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Quantification of pollen cysteine contents by PITC derivatization

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ABSTRACT

This protocol quantifies cysteine residues from small amounts of pollen (10 mg) after phenylisothiocyanate (PITC) derivatization (adapted from Manneberg et al 1995). Quantification of cysteine by other methods is difficult because of its instability during amino acid analysis. Samples of anther pollen, corbicular pollen, or stored pollen from bee colonies are completely digested in strong acid. Cysteine residues are completely oxidized to cysteic acid then derivatized with PITC before HPLC UV VIS analysis.

Manneberg, M., Lahm, H., & Fountoulakis, M. (1995). Quantification of cysteine residues following oxidation to cysteic acid in the presence of sodium azide. *Analytical Biochemistry, 231*, 349-353. https://doi.org/10.1006/abio.1995.9988

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ATTACHMENTS

DeGrandi Hoffman et al 2018 JIP 109 114 Connecting the nutrient composition of seasonal pollens.pdf

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

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GUIDELINES

- Sample contents are quantified by comparison to known amounts of cysteine external standards..
- Process external standards by exactly the same steps as samples.
- To avoid degradation, store pollen samples before processing in the dark in a deep freezer (-80°C).
- PITC derivatives degrade rapidly in the light. Keep derivatized samples and standards covered in the dark at all steps from PITC derivatization through HPLC analysis.
- This method can be combined with other amino acid analyses (EZ FAAST acid and base hydrolysis) to provide a full profile of sample amino acid contents.

MATERIALS

Consumables and benchtop equipment

crimp cap vials, 2 mL 12 mm x 32 mm (Thermo Scientific 200 000)
crimp caps, 11 mm white aluminum PFTE (Thermo Scientific 502 070)
HPLC vials, 1.5 mL 12 mm x 32 mm (Sun Sri 500 778)
HPLC caps, 11 mm (Sun Sri 501 348)
P200 and P1000 pipettors and pipette tips
aluminum foil
aluminum blocks (capable of fitting 12 mm crimp cap vials)
crimper and decrimper, for 12 mm vials

Instruments

digital hot plate (HP 30A digital hot plate, Torrey Pines Scientific)
freeze dryer (Labconco 6L freeze dryer)
HPLC PDA (Thermo Spectra System AS 3000 HPLC; Finnegan Surveyor PDA UV VIS detector)
Waters Pico Tag column (3.9 mm x 150 mm), Waters Corporation, Milford, MA, USA
microcentrifuge
vacuum centrifuge (Savant SpeedVac SPD 2010, ThermoScientific)

Chemicals

acetonitrile
concentrated hydrochloric acid (12.1M)
cysteine (external standard)
disodium hydrogen phosphate
methanol
phenol
phenylisothiocyanate (PITC)
sodium acetate
sodium azide (NaN_3)
triethylamine (TEA)
water, DI or HPLC grade

SAFETY WARNINGS

Read the SDS safety sheets for all chemicals used in this assay, especially acetonitrile, hydrochloric acid, phenol, PITC, sodium azide, and TEA. Wear full eye protection, lab coats, and gloves and work in a ventilated hood while handling chemicals. Wear additional protection (arms sleeves, face shield, and two layers of gloves) when handling sodium azide or concentrated hydrochloric acid. Sodium azide is a highly toxic neurotoxin that can readily penetrate through skin contact. Always add strong acids to water to avoid violent overheating. Store and dispose of hazardous wastes and chemicals in accordance with national, state, and local laws.

BEFORE START INSTRUCTIONS

The optimal UV VIS wavelength used for HPLC peak quantification may be affected by interference from other plant compounds in the pollen. Compare a processed representative sample of novel pollens against external standards before processing multiple samples to determine if the targeted peak wavelength is obscured.

Acid Hydrolysis and Oxidation of Pollen Samples

1 Dry down pollen samples in a freeze dryer.

2 Create a cysteine external standard in water. Free cysteine is nearly insoluble in water and should be prepared at dilute concentrations. Make a 1 µg cysteine/mL ethanolic water stock solution. Start by dissolving cysteine in ethanol at 10x final concentration, then dilute the concentrated ethanolic solution in DI water. Use the external standard to create a standard curve from 0 µg to 100 µg cysteine (0, 20, 40, 60, 80, or 100 µL stock solution added to a crimp cap vial and dried down like the samples).

