Fatty Acid Mass Spectrometry Protocol
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Synopsis:

This protocol describes the standard method for extracting and quantifying free fatty acids and total fatty acids via negative ion chemical ionization GC-MS. Samples can be cells, media, plasma, or tissue. Methanol, HCl and deuterated internal standards are added to cell or media samples that are then extracted with iso-octane and derivatized to pentafluorobenzyl esters for GC analysis. Samples are then analyzed by GC-MS along with a standard curve consisting of unlabeled primary fatty acid standards mixed with the same deuterated internal standards added to the samples. The ratios of unlabeled to labeled standard are measured for the standard curve and used to determine unlabeled analyte levels for samples. All operating parameters for the instrument are contained in the raw data files, all other conditions are listed here.

Storage: All samples and standards are stored under argon at -20°C.

Internal Standards

The internal standard contains 25 ng (0.25 ng/µl) of each of the following deuterated fatty acids in 100% ethanol:

Name	Abbrev.	Company	Amt. (mg)	Cat. #
Lauric Acid (d3)	12:0	CDN Isotopes	100	D-4027
Myristic Acid (d3)	14:0	Cambridge Isotopes	100	DLM-1039-0.1
Pentadecanoic Acid (d3)	15:0	CDN Isotopes	100	D-5258
Palmitic Acid (d3)	16:0	CDN Isotopes	100	D-1655
Heptadecanoic Acid (d3)	17:0	CDN Isotopes	100	D-5255
Stearic Acid (d3)	18:0	CDN Isotopes	100	D-1825
Oleic Acid (d2)	18:1	Cambridge Isotopes	100	DLM-689-0.1
Arachidic Acid (d3)	20:0	CDN Isotopes	0.1/200uL	D-5254
Arachidonic Acid (d8)	20:4	Cayman Chemical	1/100uL	390010
Eicosapentaenoic Acid (d5)	20:5	Cayman Chemical	100	10005056
Behenic Acid (d3)	22:0	CDN Isotopes	0.1/200uL	D-5708
Docosahexaenoic Acid (d5)	22:6	Cayman Chemical	100	10005057
Lignoceric Acid (d4)	24:0	CDN Isotopes	100	D-6167
Cerotic Acid (d4)	26:0	CDN Isotopes	100	D-6145

Stocks are maintained at 10X concentration (2.5 ng/ul) in 100% ethanol and diluted to working strength at extraction time. 100 µl of 1X internal standard mix is added to each sample just prior to extraction.

Primary Standards

The primary standard contains unlabeled analytes at known concentrations. A concentrated stock at 10 ng/ul in 100% ethanol is made and serial diluted to create a standard curve.

Name	Abbrev.	Company	Amt. (mg)	Cat. #
Lauric Acid	12:0	NuChek	100	N-12-A
Myristic Acid	14:0	NuChek	100	N-14-A
Pentadecanoic Acid	15:0	NuChek	100	N-15-A
Palmitic Acid	16:0	NuChek	100	N-16-A
Palmitoleic Acid	16:1	NuChek	100	U-40-A
Heptadecanoic Acid	17:0	NuChek	100	N-17-A
Heptadecaenoic Acid	17:1	NuChek	100	U-42-A
Stearic Acid	18:0	NuChek	100	N-18-A
Oleic Acid	18:1	NuChek	100	U-46-A
Linoleic Acid	18:2	NuChek	100	U-59-A
α-linolenic Acid	18:3 N-3	NuChek	100	U-62-A
γ-linolenic Acid	18:3 N-6	NuChek	100	U-63-A
Stearidonic Acid	18:4	Cayman	50	90320
Arachidic Acid	20:0	NuChek	100	N-20-A
Gondolic Acid	20:1	NuChek	100	U-65-A
Eicosadienoic Acid	20:2	NuChek	100	U-68-A
Eicosatrienoic Acid	20:3 N-3	NuChek	100	U-79-A
Dihomo-g-linolenic	20:3 N-6	NuChek	100	U-69_A
Eicosatrienoic Acid	20:3 N-9	Cayman	10	90190
Arachidonic Acid	20:4	NuChek	100	U-71-A
Eicosapentaenoic Acid	20:5	NuChek	100	U-99-A
Behenic Acid	22:0	NuChek	100	N-22-A
Docosaenoic Acid	22:1	NuChek	100	U-79-A
Docosadienoic Acid	22:2	NuChek	100	U-81-A
Docosatrienoic Acid	22:3	NuChek	100	U-82-A
Adrenic Acid	22:4	NuChek	100	U-83-A
Docosapentaenoic Acid	22:5 N-3	NuChek	100	U-101-A
Docosapentaenoic Acid	22:5 N-6	NuChek	100	U-102-A
Docosahexaenoic Acid	22:6	NuChek	100	U-84-A
Tricosanoic Acid	23:0	NuChek	100	N-23-A
Tricosenoic Acid	23:1	NuChek	100	U-87-A
Lignoceric Acid	24:0	NuChek	100	N-24-A
Nervonic Acid	24:1	NuChek	100	U-88-A
Cerotic Acid	26:0	Cayman	100	13354

Standard Curve

Standard curve samples are created by adding 50 µl of each primary standard dilution to 100 µl internal standard in washed 10 mm x 75 mm glass tubes.

