NONTARGETED METABOLOMICS (REVERSE PHASE)

Analysis of Foods Standard Operating Procedure (SOP)

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1.0 Overview

The PTFI Nontargeted Metabolomics (Reverse Phase) Standard Operating Procedure (SOP) of Food Analysis details the extraction of small molecules from food using solid-phase extraction (SPE) prior to analysis of food using a Reverse Phase Liquid Chromatography-Mass Spectrometry (LC-MS) Method.

Documents Contents:

2.0 Prerequisites

PTFI Nontargeted Metabolomics Onboarding

Before beginning the PTFI Nontargeted Metabolomics (Reverse Phase) of Food, all steps of PTFI Onboarding for Nontargeted Metabolomics (Reverse Phase) **must** be completed and approved by the PTFI Metabolomics Technical Expert.

Food Processing

Before undergoing the PTFI Nontargeted Metabolomics of Food, all standardized PTFI Access and Benefit Sharing, Metadata, and Intake Processing steps **must** be completed for all foods.

Safety

During small molecule extraction, you **must** wear appropriate Personal Protection Equipment. Any organic solvents or acids **must** be used in a fume hood.

3.0 Materials

The materials and equipment listed below are **required** to complete this protocol.

Reagents

- Water (H_2O), LC-MS grade or equivalent
- Methanol (MeOH), LC-MS grade or equivalent
- Acetonitrile (ACN), LC-MS grade or equivalent
- Formic Acid (FA), LC-MS grade or equivalent

Standards

- Reverse Phase (RP) Internal Retention Time Standard (IRTS) Mix

Foods

- Lyophilized and homogenized foods for analysis, with corresponding PTFI Unique IDs
- Locally procured carrot (carrot quality check (QC))

Consumables

- Agilent SPE Captiva EMR-Lipid Cartridges, 1mL, 40mg, 5190-1002
- Agilent Zorbax SB-Aq RRHD 2.1 x 100 mm 1.8-Micron, 858700-914
- Agilent Inline Filter: 0.3 µm, 2 mm ID, 5067-6189
- 2 mL autosampler vials with caps
- 1.7 mL microcentrifuge tubes
- 150 µL glass inserts with pre-installed springs
- 100-1000 µL pipette tips
- 10-100 µL pipette tips

Equipment

- High-resolution LC-MS Instrument
- One 50-250 mL glass bottle
- One 100-1000 µL pipet
- One 10-100 µL pipet
- Two 1000 mL glass bottles
- Vortexer
- Centrifuge (for use with 2mL glass vials at 1000 RCF and 1.7 mL microcentrifuge tubes at 14000 RCF)
- Analytical Balance
- Nitrogen Evaporator
- Positive Pressure Manifold
- -80C Freezer
- 4C Refrigerator

4.0 Preparation of Reagents

To support reagent preparation, consult section 9.0 Appendix and the Food Extraction Calculator where referenced.

80% MeOH

1. _____ Prepare the required volume of 80% MeOH (*See Note 1*).
2._____ Cap and invert the mixture a few times to mix.
3._____ This solution can be stored in the refrigerator for later use. Allow the solution to come to room temperature before use.

Notes:

1. To determine the appropriate amount of 80% MeOH, see Section 9.0 Appendix and the PTFI Nontargeted Metabolomics (Reverse Phase) Food Extraction Calculator.

Crash Solution (1% FA in 5:1 ACN: H_2O)

1. _____ Prepare the required volume of Crash Solution (*see Note 1*).
2. _____ Cap and invert the mixture a few times to mix.
3. _____ This solution can be stored in the refrigerator for later use. Allow the solution to come to room temperature before use.

Notes:

1. To determine the appropriate amount of Crash Solution, see Section 9.0 Appendix and the PTFI Nontargeted Metabolomics (Reverse Phase) Food Extraction Calculator.

Reverse Phase (RP) Internal Retention Time Standard (IRTS) Mix

1. _____ Add 1.1 mL of 80% MeOH to the RP IRTS vial.
2. _____ Cap and vortex for 10 minutes or until fully dissolved.
3. _____ Centrifuge the vial at 1000 RCF at room temperature for 10 minutes.
4. _____ This solution can be stored at -80C for later use. Allow the solution to come to room temperature before use (*see Note 1*).

