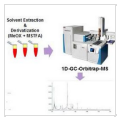

Oct 09, 2019

Metabolite Extraction and Derivatization of Plasma/ Serum Samples for High Resolution GC-MS-based Metabolomics

Biswapriya Misra[1], Michael Olivier[1]

[1]Wake Forest School of Medicine

1 *Works for me* dx.doi.org/10.17504/protocols.io.723hqgn

Metabolomics Protocols & Workflows
Tech. support email: **bbmisraccb@gmail.com**

Biswapriya Misra
Wake Forest School of Medicine

ABSTRACT

This protocol provides details on preparation of human and non-human primate blood plasma/ serum extraction, and derivatization for GC-MS based metabolomics data acquisition.

EXTERNAL LINK

https://onlinelibrary.wiley.com/doi/abs/10.1002/rcm.8197

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Misra, B.B., Bassey, E., Bishop, A.C., Kusel, D.T., Cox, L.A. and Olivier, M., 2018. High-resolution gas chromatography/mass spectrometry metabolomics of non-human primate serum. Rapid Communications in Mass Spectrometry, 32(17), pp.1497-1506.

GUIDELINES

1. Avoid freeze thawing of plasma/ serum samples.

2. I prefer to analyze plasma/ serum samples in this order of preference, when available from various source/s investigators: serum (i.e., has no additives)> EDTA-plasma > heparin-Plasma > and NEVER ever the citrate-Plasma (i..e, this citrate can mess up the citrate quantification as well as other TCA cycle metabolites), and never BIO-PAX tub collected materials (i.e, lysis will cause RBC/ WBC contents to contaminate the pure plasma/ serum).

3. No matter what downstream biological question is addressed, prefer to collect and analyze "fasted plasma/ serum" samples to avoid confounders from diet, which has an enormous impact on serum/ plasma metabolites.

4. SUPER IMPORTANT: If you see a red tinge in serum/ plasma avoid it's inclusion in analysis (and inform the source/ PI/ investigator) which means it has lysed blood cell content (i.e., red from hemoglobin) and the measurements would be unreliable and unhelpful altogether.

5. The described protocol for sample preparation and derivatization is a detailed version and adapted from existing literature i.e., :

(a) Lisec, J., Schauer, N., Kopka, J., Willmitzer, L. and Fernie, A.R., 2006. Gas chromatography mass spectrometry–based metabolite profiling in plants. *Nature protocols*, *1*(1), p.387. and

(b) Fiehn, O., 2016. Metabolomics by gas chromatography–mass spectrometry: Combined targeted and untargeted profiling. *Current protocols in molecular biology*, *114*(1), pp.30-4.

while details on the solvent mixtures used for extraction and the extraction steps are available:

(c) Fiehn, O., Wohlgemuth, G., Scholz, M., Kind, T., Lee, D.Y., Lu, Y., Moon, S. and Nikolau, B., 2008. Quality control for plant metabolomics: reporting MSI-compliant studies. *The Plant Journal*, *53*(4), pp.691-704.

MATERIALS TEXT

Acetonitrile, isopropanol, and pyridine, methoxyamine hydrochloride (MeOX), 1% TMCS in *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide

(MSTFA), adonitol,

SAFETY WARNINGS

Change gloves regularly esp. when suspecting contamination.
Perform all extractions and derivatization in Chemical Hood (not Laminar Flow Hood)
Clean the Chemical Hood work bench with 70% ethanol to get rid of all posisble chemicals/ spills/ DNA/ Protein from previous experiments.
1.5 mL microcentrifuge tubes need not be autoclaved as long as they are from fresh packets. Use filter tips whenever possible.
Avoid breathing, sneezing, coughing over the samples at any point of time and talk less to avoid salivary droplets/ aerosols to get inside samples.
Get permanent markers, stop watches (timers), Para films on the sides.
Keep all solutions chilled/ on ice always, EXCEPT- Pyridine, MeOX, MSTFA
Dehydrate Pyridine using Sodium Sulfate columns if old/ needed or always keep it sealed under silica-chamber.
Microcentrifuge tubes (1.5 ml, Fisher brand, colorless, low-leaching tubes; protein/ DBA lo-bind does not help, as we are analyzing metabolites and their binding properties ar very different!) should be pre-chilled on ice before transferring solutions. **NEVER hold the microcentrifuge tubes with samples inside from bottom with sample in it! Metabolites are susceptible to temperature and thermal degradation**
Avoid using perfumes, strong deoderants, medications, flavored food items, confectionaries working in the GC-MS laboratory.
Label all aliquots, tubes, with sample ID and dates all the time.

BEFORE STARTING

- *Before day starts, check the AVAILABILITY of the GC-MS system in first place. As derivatized (methoxyaminated and silylated samples) samples are only stable enough for 24-48 h and their performance decreases there after, better to post-pone sample prpearation than storing the samples in -80 C etc. (not recommended as moisture degrades silylated compounds).*
- *See that the carrier gasses such as **Helium, Nitrogen etc.** are at optimal levels in the system.*
- *Ensure that the GC-MS system is in optimal condition, i.e., perform routine clean-up and maintenance, i.e, LEAK CHECK (using the PFTBA), TUNE, and MASS CALIBRATION on the day of analysis/ prior to the submission que.*
- *Ensure that the column is in optimal shape by running you favorite RT/ RI calibration standards and blank SOLVENT runs.*
- *Ensure that new LINER and new SEPTUM are in place. They need to be changed after evry 24 hours of run or 10-20 samples for best reproducible results.*
- *Prepare fresh stock on MeOX on the day of sample preparation/ derivatization and is stable for a week only.*
- *Prepare the acquisition que prior to the sample preparation.*
- *Label the Blank tubes as B1, B2, B3, B4, B5....Bn.*
- *Label the Reagent Blank tubes as R1, R2, R3,....Rn.*
- *Label the sample tubes as S1, S2, S3...Sn or with sample codes/ LIMS system generated codes etc.*
- *Label the QC or pooled QCs as QC1, QC2, QC3, ...QCn etc.*
- **10 µl FAME** (Supelco, -20 C stored) standards can be used if needed as Retention Time/ Index (LRI) marker or n-hydrocarbon

mixtures can be used as Kovat's Index markers.

