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FAME analysis of pollen fatty acids

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We use this protocol and it's working

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Abstract

This protocol uses FAME (Fatty Acid Methyl Esterification) techniques adapted from Seppänen-Laasko et al., 2002 to quantify fatty acid contents in small samples (10 mg) of anther pollen, corbicular pollen, or stored pollen. Pollen is initially fractured by cell disruption and fatty acids are extracted by Folch extraction/partition. Fatty acids are converted to their fatty acid methyl ester (FAME) equivalents by acid methylation then separated and analyzed by GCMS in SIM mode.

Seppänen-Laasko, T., Laakso, I., & Hiltunen, R. (2002). Analysis of fatty acids by gas chromatography and its relevance to research on health and nutrition. *Analytica Chimica Acta*, *465*, 39-62. [https://doi.org/10.1016/S0003-2670\(02\)00397-5](https://doi.org/10.1016/S0003-2670(02)00397-5)

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Grand Challenge - Assessing the Nutrient Contents of Pollen for Bees

Attachments



DeGrandi Hoffman et ...

1.1MB

Guidelines

Synthetic external standards (ESTD) are needed for each fatty acid of interest. Both fatty acid and their fatty acid methyl ester equivalents provide information for identification and quantification. Underivatized fatty acids authentic standards can be fully processed alongside samples to allow compound quantification and estimates of compound losses. Fully methylated fatty acid methyl ester (FAME) authentic standards can be used to confirm compound identity, retention time, and mass spectral characteristics.

A synthetic internal standard (ISTD) fatty acid is needed to quantify compound amounts by providing estimates of the fraction of FAME products recovered and present in the GCMS injection. Choose an internal standard that does not naturally occur in the samples, does not significantly interfere with pollen fatty acid compound peaks (at a particular retention time and m/z ion range), and is not obscured by background contaminants.

- Fatty acid external standards are processed exactly the same as the samples to account for losses.
- FAME external standards are not processed alongside samples but are analyzed directly by GCMS.
- Internal standard is added during the initial Folch extraction/partition steps.
- Unprocessed samples should be stored for long periods in a -80°C to reduce losses of unsaturated fatty acids.
- The approximate working range of FAME is approximately 20 ng to 1 mg fatty acid materials.
- The 10 mg sample mass is based on average fatty acid contents for pollens. Adjust upwards or downwards as needed.

This method for quantification of individual fatty acid compounds from pollen can be coupled with methods to quantify total lipid contents of pollen (i.e. Chromic Acid Assay for Quantification of Total Lipids in Pollen). Subsamples of Folch extracts of pollen homogenates can be divided between these two methods to quantify both metrics.

Materials

Consumables and benchtop equipment

homogenizer tubes, 2 mL (BioSpec 10832)
zirconia beads, 1.0 mm (BioSpec 11079110ZX)
crimp cap vials, 12 mm x 32 mm (Thermo Scientific 200 000)
crimp caps, 11 mm red rubber (Wheaton W224211-01SP)
screw cap vials, 12 mm x 32 mm (Thermo Scientific C5000-1W)
screw caps, 11 mm red rubber (Fisherbrand 501 347)
glass vial inserts, 200 uL (ThermoFisher 500 304)
scintillation vials, 20 mL (VWR 66022-128)
Eppendorf tubes, 2 mL
P200 pipettor and pipette tips
P1000 pipettor and pipette tips
aluminum foil
aluminum blocks (capable of fitting 12 mm crimp cap vials)
crimper and decrimper for 12 mm vials

Instruments

cell disruptor homogenizer (Mini BeadBeater 96, BioSpec)
digital hot plate (HP 30A digital hot plate, Torrey Pines Scientific)
microcentrifuge
vacuum centrifuge (Savant SpeedVac SPD 2010, ThermoScientific)
EI GCMS with chromatography analysis software

Chemicals (use at least reagent grade except where noted)

chloroform
cis-10-heptadecenoic acid (ISTD)
concentrated hydrochloric acid
hexane, GCMS or HPLC grade
methanol, GCMS or HPLC grade
potassium chloride
water, HPLC grade
toluene

2:1 (v/v) chloroform: methanol (Folch solution)
0.25% KCl solution in DI water
methanolic-HCl reagent (made as described in steps)

Synthetic external standard (ESTD) fatty acids will be needed for each fatty acid of interest.

A synthetic internal standard (ISTD) fatty acid will be needed to quantify compound amounts by providing estimates of the fraction of FAME products recovered and present in the GCMS injection. Choose an internal

standard that does not significantly interfere with pollen fatty acid compound peaks and is not obscured by contaminants.

