Biomarker Analytical Laboratories

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Title: SOP for metabolite profiling of seminal plasma via GC Orbitrap
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Summary

Seminal plasma chemical profiles provide distinct and metabolic phenotypes suited for fertility study monitoring. Profiling via GC-MS is favourable as it provides robust measures across many core primary metabolic pathways; including amino acids, TCA metabolites, carbohydrates and some lipids (e.g. steroids & fatty acid). A protocol for simple preparation of seminal plasma samples intended to undergo GC-MS analysis is described and enables large-scale semi-quantitative profiling.

Preparation

Safety information

- Seminal plasma is classified as a biohazardous material & thus all items in contact (e.g. pipette tips, vials) should be disposed of appropriately.
- All work should be conducted in fume hood.
    - MeOx: Danger - corrosive, harmful, health hazard, environmental hazard
    - Pyridine: Danger - harmful, flammable
    - MSTFA: Warning - harmful, flammable
    - C_7-C_{40} alkane series (heptane) – flammable, harmful, health hazard, environmental hazard

Equipment

- 10 µL, 100 µL & 1 mL pipettes
- Centrifugal evaporator
- Storage boxes for 2 mL vials
- Heat block for glass vials
- Mini vortex
- Centrifuge with rotor for 1.5 mL Eppendorf vials
- Box with ice

Consumables & glassware

- 2 mL screw cap glass autosampler vials with built-in 200 µL insert and caps (PTFE septa)

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- 4 mL screw cap glass vial and cap
- 1.5 mL Eppendorf vial
- 10 µL, 100 µL & 1 mL tips
- Labels for 1.5 and 2 mL vials

Reagents

- Seminal plasma stored in -80 °C freezer and thawed at room temperature
- Methanol, LC-MS grade
- Chloroform, LC-MS grade
- GC grade anhydrous pyridine >99% purity
    - Pyridine will discolour to yellow if old or impure.
- C_7-C_{40} alkane series mix (10 µg/mL in pyrimidin/or isooctane)
- PCBs mix (1 µg/mL in pyridine/or isooctane)
- Methoxyamine hydrochloride (MeOx) > 97.5%
    - MeOx reacts with carbonyl groups (e.g. ketones, aldehydes) to form oxime and prevent numerous enol forms that gives rise to many peaks, especially for reducing sugars.
- N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) > 98%
    - MSTFA replaces free hydroxyl groups with trimethylsilyl (TMS) to reduce polarity and increase volatility (alcohol > phenol > carboxylic acid > amine > amide).
    - Alternatively can use MSTFA + 1% trimethylchlorosilane (TMCS) as an extra catalyst if many sites observed to be unreacted. (Typically, does not make a difference when using pyridine).

<u>MeOx solution</u> (20 mg/mL in pyridine)

- For 3 mL of solution, weigh 60 mg methoxyamine hydrochloride into glass tube.
- Add 3 mL of pyridine and vortex until dissolved.
    - Solution can be kept for 3 days at room temp. Store double wrapped (e.g. parafilm cap and place glass tube in plastic falcon).

<u>Standards</u>

[$^2H_4$] Succinic acid (99% isotope enrichment) – corrosive, harmful

[$^2H_{27}$] Myristic acid (98% isotope enrichment) - harmful

<u>Procedure</u>

Standard preparation

1. Prepare 1 mL alikvot of stock [$^2H_4$] Succinic acid c = 100 µg/mL in methanol
2. Prepare 1 mL alikvot of stock [$^2H_{27}$] Myristic acid c = 100 µg/mL in chloroform

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3. Pipette directly or dry the internal reference standard mix down in Centrifugal evaporator at laboratory temperature, store in -80 °C and reconstitute in the solvent before analysis.

Sample preparation

1. NOTE: Store seminal plasma samples at – 80 °C in a glass tubes
2. Refrigerate sample at laboratory temperature
3. Vortexed for 10 seconds, centrifuge for 10 minutes at 4000 RPM
4. Pipette 10 µL of the supernatant to 1.5 mL glass vials with built-in 200 µL insert
5. Pipette 10 µL of $[^2H_4]$ Succinic acid c = 100 µg/mL in methanol
6. Pipette 10 µL of $[^2H_{27}]$ Myristic acid c = 100 µg/mL in chloroform
7. For every 10 samples prepare 1 QC sample (pool of seminal plasma), 1 processing blank (pipetting 10 µL of milliQ water). QC sample and processing blank are prepared with samples
8. Dry the sample down in Centrifugal evaporator at laboratory temperature, store in – 80 °C
9. Derivatize dried samples

Sample derivatisation

1. Remove samples from freezer, decap and leave for 20 minutes in fume hood.
    a. Moisture prevents derivatisation reaction.
2. Pre-heat dry heat block to 60 °C.
3. Add 30 µL MeOX solution to each dried sample
4. Cap immediately and incubate in a heating block for 60 minutes at 60 °C
5. Add 70 µL of MSTFA to each sample
6. Cap immediately and incubate in a heating block for 60 minutes at 60 °C
7. After the time has elapsed cooled down samples for 2 minutes on ice
    - Cap and measure on GC-Q Exactive immediately or maximum within 24 hours/ If required, replace vials caps prior to re-injection of sample.

GC-MS analysis

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2. Before measurement replace liner and septa per ~150-200 samples
3. Calibrate and Tune MS
4. Input sequence
5. Start GC-MS analysis run
6. Check initial blanks, alkane series, PCBs series and QC samples.
    - If problems, stop run, wrap samples in parafilm and store at -20 °C whilst troubleshooting.
    - Can do initial checks of blanks / alkane series/PCBs series prior to derivatisation if samples are precious.

