarity –	Positive
Scan mode –	Profile
Ion source –	Turbo Spray
Neutral Loss –	190.1 Da
Resolution Q1 –	Unit
Resolution of Q3	Unit
Intensity threshold –	0.00 cps
Settling Time –	5.000 ms
MR pause –	5.0070 ms
MCA –	No
Center/Width –	No
Step size –	0.1 amu
Scan range –	500-1200 amu
Scan time –	5 s

Mass Spectrometer Parameters

Cur –	10.0
IS –	5500.0
TEM –	300.0
GS1 –	40.0
GS2 –	20.0
ihe –	ON
CAD –	7.0
DP –	85.0
EP –	9.0
CE –	52.0
CXP –	6.0

Monoacylglycerols Ions

MAG (CN:DB)	**Formula**	**Molecular Mass**	**Mass** $[NH_4]^+$	**Mass** $[NH_4\text{-DFPI}]^+$
14:1	$C_{17}H_{32}O_4$	300.2	318.3	628.3
14:0	$C_{17}H_{34}O_4$	302.2	320.3	630.3
15:1	$C_{18}H_{34}0_4$	314.3	332.3	642.3
15:0	$C_{18}H_{36}0_4$	316.3	334.3	644.3
16:2	$C_{19}H_{34}0_4$	326.3	344.3	654.3
16:1	$C_{19}H_{36}0_4$	328.3	346.3	656.3
16:0	$C_{19}H_{38}0_4$	330.3	348.3	658.3
17:1	$C_{20}H_{38}0_4$	342.3	360.3	670.3
17:0	$C_{20}H_{40}0_4$	344.3	362.3	672.3
18:3	$C_{21}H_{36}0_4$	352.3	370.3	680.3
18:2	$C_{21}H_{38}0_4$	354.3	372.3	682.3
18:1	$C_{21}H_{40}0_4$	356.3	374.3	684.3
18:0	$C_{21}H_{42}0_4$	358.3	376.3	686.3
20:4	$C_{23}H_{38}0_4$	378.3	396.3	706.3
20:3	$C_{23}H_{40}0_4$	380.3	398.3	708.3
20:2	$C_{23}H_{42}0_4$	382.3	400.3	710.3
20:1	$C_{23}H_{44}0_4$	384.4	402.4	712.4
20:0	$C_{23}H_{46}0_4$	386.3	404.3	714.3
22:6	$C_{25}H_{38}0_4$	402.3	420.3	730.3
22:5	$C_{25}H_{40}0_4$	404.3	422.3	732.3
22:4	$C_{25}H_40_4$	406.3	424.3	734.3
22:2	$C_{25}H_{46}0_4$	410.3	428.3	738.3
22:1	$C_{25}H_{48}0_4$	412.3	430.3	740.3
22:0	$C_{25}H_{50}0_4$	414.3	432.3	742.3