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Protocol status: Working
We use this protocol and it's working

Hemlock Sample Analysis for Headspace Terpenes, Liquid Analysis, and Sugars

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ABSTRACT

This protocol was developed by the Cernak Lab at the University of Michigan to analyze the terpene concentration and content of various species of hemlock trees using Gas-Chromatography Mass-Spectrometry (GC-MS). It details the procedure one samples have been collected, starting with the sample preparation. The procedure, as well as the GC-MS setting are included for analysis of volatile terpenes using a headspace autosampler, liquid analysis of all compounds (including non-volatile terpenes), and analysis of sugars.

The procedure for analyzing hemlock using a tissuelyser is also attached to this protocol. This method was developed by the Kersten lab at the University of Michigan and is used for Liquid-Chromatography Mass-Spectrometry (LC-MS) analysis.

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Sample Preparation

1 Pick off the leaves of the hemlock stem using your fingers and gloved hands.

2 Weigh approximately 0.1 g of each sample of hemlock leaves and put the sample into a headspace vial compatible with the autosampler of your GC-MS.

3 Add 1000 microliters (1mL) of the internal standard into each vial (heptyl acetate 0.05% v/v in ethanol).

4 Clamp the PTFE lid onto each vial using a lid clamping tool.

5 Sonicate the vials in a water bath at a temperature of 30°C for 30 minutes. Wipe off any excess water on the outside of the vials before beginning GC analysis.

Headspace analysis (Terpenes):

6 Run the samples through the GC-MS machine with the settings below:

- Column: Agilent 19091S-433UI: 2762943H HP-5MS UI
- Temperature range of column: -60°C to 325°C
- Dimensions: 30m x 250 micrometers x 0.25 micrometers
- Analysis using MSD Detector
- Injection volume: 1 µL (in gas phase)

- Incubation temperature: 80°C
- Syringe temperature: 90°C

Oven program:

1. Start at 40°C, hold for 2 min
2. Increase at rate of 17°C/min until 150°C
3. Hold at 150°C for 1 min
4. Increase at rate of 38°C/min until 250°C
5. Hold at 250°C for 3 min
6. Total run time: 19 min
7. Mode: Split
8. Split ratio: 40:1
9. Helium Gas flow rate: 0.9 mL/min

Each sample must be run through the GC-MS at least three times. After every 10 runs, make sure to wash out the GC-MS by running a vial filled with some cleaning solution.

Extract all data by using the built-in RTE integrator to find the peak areas for all samples.

7

Liquid Analysis (Terpenes and other compounds):

8 You may use the same 0.1g sample that you weighed out in the beginning, or repeat the sample preparation if needed.

Remove 1000microliters of the liquid extract using a pipette and add it into a 2mL glass vial (made for GCMS liquid injection) for each sample. If there are some sediments or particles floating around, use a 2-3 mL syringe to extract the liquid in each vial. Add a PTFE 0.45 µm syringe filter. An image of the set-up is provided below:

Demonstration of syringe and filter set-up for liquid extraction

Inject liquid through filter into GC liquid injection vials for direct injection.

If the headspace analysis indicates that the concentration of terpenes is too high, then dilute as needed with a 1:1 dilution ratio.

9 Run the samples using liquid injection and the following GC settings:

- Column: Agilent 19091S-433UI: 2762943H HP-5MS UI
- Temperature range of column: -60°C to 325°C
- Dimensions: 30m x 250 micrometers x 0.25 micrometers
- Analysis using MSD Detector
- Injection volume: 1 µL (in liquid phase)

Oven program:

1. Start at 50°C, hold for 2 min
2. Increase at rate of 5°C/min until 285°C
3. Hold at 285°C for 25 min
4. Mode: Split
5. Split ratio: 20:1
6. Helium Gas flow rate: 0.9 mL/min

Extract and record data

Sugar Analysis

10 You may use the same 0.1g sample that you weighed out in the beginning, or repeat the sample preparation if needed.

Add 200 microliters internal standard into each vial (0.05% Mannitol solution as IS in 50:50 water:methanol solvent).