Cysteine oxidizes rapidly at room temperature.Use fresh external samples.

Cysteine external standards are processed in the same exact method as the samples (except for initial formation) to account for losses.

Consider comparing your cysteine external standards against cysteic acid standards (the oxidized substrate derivatized by PITC) to validate your method processing.

3 Weigh out 10.0 mg of pollen into an amber crimp cap vial.

4 Add the following solutions sequentially to the vial: 780 µL 6M HCl, 20 µL 1% phenol in water, 100 µL 12M HCl, and 100 µL 8% sodium azide in water. Avoid spilling reagent drops on the vial lip where the crimp cap will seal the vial.

The reagents here include strong acids, corrosives, and highly toxic compounds capable of entering human tissue through the skin. Wear full protective clothing and gear that cover all your skin.

5 Allow the vials to cool in their heating blocks to room temperature. Briefly spin the vials down for a few seconds in a vacuum centrifuge (**without vacuum**) to bring down condensation.

6 Use a crimper to seal the vial with a PTFE aluminum crimp cap. Double crimp the cap at two different angles to ensure a tight seal.

7 Incubate the vials in a heating block on a hot plate at 70°C for 24 hours. Cover the heating block with aluminum foil to reduce light and convective heat loss.

8 Transfer 25 µL acid hydrosylate to an amber crimp vial and add 50 µL of redrying solution (2:2:1 methanol: water: TEA (triethylamine)). Vortex for 10 seconds and dry down in a vacuum centrifuge.

Save the remaining acid hydrosylate in a freezer as backup material in case the reaction fails.

9 Add another 50 µL of redrying solution to the vial, vortex for 10 seconds and dry down again in a vacuum centrifuge.

PITC Derivatization and HPLC Preparation

10 *The samples and reagents are light sensitive from the next step until HPLC analysis. Keep the samples covered and in the dark when not actively being handled.*

Add 50 µL derivatizing reagent (7:1:1:1 methanol: TEA: water: PITC (phenylisothiocyanate)) to each vial, vortex for 10 seconds and incubate at room temperature (25°C) for 20 minutes.

11 Dry down the vial in a vacuum centrifuge.

12 Add 50 µL methanol to each vial and vortex for 10 seconds. Dry down the vial again in a vacuum centrifuge.

13 Add another 50 µL methanol to each vial and vortex for 10 seconds. Dry down the vial again in a vacuum centrifuge.

14 Dissolve the dried material in 200 µL 95:5 5 mM disodium hydrogen phosphate (pH 7.4): acetonitrile in preparation for injection on the HPLC.

HPLC Separation and Peak Analysis

15 Analyze the cysteine contents of samples and external standards as PITC-derivatized cysteic acid by HPLC UV VIS (Thermo Spectra System AS 3000 HPLC with a Finnegan Surveyor PDA UV VIS detector). Inject 10 µl on a Waters Pico Tag column (3.9 mm x 150 mm) and separate with a step gradient from 100% sodium acetate buffer (150 mM sodium acetate with 6% acetonitrile and 0.05% TEA) to 18:28:54 water: acetonitrile: sodium acetate buffer in 5.5 minutes, then to 40:60 water: acetonitrile in 10.0 minutes, followed by a 2.5 minute hold.

16 Identify the retention time for PITC-derivatized cysteic acid by examining the chromatograms for the external standards. The optimal UV VIS wavelength selected for peak quantification will vary

depending on interference from other compounds present in the pollen extract.

17 The amount of PITC-derivatized cysteic acid present in samples is calculated by comparison of retention times and peak areas with known amounts of oxidized/derivatized cysteine external standards. Pollen cysteine contents can then be calculated as the amount of cysteine per given mass of pollen material. Steps that were performed differently between standards and samples (i.e. dilutions, taking only a fraction of the total sample) need corrections in calculations.