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Chromic acid assay for quantification of total lipids in pollen

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We use this protocol and it's working

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Abstract

This protocol quantifies total lipid contents of pollen through colorimetric oxidation of lipids by chromic acid (adapted from Amenta, 1970). Small pollen samples (10 mg) are fractured by cell homogenization and lipids are extracted by Folch extraction. Lipid amounts are measured by colorimetric changes when hexavalent (+6 oxidation state) chromium ion is reduced to a trivalent (+3 oxidation state) state during lipid oxidation. The accuracy of the assay depends on how thoroughly the initial extraction excludes non-lipid compounds such as sugars and proteins. A considerable drawback of this method is the toxicity, carcinogenicity, and environmental toxicity of the hexavalent chromium reagents used in this assay.

Amenta, J.S. (1970). A rapid extraction and quantification of total lipids and lipid fractions in blood and feces. *Clinical Chemistry, 16,* 339-346.

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Carl Hayden Bee Research Center, USDA-ARS PWA, Tucson, Arizona, USA
Grand Challenge - Assessing the Nutrient Contents of Pollen for Bees

Attachments

DeGrandi Hoffman et ...

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Guidelines

- Sample contents are quantified by comparison against known amounts of lipid external standards.
- The working range of this assay is approximately 50 µg to 1000 µg lipid material.
- The 10 mg sample mass in this protocol is based on dried sample estimates within this range. Adjust sample mass upwards or downwards as needed.
- Unprocessed samples can be stored in a -20°C freezer for months.
- Both samples and external standard reaction solutions are run in triplicate on a 96 well plate.

This method for quantification of total lipid contents from pollen can be coupled with methods to quantify individual fatty acid compounds of pollen (i.e. FAME Analysis For Pollen Fatty Acids). Subsamples of Folch extracts of pollen homogenates can be divided between these two methods to obtain both metrics.

Materials

Please note that two separate sets of benchtop equipment should be made for use in the lipid extraction (not chromic acid-dedicated) and chromic acid reaction (chromic acid-dedicated). Equipment used in the chromic acid reaction steps is contaminated and should be kept completely separate from equipment used in normal laboratory work.

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 aluminum PFTE (Thermo Scientific 502 070)
P200 pipettor and pipette tips (not chromic acid dedicated)
P1000 pipettor and pipette tips (not chromic acid dedicated)
P200 and P1000 filter tips
aluminum foil
aluminum blocks (chromic acid dedicated, capable of fitting 12 mm crimp cap vials)
crimper and decrimper, for 12 mm vials (chromic acid dedicated)
P1000 pipettor (chromic acid-dedicated)
P1000 filter tips (chromic acid dedicated - use filtered tips to limit pipetteman contamination)
96 well flat bottom plates (Costar 9018)

Instruments
cell disruptor homogenizer (Mini BeadBeater 96, BioSpec)
digital hot plate (HP 30A digital hot plate, Torrey Pines Scientific)
microcentrifuge
microplate reader (BioTek microplate reader Synergy HT and Gen5 program, Bio Tek)
vacuum centrifuge (Savant SpeedVac SPD 2010, ThermoScientific)

Safety and disposal
benchtop liner paper (disposed of as hexavalent chromium waste after each session)
duct tape (to tape down liner paper, disposed of as hexavalent chromium waste after each session)
dedicated storage containers for hexavalent chromium chemical waste and contaminated equipment/consumables
- liquid wastes for waste reagents, reaction vials, and reaction 96 well plates
- lab consumables and benchtop tools dedicated for use in the chromic acid reaction
- consumable wastes (pipette tips, vial caps, benchtop liner paper, gloves, protective arm sleeves)

Chemicals (use at least reagent grade)
chloroform
concentrated sulfuric acid
DI water or HPLC grade water
methanol

oleic acid
potassium chloride
potassium dichromate

2:1 (v/v) chloroform: methanol (Folch solution)
0.25% KCl solution in DI water
1 mg/mL oleic acid standard solution (in 2:1 chloroform: methanol, stock concentrate for standard curve)
chromic acid reagent (potassium dichromate and sulfuric acid prepared as described in steps)

Troubleshooting

Safety warnings

Read the SDS for all chemicals used in this assay, especially sulfuric acid and potassium dichromate.Store and dispose of hazardous wastes and chemicals in accordance with national, state, and local laws. Read the appropriate safety, environmental hazard, and chemical disposal literature on the special hazards of chromic acid, chromates, and hexavalent chromium compounds. Chromic acid presents special hazards not present in its individual precursors. These compounds require special hazardous disposal as hexavalent chromium waste.

Potassium dichromate is a powerful oxidizer and severe corrosive with known toxic, carcinogenic, mutagenic, and teratogenic effects. This chemical may react violently as an oxidizer with organic materials. Chromic acid is a powerful oxidizer that includes the hazards of both sulfuric acid and potassium dichromate. Burns from chromic acid are rapid and do not heal well at all. Both sulfuric acid and chromic acid are capable of causing severe corrosive burns and will burn through most clothes and protective gear including gloves and lab coats. Immediately attend to spills on yourself or in your working area and remove and clean affected materials.