GC sequence setup

1. Pyridine blank
2. Alkanes
3. PCBs
4. Pyridine blank
5. Pyridine blank
6. QC_dilution series in 3 replicates (same sample preparation as QC with 2x, 4x, 8x, 16x, 32x, 64x dilution & spiked with labeled standards same as QC (samples)
7. Procedural blank
8. QC sample
9. Samples 1-10
10. Procedural blank
11. QC sample
12. Sample 11-20
13. Procedural blank
14. End with Alkanes, PCBs and 3 pyridine blanks

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GC-MS method setup

Autosampler settings

TriPlus RSH Autosampler		Comments
General		
Injection port		
Injector	Injector A (SSL)	
Type	Single	
Injection mode		
Mode	Basic	
Rapid mode		
Rapid mode	Disable	
Syringe type		
Syringe volume (µL)	10	
Needle length (mm)	57	
Sampling		
Sample volume (µL)	2	*Set in method sequence to be 1 uL*
Plunger strokes	0	
Air and filling mode	Custom	
Air volume (µL)	0	
Filling volume (µL)	0	
Sampling depth in vials		
Sample vial depth (mm)	\	
Bottom sense	Yes	
Height from bottom (mm)	1.5	
Injection		
Injection depth	Custom	
Pre-injection dwell time (s)	0	
Post-injection dwell time (s)	0.1	
Fast injection	Off	
Injection depth (mm)	45	
Penetration speed (mm/s)	90	
Injection Speed (µL/s)	50.0	
Sample viscosity		
Sample type	Custom	
Sample pullup speed (µL/s)	0.4	
Delay after plunger strokes (s)	1.5	
Viscosity delay (s)	2	

Washes		Solvent A: nonane
Washes		Solvent B: toluene
Number of solvents(s)	Multiple	Solvent C: acetone
Wash station	Standard wash station	Solvent D: nonane
Pre-injection		E position: waste

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Solvent	A	\	\	\
Cycles		4		
Solvent volume (uL)		7.0		
Rinse				
Rinses		0		
Rinse Volume (Ul)		1		
Post-injection				
Solvent	\	B	C	D
Cycles		7		
Solvent volume (µL)		7.0		
Sync				
GC synchro start				
Synchro type		Standard		
Advanced				
Advanced parameters				
Wash solvent depth (mm)		45		
Waste depth (mm)		10		
Needle speed in vial (mm/s)		10		
Solvent filling speed (µL/s)		1		
Bubble elimin. pullup (µL/s)		5		
Delay between strokes (s)		2.0		

GC settings

TRACE 1310 Series GC

Oven

Ramps

	Rate (°C/min)	Temp (°C)	Hold time (min)
Initial		80	0.5
1	40	200	0.5
2	40	260	0.5
3	55	330	4.0

Options

Max temp.	340 °C
Prep-run timeout	10 min
Equilibration time	1 min
Ready delay	0.00 min
Oven on	Yes

Comments

Trace 1310
Column: Rxi-5SilMS (15m x 0.25 mm x 0.25 um) with 2m guard (0.25 mm) at transfer & 2m guard (0.53 mm) at inlet.

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S/SL (front)			Septa: Merlin microseal Liner: Restek Topaz 4.0mm precision liner w/ wool
S/SL mode	Split		
Inlet			
Temp	On	290 °C	
Split flow	On	24.0 mL/min	
Split ratio	20		
Splitless time	0.80 min		
Surge			
Surge pressure	140.00 kPA		
Surge duration	0.80 min		
Septum purge			
Purge flow	3.0 mL/min		
Constant septum purge	Yes		
Stop purge for	\		
Carrier mode	Constant flow		
Carrier flow			
Flow	On	1.2 mL/min	
Carrier options			
Vacuum compensation	On		
Carrier gas saver	On		
Gas saver flow	15.0 mL/min		
Gas saver time	3.00 min		
Aux. Temperatures			
Auxilliary temperature control			
Transfer Line 1	On	280 °C	
Transfer Line 2	On	280 °C	

MS settings

Q Exactive GC-Orbitrap MS				**Comments**
Global Settings				
User role			Advanced	
Use lock masses			off	
Lock mass injection			\	
Time				
Method duration			11.5 min	
Customized Tolerances (+/-)				
Lock Masses			\	
Inlcusion			\	
Exclusion	—		\	

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Mass Tags	—	\
Dynamic Exclusion		\
EI/CI Source		
Filament on delay		2.00 min
MS transfer line temp		250 °C
Ion source temp		280 °C
Ionization mode		EI
CI gas type		None
CI gas flow		0.00 mL/min
Use tune emission current		FALSE
Emission current		50.0 µA
Use tune electon energy		FALSE
Electron energy		70 eV
Use tune file C-Trap energy offset		TRUE
C-Trap energy offset		0.0 V
Cal gas level		Off
Properties of Full MS-SIM		
Experiment		Full MS — SIM
General		
Runtime		2 to 11.5 min
Polarity		Positive
Full MS		
Microscans		1
Resolution		60,000
AGC target		1.00E+06
Maximum IT		auto
Scan range		70 to 700 m/z
Spectrum data type		Profile
Setup		
Tunefiles		
General		
Switch Count 0		
Base Tunefile C:\Xcalibur\methods\EI_20200605_EP-IT50_250_280.mstune		**Update each acquisition - Tune to TIC**
Tune scan range		70-700
Res		60,000
AGC		1.00E+06
Max inject		auto
MS transfer		250
Ion source		280

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