PROTOCOL OVERVIEW BASED ON Lisec et al., 2006, Fiehn , 2016

1 Freshly thawed aliquots of serum or plasma (30 µL) samples from non-human primate (i.e., baboons, vervets) are subjected to sequential solvent extraction once each with 1 mL of acetonitrile: isopropanol: water (3:3:2, v/v ratio) stord in -20 C and 500 µL of acetonitrile: water (1:1, v/v) ratio mixtures stored at 4 °C.[22]

2 Adonitol (2 µL from 10 mg/mL stock in methanol, stored in -20 C) is added to each aliquots as internal standard to the sample tube prior to the start of extraction.

3 The pooled extracts (~ 1500 µL) from the two steps are dried under vacuum at 4 °C prior to chemical derivatization.

4 Dummy extractions are also performed on blank microcentrifuge tubes that serve as extraction blanks to account for background (extraction solvent, derivatization reagents) noise and other sources of contamination (septa, liner, column, vials, handling etc.).

5 Blanks are only used to see that no carry overs occurred during randomized run orders and to manually filter out contaminating chemicals from the combined list of features.

6 Dried samples are then sequentially derivatized with methoxyamine hydrochloride (MeOX) and 1% TMCS in *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA) as described elsewhere.[23, 24]

7 The steps involve the addition of 10 µL of MeOX (20 mg/mL stock in pyridine, freshly prepared and stable for a week at maximum) in pyridine incubated under shaking at 55 °C for 60 min followed by trimethylsilylation at 60 °C for 60 min after adding 90 µL MSTFA.

DETAILED PROTOCOL:

8 Prepare **methoxyamine (MeOX,** Sigma/ Supelco): Prepare a 20 mg/ml MeOX solution in Pyridine; vortex and then Thermomix 3m at 60 °C for 15 min to dissolve; *1 ml is good for a week only if stored in desiccator at room temperature*.

9 Prepare **Adonitol (synonym, ribitol)** (Sigma) stock: 2 mg/ ml (Prepare 10 ml, use 20 mg in methanol, i.e., 20 mg/ ml): Store 1m in −20°C for months! *Used to (i) monitor extraction efficiency, and (ii) as an internal standard to normalize peak area.*

10 Prepare **Extraction Solvent I: 3:3:2 (v/v/v) acetonitrile/isopropanol/water:** Make 100 ml (or, as needed): Store in 1m −20°C

11 Prepare **Extraction Solvent II**: **1:1 (v/v) acetonitrile/water** : Make 50 ml (or, as needed) : Store in **-20 °** 1m

12 Retrieve plasma/ serum samples form -80 C freezer on to ice/ dry ice. Can take 30 min to 1 hr for thawing.

13 Add **2.5µl** adonitol (from stock) to each 'thawed' sample (i.e., 30 µL plasma/ serum) kept on ice. Hence forth, called "sample".

14 Add **1 ml** cooled extraction solvent to one 1.5 mL microfuge tube containing an aliquot of the sample (30 µl) and 'dummy' control (B1, empty tube).

15 The rest of the tubes should be kept ice chilled (dry ice) at 4°C on ice.

16 Vigorously vortex for **10 sec** and on Thermomixer shake for **5 min at 4°C** (**1000 rpm** shaking needed)

17 Centrifuge for **2 min** at **14,000 × g**, room temperature.

18 Aliquot two (**x2**) **450 µl** portions of the supernatant to separate tubes: one for analysis and one for a backup in -80 C.

19 Evaporate both aliquots to complete dryness using a ***speed vacuum concentrator***. Preferably operated at 4 C. ***Store the backup aliquot at −20°C for up to 4 weeks.***

20 Resuspend the other dried aliquot in **450 µL** nitrogen-degassed 50:50 (v/v) acetonitrile/water at room temperature.

21 Centrifuge for **2 min** at **14,000 × g**, room temperature.

22 Transfer supernatant (**400 µL**) to a fresh **1.5-ml** polypropylene microcentrifuge tube and evaporate to dryness in a Speedvac evaporator.

23 Add **10 µl** MeOX solution to samples [Stock **20 mg/ ml** in **Pyridine**]. *Slow* vortex for **10 sec**. 01s

24 Use Thermomixer to heat for **1 hr at 55°C.** (no shaking needed)

25 Add **90 µl** MSTFA[Sigma, Original bottle] to each sample and QC. *Slow* vortex for **10 sec.** 01s

26 Use Thermomixer to heat for **1 hr at 60°C.** (no shaking needed)

27 Collect the tubes, vortex slowly for for 10 seconds, centrifuge for 30 seconds and collect the supernatant into autosampler vials (with 250 uL inserts, screw capped, Amber color); and submit to the GC-MS autosampler. 2m