Plumage pigments identification by High-performance liquid chromatography

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Abstract

As one of the most recognizable characteristics in birds, plumage colour has a high impact on understanding the evolution and mechanisms of colouration. Feather and skin are ideal tissues to explore the genomics and complexity of colour patterns in vertebrates. Two species of the genus Chrysolophus, golden pheasant (Chrysolophus pictus) and Lady Amherst's pheasant (Chrysolophus amherstiae), exhibit brilliant colours in their plumage, but with extreme phenotypic differences, making these two species great models to investigate plumage colouration mechanisms in birds. Here we provide details of the protocols used for plumage pigments identification by High-performance liquid chromatography. The fat-soluble pigments (carotenoids) and oxidation products of melanins (TTCA and PTCA) can be qualitatively and quantificationally analyzed by HPLC with the corresponding standards, respectively.

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Guidelines

Carotenoids should be extracted from feathers by a thermochemical treatment. The melanins from feathers should be treated under alkaline H2O2 conditions to obtain oxidation products.

Protocol

Carotenoids extraction

Step 1.

Each type of coloured feather was soaked in ethanol and hexanes in turn so that surface lipids were removed.

Step 2.

Pigmented barbs were trimmed into pieces, weighed and placed into a 10ml glass tube which can be capped tightly, and then 1ml acidified pyridine was infused to cover the pieces.

Step 3.
The tube was filled with argon gas and placed in a 368K water bath for 3 h.

Step 4.
After cooling to room temperature, 2 ml pure water and 1ml hexane: tert-butyl methyl ether (1:1) was added into the tube in order to separate the carotenoid pigments from the solution.

Step 5.
The mixture was vigorously shaken for 2 min and centrifuged at 3000 rpm for 5 min.

Step 6.
The supernatant was transferred to a clean tube, dried under nitrogen gas, and stored at 193 K for subsequent analysis.

Carotenoids identification by HPLC

Step 7.
Dried pigment was dissolved in HPLC mobile phase (methanol/acetonitrile, 50:50 v/v, +0.05% triethylamine), filtered through a nylon syringe filter and injected into an SIL-20A HPLC system (Shimadzu, Japan) equipped with a YMC C-30 Carotenoid column (5 µm, 4.6 mm × 250 mm; YMC, Japan) and an SPD-20A UV/Vis detector (Shimadzu, Japan). An isocratic system at a flow rate of 1.5 ml/min for 40 min was programmed and data were collected at 450 nm.

Melanins oxidation

Step 8.
A total of 20 mg barbs were weighed and ground into powder in liquid nitrogen.

Step 9.
After warming to room temperature, 2 ml pure water was infused to the powder and 200 µl suspensions were placed into a 10ml glass tube, to which 750 µl 1 mol/ l K_2CO_3 and 50 µl 30% H_2O_2 were added.

Step 10.
After the mixtures were shaken at 298K for 20 h, 100 µl 10% Na_2SO_3 and 280 µl 6 mol/l HCl were used to decompose the residual H_2O_2 and acidify the solution, respectively.

Melanins identification by HPLC

Step 11.
The reaction products then were centrifuged at 4000 g for 1 min and an aliquot of each supernatant was injected into the same HPLC system except equipped with a Wondasil C18 column (5 µm, 4.6 mm × 250 mm; GL Sciences, Japan). TTCA and PTCA were analyzed with a mobile phase of 0.1 mol/l potassium phosphate buffer (pH 2.1) / methanol, 99:1 (v/v) at 318K at a flow rate of 0.7 ml/ min, and data were collected at 269 nm.

Warnings

The sample pretreatment process should be completed in the draught cupboard.