Notes:

1. A single vial of IRTS mix is sufficient for 100 food samples. See Section 9.0 Appendix and the PTFI Nontargeted Metabolomics (Reverse Phase) Food Extraction Calculator.

Mobile Phase A: H2O with 0.1% FA

1. _____ Add 1000 mL of water to a solvent bottle.
2. _____ Add 1 mL of Formic Acid to the water.
3. _____ Cap and invert the mixture a few times to mix.

Mobile Phase B: ACN with 0.1% FA

1. _____ Add 1000 mL of acetonitrile to a solvent bottle.
2. _____ Add 1 mL of Formic Acid to the acetonitrile.
3. _____ Cap and invert the mixture a few times to mix.

LC Wash Solution: 50:50 MeOH: H2O

1. _____ Add 500 mL of methanol to a solvent bottle.
2. _____ Add 500 mL of water to a solvent bottle.

3. _____ Cap and invert the mixture a few times to mix.

5.0 Extraction

5.1 Pre-Extraction

1. _____ For each sample, label 1.7 mL microcentrifuge tubes with PTFI Unique ID. Also label tubes for carrot QCs and IRTS blanks.
2. _____ For each extraction, label autosampler vials as "NTM-RP" along with PTFI Unique ID. Also label tubes for carrot QCs and IRTS blanks. These samples will be used for LC-MS analysis.
3._____Allow the 80% MeOH and Crash Solution to reach room temperature if stored in the refrigerator (~30 minutes).

4._____Allow the IRTS Solution to reach room temperature if stored in the -80C freezer (approximately 30 minutes).

5. _____ Remove the lyophilized and homogenized samples and carrot QC samples from the -80C freezer and allow them to reach room temperature (~30 minutes).

5.2 Conditioning Captiva Columns

1. _____ Place the Captiva columns on the pressure manifold's column rack. Place test tubes in the manifold to collect the waste eluent.
2._____ Add 120 µL of 80% MeOH to each Captiva column.
3._____ Subsequently, add 480 µL of the Crash Solution to each Captiva column.
4._____ Start the nitrogen flow to the pressure manifold.
5._____ Adjust the nitrogen gas flow such that only 1 drop of solvent is flowing through each column not more than once every 3 seconds.
6._____ Once the solution has passed through all Captiva columns, continue flowing nitrogen through the columns for an additional 10 minutes.
7. _____ Visually inspect the columns to make sure they are dry.
8._____ Turn off the nitrogen flow.
9._____ The columns are now prepped and ready to be used for extractions (*See Note 1*).

Notes:

1. *Conditioning of captiva columns should be performed immediately before extracting samples.*