Put this solution under nitrogen gas until the liquid evaporates. This process takes around 40-60 mins and you should be left with dry 2mL liquid injection vials with compounds (green in color) adhered to the walls of the vial.

11 Prepare a silylation reagent (Acetonitrile, 1,1, 1, 3, 3 3-hexamethyldisiloxane (HMDSO), and Tetramethylsilane (TMS) in the ratios 9:3:1 respectively).

Once evaporation is complete, add 200 microliters of the silylation reagent, and sonicate until a homogenous solution is achieved.

Then add 800 microliters of acetonitrile to each sample. Sonicate once again to get a homogenous mixture. Do this for every sample.

12 Use a 2-3 mL syringe (or a 6mL if a smaller one is not available) to filter the liquid in all samples. Add a PTFE 0.45 µm filter onto the end of the syringe and filter the samples (Pour the contents of each sample into the syringe, and pump out filtrate into a new liquid injection GC vial. Make sure to change syringes and filters for each sample).

Set-up of syringe filter for sugar analysis

13 Seal all vials with their caps and run a liquid injection into the GC-MS with the settings below (same GC column as the liquid analysis):

Oven program:
1. Start at 50°C, hold for 3 min

2. Increase at rate of 5°C/min until 285°C
3. Hold at 285°C for 25 min
4. Total run time: 65 min
5. Mode: Split
6. Split ratio: 30:1
7. Helium Gas flow rate: 0.9mL/min

Extract and record data.

Tissuelyser Protocol (for LCMS)

14 Preheat waterbath to 60 °C.

15 Separate needles & stem tissue.

16 Weigh stem tissue (target mass: 0.1 g) and leaf tissue (target mass: 0.2 g). a. Document sample weight in a table for sample vials.

17 Add stem & leaf tissue to separate metal tissuelyser tubes.
1. Add one steel ball to each tube.
2. Add a clean (washed or new) O-ring (red rubber ring) to the lid.
3. Close the tube with the lid.

18 Freeze the sample tubes for 30 min in -80 °C freezer or on dry ice.

19 Grind the samples in tissuelyser (regular plastic adaptor) for 20 s for 3 times @ 6 m/s speed. Grind 12 samples at a time to avoid adding too much weight to the Tissuelyzer.

20 Add 80% methanol to the tubes at adjusted concentration of a. 100mg/mL for stem samples, b. 200mg/mL for needle samples. Calculate the solvent amounts for the target concentrations

based on the sample weights from step 3.

21 Grind the samples in Tissuelyzer (regular adaptor) for 20 s for 2 times @ 6 m/s speed. Grind 12 samples at a time to avoid adding too much weight to the Tissulyzer.

22 Incubate samples in metal tubes @ 60C for 10min.

23 Centrifuge samples for 5min @ 16000rpm in tabletop centrifuge. Centrifuge 12 samples at a time to avoid adding too much weight to the Centrifuge.

24 Filter samples through Whatman syringeless filters. Use glass Pasteur pipettes for liquid transfer.

25 Add samples to LCMS vials & cap. Use glass Pasteur pipettes for liquid transfer. a. For low-volume extracts, use low-volume LCMS tubes. The target volume should be >50 µL to allow for multiple data acquisition experiments (negative and positive ion mode, low- and high-m/z-range).

26 Run samples on LCMS or store @ -80C until LCMS analysis.

27 Quality control sample

For quantitative analysis, it is recommended to prepare one quality control (QC) sample (stem & leaf each) from one common hemlock plant sample for every large sample preparation with a target QC sample volume of 500 µL. Needles and stem parts of the common hemlock plant sample should be aliquoted into 0.2g and 0.1g samples, respectively, in 2mL vials to avoid freeze-thawing of the common hemlock sample. The aliquoted common hemlock samples are stored in the -80C freezer. The QC sample is used as an external LCMS standard in the data acquisiton to monitor signal intensity shifts over long LCMS sequences.