Fatty Acid Mass Spectrometry Protocol
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Synopsis:
This protocol describes the standard method for extracting and quantifying free fatty acids found in cells and media via negative ion chemical ionization GC-MS. 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:

Compound	Deuteration	Supplier	Catalog number
Lauric Acid (12:0)	d_3	CDN Isotopes	D-4027
Myristic Acid (14:0)	d_3	Cambridge Isotopes	DLM-1039-0.1
Pentadecanoic Acid (15:0)	d_3	CDN Isotopes	D-5258
Palmitic Acid (16:0)	d_3	CDN Isotopes	D-1655
Heptadecanoic Acid (17:0)	d_3	CDN Isotopes	D-5255
Linoleic Acid (18:2)	d_4	Cayman Chemical	390150
Oleic Acid (18:1)	d_2	Cambridge Isotopes	DLM-689-0.1
Stearic Acid (18:0)	d_3	CDN Isotopes	D-1825
Eicosapentaenoic Acid (20:5)	d_5	Cayman Chemical	10005056
Arachidonic Acid (20:4)	d_8	Cayman Chemical	390010
Arachidic Acid (20:0)	d_3	CDN Isotopes	D-5254
Docosahexaenoic Acid (22:6)	d_5	Cayman Chemical	10005057
Behenic Acid (22:0)	d_3	CDN Isotopes	D-5708
Lignoceric Acid (24:0)	d_4	CDN Isotopes	D-6167
Hexacosanoic Acid (26:0)	d_4	CDN Isotopes	D-6145

Stocks are maintained at 10X concentration (2.5 ng/μl) 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/μl in 100% ethanol is made and serial diluted to create a standard curve.

Compound	Supplier	Catalog number
Lauric Acid (12:0)	Cayman Chemical	10006626
Myristic Acid (14:0)	Sigma Aldrich	70079
Pentadecanoic Acid (15:0)	Sigma Aldrich	P3600
Palmitoleic Acid (16:1)	Sigma Aldrich	76169
Palmitic Acid (16:0)	Cayman Chemical	10006627
Heptadecaenoic Acid (17:1)	Sigma Aldrich	H8896
Heptadecanoic Acid (17:0)	Sigma Aldrich	51610
Stearidonic Acid (18:4)	Cayman Chemical	90320
α-linolenic Acid (18:3)	Cayman Chemical	90210
γ-linolenic Acid (18:3)	Cayman Chemical	90220

Linoleic Acid (18:2)	Cayman Chemical	90150
Oleic Acid (18:1)	Cayman Chemical	90260
Stearic Acid (18:0)	Sigma Aldrich	85679
Eicosapentaenoic Acid (20:5)	Cayman Chemical	90110
Arachidonic Acid (20:4)	Cayman Chemical	90010
Eicosatrienoic Acid (20:3, n-3)	Cayman Chemical	90192
Dihomo-γ-linolenic Acid (20:3)	Cayman Chemical	90230
Eicosatrienoic Acid (20:3, n-9)	Cayman Chemical	90190
Eicosadienoic Acid (20:2)	Cayman Chemical	90330
Arachidic Acid (20:0)	Sigma Aldrich	10930
Docosahexaenoic Acid (22:6)	Cayman Chemical	90310
Docosapentaenoic Acid (22:5)	Cayman Chemical	90165
Adrenic Acid (22:4)	Cayman Chemical	90300
Docosatrienoic Acid (22:3)	Cayman Chemical	90170
Docosadienoic Acid (22:2)	Sigma Aldrich	D4159
Docosaenoic Acid (22:1)	Cayman Chemical	90175
Behenic Acid (22:0)	Sigma Aldrich	11909
Tricosanoic Acid (23:0)	Sigma Aldrich	T6543
Nervonic Acid (24:1)	Sigma Aldrich	N1514
Lignoceric Acid (24:0)	Sigma Aldrich	L6641
Cerotic Acid (26:0)	Sigma Aldrich	H0388

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) 4.70 to 6.15 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) 6.15 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 8.90 minutes
Ions: 275, 277, 279, 281, 283, 286
Analytes: 18:4, 18:3, 18:2, 18:1, 18:2-d_4, 18:1-d_2, 18:0, 18:0-d_3

Group 5 (20 carbons) 8.90 to 9.85 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) 9.85 to 11.10 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) 11.10 to 12.40 minutes
Ions: 353, 365, 367, 371
Analytes: 23:0, 24:1, 24:0, 24:0-d_4

Group 8 (26 carbons) 12.40 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

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 Extraction

Free fatty acids are extracted from either cells or media by iso-octane. Cell number should be kept below 2 million (common use is 0.5 million cells). Addition of two volumes methanol lyses the cells and the mixture is acidified with HCl to 25 mM final concentration. For media, 0.5 ml media is mixed with 1 volume methanol and acidified with HCl to 25 mM final concentration. Internal standard is added and the sample is extracted twice with iso-octane and the upper layers pooled. Extracted fatty acids are dried down under vacuum and derivatized with pentafluorobenzyl bromide, dried down under vacuum and dissolved in 50 μl iso-octane. 1 μl derivatized sample is loaded onto the GC-MS for analysis.