Primary Standard Concentration (ng/µl)	µl used	Total ng	µl internal standard
10.0	50	500.0	100

3.0	50	150.0	100
1.0	50	50.0	100
0.3	50	15.0	100
0.1	50	5.0	100
0.03	50	1.5	100
0.01	50	0.5	100
0.003	50	0.15	100

The samples are dried down under vacuum using a speedvac and derivatized by adding 25 µl 1% pentafluorobenzyl bromide in acetonitrile and 1% diisopropylethylamine in acetonitrile. After incubation at room temperature for 20 minutes, standard curve samples are dried under vacuum in a speedvac and 50 µl iso-octane is added to dissolve the samples. 1 µl is injected for GC-MS analysis.

GC conditions:

Agilent 6890N Gas Chromatograph
0.9 ml/min (50 cm/sec) Helium carrier gas (ultra high purity)
Zebron ZB-1 100% dimethylpolysiloxane column (15 m x 0.25 mm ID x 0.10 mm film thickness)
250°C injector temp
pulsed splitless mode (25 psi pulse)
280°C sample transfer line

Gradient:

150°C start
10°C/min to 270°C
40°C/min to 310°C, 1 minute hold

Mass Spectrometer conditions:

Agilent 5975 Mass Selective Detector
Methane reagent gas (ultra high purity)
Reagent gas flow = 40%
Negative ion chemical ionization
1 minute solvent delay
150°C Quad temp
280°C Source temp
200 eV
Selected Ion Monitoring (SIM), low resolution
10 ms dwell time on all ions

SIM ions and groups:

Group 1 (9 to 12 carbons): 1.00 to 4.70 minutes
Ions: 157, 171, 185, 199, 202, 213
Analytes: 9:0, 10:0, 11:0, 12:0, 12:0-d_3, 13:0

Group 2 (14 to 15 carbons) 4.90 to 6.25 minutes
Ions: 227, 230, 239, 241, 244
Analytes: 14:0, 14:0-d_3, 15:1, 15:0, 15:0-d_3

Group 3 (16 to 17 carbons) 6.25 to 7.70 minutes
Ions: 253, 255, 258, 267, 269, 272
Analytes: 16:1, 16:0, 16:0-d_3, 17:1, 17:0, 17:0-d_3

Group 4 (18 carbons) 7.70 to 8.80 minutes
Ions: 275, 277, 279, 281, 283, 286
Analytes: 18:4, 18:3, 18:2, 18:1, 18:1-d_2, 18:0, 18:0-d_3

Group 5 (20 carbons) 8.80 to 9.90 minutes
Ions: 299, 301, 303, 305, 306, 307, 309, 311, 314
Analytes: 20:6, 20:5, 20:4, 20:3, 20:5-d_5, 20:2, 20:1, 20:4-d_8, 20:0, 20:0-d_3

Group 6 (22 carbons) 9.90 to 11.30 minutes
Ions: 327, 329, 331, 332, 333, 335, 337, 339, 342
Analytes: 22:6, 22:5, 22:4, 22:6-d_5, 22:3, 22:2, 22:1, 22:0, 22:0-d_3

Group 7 (23 to 24 carbons) 11.30 to 12.60 minutes
Ions: 353, 365, 367, 371
Analytes: 23:0, 24:1, 24:0, 24:0-d_4

Group 8 (26 carbons) 12.60 to 14.00 minutes
Ions: 387, 389, 391, 393, 395, 399, 401
Analytes: 26:4, 26:3, 26:2, 26:1, 26:0, 26:0-d_4, extra ion for data export

Group Times may vary depending on column and GC/MS unit.

Data from the run is exported to NetCDF format using the "Export Data to AIA format" function (under the File menu) in the Agilent Enhanced Data Analysis program. CDF files generated here can be translated to .wiff files for data analysis by ABI Analyst using the **translat.exe** program found in the "bin" subdirectory of the main Analyst directory.

Sample Preparation:

Free fatty acids are extracted from cells, media, plasma, or tissue by iso-octane.

Cells:
Cell number should be kept below 2 million (common use is 0.5 million cells). 100 uL Internal Standard is added. Two volumes methanol lyses the cells and the mixture is acidified with HCl to 25 mM final concentration.

Media:
100uL internal standard is added to 0.5 ml media. Then samples is mixed with 1 volume methanol and acidified with HCl to 25 mM final concentration.

Plasma:
For blood plasma, 200uL is added to 300uL dPBS. Then 100uL Internal Standard is added and mixed with 1 volume methanol and acidified with HCL to 25 mM final concentration.

