

JOHN W. NEWMAN LAB

NON-ESTERIFIED FATTY ACID (NEFA) EXTRACTION & ANALYSIS PROTOCOL

Project: Rudy Ortiz WCMC Pilot & Feasibility Study

NEFA Extraction & Analysis

Plasma NEFA were isolated as previously described by Smedes (1). and Gladine et al. (2). Specifically, plasma aliquots (100 mL) were enriched with 5 mL 0.2 mg/ml butylated hydroxytoluene/EDTA in 1:1 methanol:-water, and a suite of extraction surrogates, which included deuterated-tri-palmitoyl glycerol (d31-16:0-TG; CDN Isotopes, Pointe-Claire, Quebec, Canada), deuterated distearoylphosphatidylcholine (d35-18:0-PC; Avanti Polar Lipids, Alabaster, Alabama), dodeca-(9E)-enoyl cholesterylestes (22:1n9-CE; NuChek Prep, Elysian MN) and dodecatrienoic acid (22:3n3; NuChek Prep). Lipids were then extracted with 10:8: 11 cyclohexane: 2-propanol:ammonium acetate. Briefly, enriched samples were mixed with cyclopropane/2-propanol, phases were split with ammonium acetate, the organic phase was isolated and the aqueous phase was re-extracted with cyclohexane. The combined organic total lipid extract was reduced to dryness and reconstituted in 200 μ L of 1:1 methanol/toluene and the total lipid extract was used to quantify plasma fatty acids as methyl esters by gas chromatography-mass spectrometry (GC-MS). They were spiked with 15:1n5 free acid to track methylation efficiency and brought to a final volume of 200 mL with 90:10 methanol/toluene (v/v) and left at room temperature for 30 min, before being brought to dryness. The remaining fatty acid methyl esters (FAMES) were re-constituted in 300 mL Hexane plus 10 μ L of 44 mM tricosanoate methyl ester (23:0; NuChek Prep), vortexed, and 100 μ L was transferred to a GC-MS Vial for analysis. A 1 μ L injection was analyzed by GC-MS on an Agilent 6890/5973N MSD (Agilent Technologies, San Jose, CA) with electron impact ionization and in simultaneous selected ion monitoring/full scan mode. Analytes were separated on a 30 m60.25 mm60.25 mm DB-225 ms. Analytes were quantified with ChemStation vE.02.14 (Agilent Technologies) using internal standard methodologies against a 5 to 8 pt calibration curves.

- (1) Smedes F (1999). Determination of total lipid using non-chlorinated solvents. *Analyst*. 124:1711-1718.
- (2) Gladine et al (2014). Lipid profiling following intake of the omega 3 fatty acid DHA identifies peroxidized metabolites F4-neuroprostanes as the best predictors of atherosclerosis prevention. *PLOS ONE*.

GC-MS Acquisition Method Parameters

6890 GC METHOD

OVEN

Initial temp: 64 'C (On) Maximum temp: 240 'C
Initial time: 1.00 min Equilibration time: 2.00 min
Ramps:
Rate Final temp Final time
1 33.00 196 0.00
2 4.00 240 7.00
3 0.0(off)
Post temp: 64 'C
Post time: 1.00 min
Run time: 23.00 min

FRONT INLET (SPLIT/SPLITLESS)

Mode: Splitless
Initial temp: 230 'C (On)
Pressure: 14.75 psi (On)
Purge flow: 15.0 mL/min
Purge time: 1.00 min
Total flow: 18.8 mL/min
Gas saver: On
Saver flow: 15.0 mL/min
Saver time: 3.00 min
Gas type: Helium

BACK INLET (SPLIT/SPLITLESS)

Mode: Splitless
Initial temp: 240 'C (On)
Pressure: 2.00 psi (On)
Purge flow: 31.9 mL/min
Purge time: 0.00 min
Total flow: 35.0 mL/min
Gas saver: Off
Gas type: Helium

COLUMN 1

Capillary Column
Model Number: Agilent 222-2932LTM
DB-225ms (G3900-63023)
Max temperature: 240 'C
Nominal length: 30.0 m
Nominal diameter: 250.00 um
Nominal film thickness: 0.25 um
Mode: ramped flow
Initial flow: 1.1 mL/min
Initial time: 12.00 min
Rate Final flow Final time
1 0.20 2.1 0.00
2 2.00 0.1 5.00
3 2.00 2.1 22.00
Post flow: 1.1 mL/min
Nominal init pressure: 14.76 psi
Average velocity: 26 cm/sec
Inlet: Front Inlet
Outlet: MSD
Outlet pressure: 2.00 psi

COLUMN 2

Capillary Column
Model Number: Agilent 222-2932LTM
DB-225ms (G3900-63023)
Max temperature: 240 'C
Nominal length: 30.0 m
Nominal diameter: 250.00 um
Nominal film thickness: 0.25 um
Mode: ramped pressure
Initial pressure: 2.00 psi
Initial time: 17.00 min
Rate Final pres Final time
1 30.00 32.00 5.00
2 0.0(off)
Post pressure: 2.00 psi
Nominal initial flow: 0.1 mL/min
Average velocity: 4 cm/sec
Inlet: Back Inlet
Outlet: MSD
Outlet pressure: ambient