Special handling and chemical disposal precautions are required during the chromic acid reaction steps. All materials used in the chromic acid reaction should be considered potentially contaminated by hexavalent chromate. Users should establish a dedicated set of equipment and materials for the chromic acid reaction that are kept separate from normal laboratory use.

Hexavalent chromium compounds such as potassium dichromate and chromic acid are toxic, highly corrosive, and carcinogenic and must be disposed as hazardous waste in accordance with national, federal, state, and local regulations. Use of these compounds is often restricted and may require special permission for use and disposal. All materials that come into contact with these chemicals are contaminated and must also be disposed of as hexavalent chromium hazardous waste. This includes any samples, lab consumables and safety materials (pipette tips, 96 well plates, reaction vials and caps, gloves, safety sleeves, benchtop liner paper and adhesives, chromic acid containers) that are used in the chromic acid reaction. All equipment that comes in contact with chromic acid reagents (pipettors, crimpers, decrimpers, and heating blocks) are also contaminated and should be stored separate from other laboratory equipment until eventual disposal as hexavalent chromium hazardous waste.

Use secondary containers to better confine waste materials that contain liquid chromate wastes (i.e. excess chromic acid reagents, reaction vials, detached caps, reaction 96 well plates). Store liquid wastes separately from less contaminated solid wastes (contaminated lab consumables and safety equipment).

Wear full goggles, lab coats, gloves, and work in a ventilated hood when performing the lipid extraction and dry down steps. Always wear lab coats, protective arm sleeves, a full face shield, and at least two layers of gloves when handling sulfuric acid or chromic acid solutions or potassium dichromate (chromic acid reaction steps). Protective safety equipment used with the chromic acid reaction should be

considered potentially contaminated by hexavalent chromium compounds, kept separate from other uses, and ultimately disposed of as hazardous waste.

Protect your workspace areas from hexavalent chromium contamination when working with potassium chromate and chromic acid reagents and waste. Fully line your benchtop working area with at least two layers of acid resistant benchtop liner paper secured by tape. Dispose of these materials as contaminated hazardous waste in hexavalent chromium waste after each use of chromic acid reagents.

The BeadBeater homogenizer poises a dangerous mechanical hazard if accidentally turned on when opened. Turn off and depower this instrument completely when loading and unloading vials. Secure vials tightly when loading to avoid destruction when shaking.

Before start

The reagents used in the chromic acid assay are highly toxic, carcinogenic, reactive, and environmentally hazardous. Chromic acid reagents contaminate not only samples and reagents, but also benchtop equipment, lab consumables, and safety materials, all of which require special handling, storage, and disposal as hazardous hexavalent chromium waste. Please read all safety and disposal concerns before attempting this reaction.

Folch Extraction and Partition of Lipids

1 Dry down the pollen in a freeze dryer.

2 Add 1.0 mm zirconia beads to a 2 mL homogenizer tube and fill approximately 1/4 full.

3 Add 10.0 mg pollen (about 1-2 mg lipid equivalent) to the homogenizer tube.

4 Add 1000 µL 2:1 chloroform: methanol (Folch solution) to the homogenizer tube.

5 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.

6 Add 210 µL 0.25% KCl to the homogenizer tube and briefly vortex.

7 Centrifuge the tube contents for 10 minutes at maximum speed in a microcentrifuge.

8 Two layers will form - an upper aqueous/methanolic layer and a lower chloroform/methanolic layer, with a possible boundary layer at the interface 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 each layer and record it (usually 310 µL upper layer and 690 µL lower layer). 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 backup in case the reaction fails.

Stopping point - The Folch extract can either be dried down immediately for the chromic acid assay or stored capped with a red rubber crimp cap in the -20°C freezer until later analysis.

9 *Turn on the SpeedVac vacuum centrifuge at least 45 minutes before drying samples to allow the solvent vapor trap to cool down before use.*

Make a 1.0 mg/mL oleic acid stock solution to create an oleic acid standard curve (0.0 to 1.0 mg/mL step 0.2 mg/mL). Add 9.8 mg oleic acid (11.0 µL) to 9.845 mL 2:1 chloroform: methanol in a scintillation vial and vortex for 10 seconds.

This standard stock solution can be stored sealed in a freezer for up to a few weeks without degradation.

10 Add oleic acid stock solution and Folch solution to a crimp vial to obtain oleic acid external standards. Make three vial replicates for each concentration.

oleic acid mg/mL	amount of 1.0 mg/mL oleic acid added (µL)	amount of Folch solution (2:1 chloroform: methanol) added (µL)
1.0	1000	0
0.8	800	200
0.6	600	400
0.4	400	600
0.2	200	800
0.0	0	1000

11 Reduce the sample and external standard solutions to dryness in the vacuum centrifuge.

Chromic Acid Reaction

12 *Use dedicated chromic acid equipment, consumables, and safety materials from this point forward.*
Take appropriate precautions for chromate handling and waste disposal.
All consumables and equipment that are used during the reaction should be considered contaminated and disposed of as hexavalent chromium waste.