Troubleshooting

Safety warnings

- ! Read the SDS safety sheets for all chemicals used in this protocol, especially chloroform, methanol, hydrochloric acid, and hexane. Wear full eye protection, lab coats, and gloves and work in a ventilated hood, particularly when handling strong acids. Always add strong acids slowly to solvents to avoid violent overheating. Store and dispose of hazardous wastes and chemicals in accordance with national, state, and local laws. The BeatBeater homogenizer poses a dangerous mechanical hazard if accidentally turned on when opened. Turn off and depower this instrument when loading and unloading vials into this instrument.

Before start

Pollen fatty acid compound identities should be elucidated and authentic standards obtained before attempting fatty acid quantification by FAME analysis (for guidelines, see Editors .2008. General guidelines for authors for submission of manuscripts that contain identifications and syntheses of compounds *Journal of Chemical Ecology* 34: 984-986). FAME analysis coupled with GCMS analysis can be used to separate and help elucidate fatty acid compounds in novel pollens. However, structurally similar fatty acids may be very difficult to cleanly separate or distinguish by conventional methods such as GCMS. For compound elucidation, we suggest that pollen sample preparations be compared by GCMS on multiple chromatographic systems and specialized analytical techniques be used to distinguish between structurally similar fatty acids, particularly unsaturated fatty acid isomers.

Folch Extraction and Partition of Fatty Acids

- 1 Dry down the pollen sample in a freeze dryer.
- 2 Add 1mm zirconia beads to a 2 mL homogenizer tube and fill tube 1/4 full for each pollen sample. Keep the amount the same among samples and standards.
- 3 Add 10.0 mg pollen (usually about 1-2 mg lipid equivalent) to the homogenizer tube.
- 4 Add a FAME internal standard (ISTD) in stock solution directly to the vial (for our preparations, 56 µg cis-10-heptadecenoic acid, 56 µL of a 1 µg/µL stock solution in 2:1 chloroform-methanol). Use a synthetic fatty acid that does not occur naturally in the pollen or obscure chromatographic peaks of pollen fatty acids through co-elution. Allow the ISTD carrier solvent to evaporate off.

Critical note: add the ISTD here only if the sample is being analyzed only for FAME. If the sample is to be analyzed separately for FAME and total lipids (chromic acid), the ISTD should only be added to the sample after Folch extraction solution subsample removal. Total lipid assays such as chromic acid cannot distinguish between native (sample) and synthetic (ISTD) lipids and ISTD should not be added to chromic acid subsamples.

Folch Extraction and Partition of Fatty Acids

- 5 Add 1000 µL 2:1 chloroform: methanol (Folch solution) to the homogenizer tube.
- 6 Pulverize the pollen in the homogenizer tube with the BeadBeater homogenizer for 30 seconds. Tough materials may require additional 30 second rounds of homogenization. Avoid homogenizing for more than 30 seconds at a time to avoid sample overheating. Check the homogenate under a microscope to confirm that the pollen exine wall was fractured.

Turn off the power to the BeadBeater when loading or unloading tubes. Secure the tube caps tightly to avoid sample loss.
- 7 Add 210 µL 0.25% KCl to the homogenizer tube and briefly vortex.
- 8 Centrifuge the tube contents for 10 minutes at maximum speed in a microcentrifuge.



- 9 Two layers will form - an upper aqueous/methanolic layer and a lower chloroform/methanolic layer, with a possible boundary layer between the two phases. The amount of solvent in these two phases will be different than the two reagents' volumes since some methanol goes into each layer. Estimate the volume of both layers (often 310 μL upper layer and 690 μL lower layer) and record. Pipette 160 μL of the lower chloroform/methanolic layer into a crimp cap vial being careful not to transfer any solid materials.

Save a second subsample of the lower chloroform layer as a backup in a deep freezer in case the reaction fails.

Stopping point - The Folch extract can either be dried down immediately for FAME acid methanolysis or stored capped with a red rubber crimp cap in the -80°C freezer until later.



Folch extraction with upper aqueous-methanol and lower chloroform-methanol layers. An interface composed of amphiphilic material is visible here between the two phases.

Acid Methanolysis of Fatty Acids (FAME Reaction)

- 10 *Turn on the SpeedVac vacuum centrifuge 30 min before dry down to allow the solvent vapor trap refrigerator to cool.*



Reduce the sample and external standards in the vacuum centrifuge to complete dryness (about 1 to 2 hrs).

- 11 Make a fresh solution of methanolic HCl reagent (8% HCl in methanol) every day. For 100 samples, add 3.5 mL concentrated hydrochloric acid to 14.9 mL methanol in a 20 mL scintillation vial for a total volume of 18.4 mL. For 300 samples, add 9.7 mL concentrated hydrochloric acid to 41.5 mL methanol for a total volume of 50.0 mL.