5.3 Extraction Procedure

1. _____ Remove the lyophilized and homogenized samples and carrot QC samples from the -80C freezer and allow them to reach room temperature (~30 minutes).
2._____ In the pre-labeled microcentrifuge tubes, weigh the appropriate number of carrot QC samples (55 mg ± 5 mg) for the number of batches being extracted, if not already (*See Note 1*).
3._____ In the pre-labeled microcentrifuge tubes, weigh 55 mg ± 5 mg of each lyophilized and homogenized sample, if not already.
4._____ Resuspend each sample and carrot QC using 12 µL of 80% MeOH per every 1 mg of food (*see Note 2*). For IRTS blanks, add 600 µL of 80% MeOH to empty microcentrifuge tubes.
5._____ Vortex for 10 minutes at room temperature.
6. _____ Centrifuge at 14000 RCF at room temperature for 10 minutes.
7._____ Set up the positive pressure manifold so that the column rack is placed on top of the sample rack, which contains a set of uncapped prelabeled autosampler vials labeled as “NTM-RP”.
8._____ Add 480 µL of crash solution to each Captiva column.
9. _____ Transfer 120 µL of the sample supernatant to the Captiva columns on the positive pressure manifold (*see Note 3*). Retain any remaining supernatant as a backup and store at -80C.
10._____Add 10 µL of the RP IRTS mix to each Captiva column by slowly pipetting the Reverse Phase IRTS mix up and down to mix with the solution.
11._____ Start the nitrogen flow to the positive pressure manifold.
12._____ Adjust the nitrogen gas flow such that only 1 drop of solvent is flowing through each column not more than once every 3 seconds.
13._____ Once all the liquid has passed through the Captiva columns, turn off the nitrogen flow.
14._____ Remove the “RP” vials from the manifold.
15. _____ Centrifuge at 1000 RCF at room temperature for 5 minutes.
16._____ Dry the samples using a nitrogen evaporator (*see Notes 4 and 5*).
17._____ Resuspend the samples in 80% MeOH using the optimal resuspension volume determined from onboarding experiments.
18._____ Vortex the samples for 10 minutes until completely dissolved (no precipitate remaining). Make sure all sample on the vial walls is reconstituted. Vortex for additional time if needed.
19._____ Centrifuge the samples at 1000 RCF for 5 minutes at room temperature.
20. _____ If using inserts, transfer 40 µL of the samples to a set of inserts and place them back in the “NTM-RP” vials.
21._____ Centrifuge the samples at 1,000 RCF for 5 minutes at room temperature to free any bubbles that might be trapped at the bottom of the insert.
22._____ Samples are ready for LC-MS analysis. Store at -80C until analysis.

Notes:

1. For sample batch calculations, See Section 9.0 Appendix and the PTFI Nontargeted Metabolomics (Reverse Phase) Food Extraction Calculator.
2. The PTFI Nontargeted Metabolomics (Reverse Phase) Food Extraction Calculator can be used to calculate the amount of 80% MeOH needed for each sample and carrot QC.
3. A vacuum filtration system is a suitable alternative for a positive pressure manifold.
4. A speed vacuum system is a suitable alternative for a nitrogen evaporator.
5. Samples can be left dry overnight before resuspending.

6.0 LC-MS Operating Parameters

6.1 Required Materials

- Inline filter: Agilent 0.3 µm, 2 mm ID 5067-6189 (*See Note 1*)
- Column: Agilent Zorbax SB-Aq RRHD 2.1 x 100 mm 1.8-Micron 858700-914

Notes:

1. Frits within in-line filter can be cleaned and re-used following the procedure outlined in the Nontargeted Metabolomics Reverse Phase User Guide.

6.2 LC Method

1. _____ Set 15-minute LC gradient method following time and %B Solvent amounts as specified in Table 1.
2. _____ Set flow rate to 0.6 mL/min for the entire method.
3. _____ Set column temperature to 30C for entire method
4. _____ Set autosampler temperature to 4C for entire method.
5. _____ Set injection volume to 2 to 4 µL.
6. _____ Set needle wash for 30 seconds.

Time (min.)	**% Mobile Phase A: H_2O with 0.1% FA**	**% Mobile Phase B: ACN with 0.1% FA**	**Flow rate (mL/min)**
0.0	98.2	1.8	0.600
1.0	98.2	1.8	0.600
11.0	10.0	90.0	0.600
12.8	10.0	90.0	0.600
12.9	98.2	1.8	0.600
15.0	98.2	1.8	0.600

Table 1. PTFI Reverse Phase Metabolomics Gradient

6.3 MS Method

MS parameters should be optimized for the collection of mass spectrometry data for small molecules. The below metrics should also be met for final data:

1. _____ Regardless of instrument vendor, data should be collected in MS1 mode at a scan rate of 2-3 scans per second for the entire 15-minute LC gradient.
3. _____ Set data to collect in centroid mode.
4. _____ Set mass spectrometer time for data collection to 15 min.
5. _____ Set mass spectrometer to collect MS1 data.
6. _____ Set all other instrument parameters for optimal performance for measuring small molecules.

6.4 Data Acquisition

Data should be collected in a randomized fashion following appropriate calibration and conditioning of the instrument. For each acquisition, at least two IRTS blanks **must** be collected for each ionization mode.