Turn the plate reader on at least 30 minutes before use to allow the reader to heat up.

Take all precautions needed for working with chromic acid from this step forward (see safety warnings). Use specialized protective safety gear and linings to limit

contamination. All consumables and benchtop equipment that is used from this point forward should be treated as potentially contaminated by hexavalent chromium and should be stored and disposed of as such.

Make the chromic acid reagent in a sealed glass container with a non-metallic plastic cap (approximately 600 µL per sample or standard (run in triplicate), 200 mL being sufficient for 1000 sample and standards wells on 5 plates). For 200 mL chromic acid, dissolve 1.0 g potassium dichromate in 10.0 mL DI water. Carefully dilute to 200 mL with concentrated sulfuric acid.

Critical - *Add the acid slowly to the aqueous dichromate solution while stirring and pause frequently to avoid violent overheating. Do not add water to concentrated acid, as this will likely result in violent boiling over and splashing of this concentrated acid solution.*

Chromic acid can be used for long periods after formation if properly stored in inert containers.

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13 Add 1.000 mL chromic acid reagent to the crimp vial with the chromic acid-dedicated P1000 pipettor. Be careful not to get any reagent on the vial lip or it will react with the crimp cap. Use the crimper to crimp close each vial with a PFTE aluminum crimp cap. Double crimp the cap at different angles.

14 Line the hot plate with two layers of aluminum foil to reduce spill damage. Place the crimp-sealed vials in the chromic acid-dedicated heating blocks of the hot plate. Loosely cover the block with aluminum foil to reduce convective heat loss. Heat the vials for 45 minutes at 95°C.

Prepare a working area in a hood that is fully prepared for working with chromate-containing materials and containment of chromate waste. Line all benchtop surfaces that will contain these materials with two layers of benchtop liner paper securely taped down. All containment materials should be disposed of as potentially contaminated after the reaction.

15 Turn off the heat and wait at least 5 minutes for the heating block to cool. Remove the entire block from the heater and give the block 10 more minutes to cool. Transfer the sample and standard vials to the chromate working area in the hood.

Store the sealed reactant vials in the hood chromate working area until the plate can be made and the absorbance can be read. The chromic acid reaction goes to completion

and will not change appreciably over time, but the reaction solution may lose water if the vials are left uncapped for long periods of time. Do not dispose of the vials until you obtain consistent results.

16 Once cooled, pull the crimped capped reaction vials out of the blocks and place the vials into 24 well plates in the spatial configuration desired for the 96 well plate (3 replicates per sample or standard, 6 standard concentrations and 26 samples per 96 well plate). Standards are usually included on the left side of each plate.

17 Decap the reaction vials with the decrimper and return each vial to its well plate location. Deposit the decrimped caps into a secondary waste container. Be careful to avoid chromic acid droplets caused by the decapping mechanism.

18 Load 200 µL of each standard and sample vial reaction solution to a well in a 96 well plate. Make three replicates in triplicate horizontally across the plate for each vial.

19 Place the plate into the plate reader and read the 620 nm absorbance for each well.

Calculation of Total Lipid Contents from the Plate Reader Absorbance

20 Export the 620 nm absorbance data. Calculate the average 620 nm absorbance for each sample and standard triplicate.

21 Generate a standard curve from the standards. Calculate the net 620 nm absorbance for each standard concentration as:

ave 620 nm ABS of the standard concentration - ave 620 nm ABS of the 0.0 mg/mL standard

22 Plot the net 620 nm absorbance for each standard concentration against the standard lipid concentration (mg lipid/mL). Apply a linear best fit line with a zero intercept.

23 Calculate the net 620 nm absorbance for each sample triplicate as:

ave 620 nm ABS of the sample triplicate - ave 620 nm ABS of the 0.0 mg/mL standard

If sample absorbances indicate that the samples were too concentrated (above 1.0 mg/mL), repeat the plate reading with a 1:4 diluted sample reaction solution (120 µL

sample reaction solution diluted with 480 uL chromic acid reagent, mixed well and run in triplicate). Do not dilute the external standards if diluted samples are rerun.

24 Construct a standard curve from the average net 620 nm absorbance of each standard concentration. Plot average net 620 nm absorbance of each standard concentration against lipid amounts (in ug) and make a linear best fit line through a zero intercept.

25 Use the best fit equation to calculate the amount of lipid present in each sample well. Steps that were performed differently between standards and samples (i.e. dilutions, taking only a fraction of the total sample) need corrections in calculations. Make a correction to adjust for the proportion of lower chloroform/methanolic layer taken out of the total volume of this layer (i.e. if 160 µL of 690 µL chloroform/methanolic layer was taken, then 0.23x of the total layer was taken, and the correction would be the inverse (4.31x)). If sample dilutions were performed, correct using the inverse of the dilution factor (i.e. a dilution to 1/5x the initial concentration would be require a 5x correction factor).

26 Divide the sample total lipid amount by the pollen mass to obtain the total lipid content of the pollen sample.