Note: Due to fatty acid contamination, all glassware used in the procedure should be washed with soap (Fisher Versa-Clean) and water, then thoroughly washed with dH_2O.

Extraction of cell samples

1. Using a washed 16 mm x 125 mm glass tube, two volumes methanol is added to a minimum volume of 0.25 ml sample in DPBS (additional DPBS is added to bring lower volume samples up to 0.25 ml). 1 N HCl is added to 25 mM final concentration to acidify the mixture and 100 μl internal standard is added.

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

3. Repeat step 2.

4. Dry down under vacuum using speedvac.

5. Derivatize samples by adding 25 μl 1% pentafluorobenzyl bromide in acetonitrile, and 25 μl 1% diisopropylethylamine in acetonitrile. Cap tubes, mix and let stand at room temperature for 20 minutes.

6. Dry down under vacuum using speedvac.

7. 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.

Extraction of media samples

1. Using a washed 16 mm x 125 mm glass tube, one volume methanol is added to 0.5 ml media. 1 N HCl is added to 25 mM final concentration to acidify the mixture and 100 μl internal standard is added.

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

3. Repeat step 2.

4. Dry down under vacuum using speedvac

5. Derivatize samples by adding 25 μl 1% pentafluorobenzyl bromide in acetonitrile, and 25 μl 1% diisopropylethylamine in acetonitrile. Cap tubes, mix and let stand at room temperature for 20 minutes.

6. Dry down under vacuum using speedvac.

7. 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.

Analyte Ion and Elution Table

Internal Standard

Compound	SIM Ion	Elution time (min)
Lauric Acid (12:0-d_3)	202	3.81
Myristic Acid (14:0-d_3)	230	5.33
Pentadecanoic Acid (15:0-d_3)	244	6.09
Palmitic Acid (16:0-d_3)	258	6.84
Heptadecanoic Acid (17:0-d_3)	272	7.58
Linoleic Acid (18:2-d_4)	283	8.01
Oleic Acid (18:1-d_2)	283	8.08
Stearic Acid (18:0-d_3)	286	8.30
Eicosapentaenoic Acid (20:5-d_5)	306	9.02
Arachidonic Acid (20:4-d_8)	311	8.99
Arachidic Acid (20:0-d_3)	314	9.68
Docosahexaenoic Acid (22:6-d_5)	332	10.20
Behenic Acid (22:0-d_3)	342	10.97
Lignoceric Acid (24:0-d_4)	371	12.17
Hexacosanoic Acid (26:0-d_4)	399	12.92

Primary Standard

Compound	SIM Ion	Elution time (min)
Lauric Acid (12:0)	199	3.84
Myristic Acid (14:0)	227	5.35
Pentadecanoic Acid (15:0)	241	6.11
Palmitoleic Acid (16:1)	253	6.67
Palmitic Acid (16:0)	255	6.87
Heptadecaenoic Acid (17:1)	267	7.42
Heptadecanoic Acid (17:0)	269	7.60
Stearidonic Acid (18:4)	275	7.85
α-linolenic Acid (18:3)	277	7.82
γ-linolenic Acid (18:3)	277	8.06
Linoleic Acid (18:2)	279	8.02
Oleic Acid (18:1)	281	8.08
Stearic Acid (18:0)	283	8.32
Eicosapentaenoic Acid (20:5)	301	9.05
Arachidonic Acid (20:4)	303	9.02
Eicosatrienoic Acid (20:3, n-3)	305	9.08
Dihomo-γ-linolenic Acid (20:3)	305	9.24
Eicosatrienoic Acid (20:3, n-9)	305	9.46
Eicosadienoic Acid (20:2)	307	9.43
Arachidic Acid (20:0)	311	9.69
Docosahexaenoic Acid (22:6)	327	10.23
Docosapentaenoic Acid (22:5)	329	10.42
Adrenic Acid (22:4)	331	10.39
Docosatrienoic Acid (22:3)	333	10.78
Docosadienoic Acid (22:2)	335	10.76

Docosaenoic Acid (22:1)	337	10.80
Behenic Acid (22:0)	339	10.98
Tricosanoic Acid (23:0)	353	11.60
Nervonic Acid (24:1)	365	12.04
Lignoceric Acid (24:0)	367	12.18
Cerotic Acid (26:0)	395	12.93