Always add solvent slowly to strong acids to avoid violent boiling and spattering.

- 12 Perform an acid methylation to convert fatty acids to their FAME equivalents. First dissolve the dried vial FAME extract in 100 μ L toluene.

- 13 Add 750 μ L methanol to the crimp cap vial followed by 150 μ L methanolic HCl. Seal with a red rubber crimp cap and vortex well. Incubate the vials at 45°C in heating blocks on a thermally controlled hot plate for 16 hours. Cover the heating blocks loosely with aluminum foil to reduce convective heat loss.

Double check that each crimp cap seal is tight before incubating the reaction vials.

Critical note: once the fatty acids are converted into their methyl ester equivalents (FAMEs), the compounds are slightly volatile and will be lost by drying down in a vacuum centrifuge or by prolonged uncapping.

Stopping point – The methylated FAMEs can be stored capped in the fridge or freezer immediately after the reaction but should be processed and analyzed by GC-MS within a few weeks maximum.

Post Acid Methanolysis Partition and Washing

- 14 After the incubation, allow the vial to cool to room temperature. Briefly centrifuge the sealed vials for 5 seconds in the SpeedVac vacuum centrifuge **without vacuum** to draw down any condensation.
- 15 Unseal (decrimp) the crimp cap and transfer the entire vial contents (~ 1 mL) to a new 2 mL Eppendorf tube.
- 16 Partition the methanolysis products between two solvents. Add 300 μ L DI water and 600 μ L hexane to each tube. To limit hexane solvent losses, keep the vials tightly capped except when in active use. Vortex for 5 seconds.
- 17 Centrifuge the tube contents for 10 minutes at full speed in a microcentrifuge. The contents should form two layers, an upper hexane layer and a lower methanol-water

layer. The upper hexane layer has the FAME compounds of interest. These layers will be different volumes than the amounts of water and hexane originally added as methanol will go into both layers.

- 18 Perform aqueous washes of the hexane layer to remove residual acids from the organic solvent. **Transfer as much of the top hexane layer (200 μ L) to a new 2 mL Eppendorf tube as possible, but do not pull in any water from the lower layer.** Estimate the volume transferred and the total volume of the top layer, and remove about the same volume for all samples and ESTD standards.

Phase transfers do not have to be exact or include all of the upper hexane layer, as the fraction transferred will be ultimately be calculated by the amount of ISTD recovered.

- 19 Wash the transferred hexane twice with water to remove acid residues. Add 300 μ L DI water to the tube, vortex 5 seconds, and centrifuge for 10 minutes at full speed. **Remove about 200 μ L of the aqueous layer (bottom layer) after the first wash and dispose of it, leaving the top hexane layer in the tube.** Add a second wash 300 μ L DI water to the tube, vortex, and centrifuge for 10 minutes. Leave the water in after the second wash - the hexane layer will be removed shortly.

- 20 Remove 100 μ L of the upper hexane layer into a glass insert in a screw top vial. Seal the sample with a screw top vial cap. Store the vial in the -80°C freezer until GC-MS analysis. Make sure that the screw top vial cap is on securely as hexane can escape through leaky threads.

Stopping point – for longer term storage (more than a few days), transfer the hexane layer to a crimp cap vial, seal the vial with a red rubber crimp cap, and store in the deep freezer. FAME compounds degrade with a month or two in the deep freezer.

GCMS Analysis of FAME Contents

- 21 FAME compounds in the hexane layer can be analyzed by EI GC-MS on a gas chromatograph coupled to a mass spectrometer detector (for the author's group, an Agilent 7890A gas chromatograph coupled to an Agilent 5975D mass spectrometer run by Agilent Mass Hunter software. 1 μ L sample is injected at 220°C onto a HP-5MS column (30 m x 0.25 mm x 0.25 μ m film; Agilent, Inc.) with helium as a carrier gas at 1.2 mL/min. Compounds are separated by oven temperatures programmed at $10^{\circ}\text{C}/\text{min}$ from 35°C with an initial 1 min hold to 230°C with an 8 min hold, then to 320°C with a 0.5 min final hold).
- 22 FAME compounds are elucidated by comparison of mass spectra and retention times with esterified standards. FAME compounds are quantified by comparison of characteristic mass fragments (m/z) with known amounts of authentic standards. The fraction of the total sample injected is calculated from the amount of internal standard (for the author's group, cis-10-heptadecenoic acid) recovered in each injected sample.



Obtain FAME compound retention times and characteristic mass fragments (m/z) from authentic standards. The m/z fragments are used in SIM mode to generate single ion chromatograms with compound peaks. Select m/z fragments for peak quantification that are prominent and free of background interference from contaminants.