7.0 Data Upload

Refer to the PTFI Data Upload User Guide for detailed instructions to access the cloud-based processing for Nontargeted Metabolomics. Once a Lab Portal Job is created, the metabolomics raw data can be uploaded to the Lab Portal.

8.0 Additional Resources

Any additional information supporting LC-MS data acquisition can be found in the following documents:

- LC Troubleshooting Guide
- MS Troubleshooting Guide
- Data Review/Upload Troubleshooting Guide
- RP IRTS Reference List
- Nontargeted Metabolomics (RP) User Guide
- Data Upload User Guide

For support from a PTFI Technical Expert, visit the PTFI Ecosystem Hub. Access to the PTFI Ecosystem Hub can be found on the PTFI Research Hub: https://ptfi.versobio.com

9.0 Appendix

9.1 Sample batch calculations

A "batch" is defined as a set of foods extracted at the same time. Two carrot QCs and one IRTS blank need to be extracted for every batch of samples. For example, if you are using a 48-position positive pressure manifold, a batch consists of a maximum of 45 samples + 2 carrot QC+ 1 IRTS blank (48 total extractions). However, if you are only running one batch of 48 samples on the instrument, you will need to extract two IRTS blanks to support data processing.

Calculate the number of sample batches. Example calculations for a set of 100 samples using the 48-position manifold:

$$\frac{100\ total\ samples\ in\ sample\ set}{45\ samples\ per\ batch} = 3\ batches$$

$$3\ batches \times 2\ reference\ foods = 6\ reference\ foods$$

$$3\ batches \times 1\ IRTS\ blank = 3\ IRTS\ blanks$$

$$100\ samples + 6\ reference\ foods + 3\ extraction\ blanks = 109\ extractions$$

9.1.2 Total Amount of 80% MeOH

During extraction, 120 µL is required per Captiva column for column conditioning (step 5.2.2). An additional 12 µL is required per 1 mg of sample for lyophilized food resuspension (step 5.3.5). An additional volume corresponding to the optimal resuspension volume determined from onboarding experiments (step 5.3.18) will be required for resuspension of samples.

Calculate the amount of 80% MeOH required for all extractions. Example calculations for a set of 100 samples (assume each sample is 50 mg and 40 µL optimal resuspension volume):

$$120\ \mu L + (12\ \mu L \times 50\ mg) + 40\ \mu L = 760\ \mu L\ of\ 80\ \%\ MeOH\ per\ sample$$

For a set of 100 samples, a total of 109 extractions will require:

$$109\ total\ extractions = 82.8\ mL\ 80\%\ MeOH$$

Note: 20% extra volume will be added to ensure backup solution is available if needed.

$$82.8\ mL\ 80\%\ MeOH \times 1.2 = 99.4\ mL\ 80\%\ MeOH$$

9.1.3 Total Amount of Crash Solution

During extraction, two 480 µL aliquots of Crash Solution are required per SPE Captiva column (steps 5.2.3 and 5.3.9).

Calculate the amount of Crash Solution (1% Formic acid with 5:1 ACN:Water) required for all extractions. Example calculations for a set of 100 samples (109 total extractions):

$$480\ \mu L\ +\ 480\ \mu L\ =\ 960\ \mu L\ of\ Crash\ solution\ per\ sample$$

For a set of 100 samples, a total of 109 extractions will require:

$$109\ total\ extractions\ =\ 105\ mL\ Crash\ solution$$

Note: 20% extra volume will be added to ensure backup solution is available if needed.

$$105\ mL\ Crash\ Solution \times 1.2\ =\ 126\ mL\ Crash\ solution$$

11.1.4 Total Amount of Reverse Phase IRTS

During extraction, one 10 µL aliquot of Reverse Phase (RP) Internal Retention Time Standard (IRTS) is required per SPE Captiva column (step 5.3.11).

Calculate the amount of IRTS mix required for all extractions. Example calculation for a set of 100 samples (109 total extractions):

$$10\ \mu L\ Reverse\ Phase\ IRTS \times 109\ extractions\ =\ 1090\ \mu L\ of\ RP\ IRTS$$