Tissue:
For Tissue, the amount used should be empirically determined beforehand based on tissue type used. Add 100uL Internal Standard. Then add 900uL methanol. For tougher Tissues, samples are ground/minced, then sonicated. For softer tissues, just sonication is required. Sample is then mixed with 1 volume methanol and acidified with HCL to 25 mM final concentration.

Sample Extraction:

Cells: 250uL of ~500,000 cells
Media: 0.5mL
Plasma: 0.5mL (200uL sample)
Tissue: 1mL

Procedure:

1. 1.Using a 16 mm x 125 mm glass tube, prepare the sample as stated above based on sample type. 3 extra samples with only Internal Standard and dPBS are also prepared.

2. 1mL iso-octane is added, and the sample is vortexed and centrifuged at 3000 x g for 1 minute to separate layers. The top layer is removed and transferred to a 10 mm x 75 mm glass tube.

3. Repeat step 2.

4. For Free Fatty Acids only go to Step 6. For Total Fatty Acids add 100uL internal Standard to the methanol fraction. Then add 500uL 1N KOH to the remaining methanol fraction, vortex, and Incubate for 1 hr. Then ad 500uL 1N HCL, check pH (should be under 5).

5. Repeat Steps 2 and 3.

6. Dry down under vacuum using speedvac.

7. Derivatize samples by adding 25 µl 1% pentafluorobenzyl bromide in acetonitrile, and 25 µl 1% diisopropylethylamine in acetonitrile. Cap tubes with rubber caps, vortex, and let stand at room temperature for 20 minutes.

8. Dry down under vacuum using speedvac.

9. Dissolve samples in 50 µl iso-octane and transfer to labeled sample vial with 250 µl glass insert. Cap and place samples in the GC-MS sample tray and begin analysis.

   Note: Tubes, plastic tips, and solvents all contain Fatty Acid Contamination. To minimize this, use all glassware whenever possible. Also keep tubes unwashed as to not further introduce contamination. Contamination is subtracted later using internal standards.

Analyte Ion and Elution Table:

Internal Standard

Compound	SIM Ion	Elution time (min)
Lauric Acid (12:0-d_3)	202	3.74
Myristic Acid (14:0-d_3)	230	5.27
Pentadecanoic Acid (15:0-d_3)	244	6.04
Palmitic Acid (16:0-d_3)	258	6.79
Heptadecanoic Acid (17:0-d_3)	272	7.53
Oleic Acid (18:1-d_2)	283	8.02
Stearic Acid (18:0-d_3)	286	8.25
Eicosapentaenoic Acid (20:5-d_5)	306	8.96
Arachidonic Acid (20:4-d_8)	311	8.94
Arachidic Acid (20:0-d_3)	314	9.63
Docosahexaenoic Acid (22:6-d_5)	332	10.15
Behenic Acid (22:0-d_3)	342	10.92
Lignoceric Acid (24:0-d_4)	371	12.14
Hexacosanoic Acid (26:0-d_4)	399	12.90

Primary Standard

Compound	SIM Ion	Elution time (min)
Lauric Acid (12:0)	199	3.75
Myristic Acid (14:0)	227	5.28
Pentadecanoic Acid (15:0)	241	6.04
Palmitoleic Acid (16:1)	253	6.60
Palmitic Acid (16:0)	255	6.80
Heptadecaenoic Acid (17:1)	267	7.35
Heptadecanoic Acid (17:0)	269	7.54
Stearidonic Acid (18:4)	275	7.77
□-linolenic Acid (18:3)	277	7.75
□-linolenic Acid (18:3)	277	7.99
Linoleic Acid (18:2)	279	7.96
Oleic Acid (18:1)	281	8.02
Stearic Acid (18:0)	283	8.25
Eicosapentaenoic Acid (20:5)	301	8.97
Arachidonic Acid (20:4)	303	8.95
Eicosatrienoic Acid (20:3, n-3)	305	9.01
Dihomo-□-linolenic Acid (20:3)	305	9.17
Eicosatrienoic Acid (20:3, n-9)	305	9.40
Eicosadienoic Acid (20:2)	307	9.37
Gondolic Acid (20:1)	309	9.42
Arachidic Acid (20:0)	311	9.63
Docosahexaenoic Acid (22:6)	327	10.16

Docosapentaenoic Acid (22:5, n-3)	329	10.13
Docosapentaenoic Acid (22:5, n-6)	329	10.35
Adrenic Acid (22:4)	331	10.32
Docosatrienoic Acid (22:3)	333	10.73
Docosadienoic Acid (22:2)	335	10.70
Docosaenoic Acid (22:1)	337	10.74
Behenic Acid (22:0)	339	10.92
Tricosanoic Acid (23:0)	353	11.54
Nervonic Acid (24:1)	365	11.98
Lignoceric Acid (24:0)	367	12.14
Cerotic Acid (26:0)	395	12.91