XANTHOPHYLLS

PRINCIPLE

Pigments are extracted from the sample with a mixed solvent and adsorbed on a magnesia-filter aid mixture contained in a chromatographic column. Carotenes and xanthophylls are eluted separately and determined spectrophotometrically.

SCOPE

The method applies to feedstuffs produced by the corn wet milling industry. With modification of sample size and extraction procedure, it can be applied to corn, xanthophyll concentrates and xanthophyll enriched feeds.

SPECIAL APPARATUS

1. Chromatography Assembly: The chromatographic column is constructed of Pyrex glass. The upper section (main body) is 12.5 mm inside diameter and 30 cm long with a flared top. The bottom section is a 2 mm inside diameter capillary tube about 10 cm long, which extends into the neck of a volumetric flask during operation.

   Pigment extract and eluents are forced through the chromatographic column with a vacuum filtration device such as a Fisher Scientific Co. "FILTRATOR", or equivalent. The column is attached to the FILTRATOR by means of a rubber stopper, so that the bottom capillary section extends into the neck of a volumetric flask.

2. Spectrophotometer: Use a modern instrument capable of continuously variable wavelength in the visible spectrum, and operating with a narrow slit width. Use 1 cm matching cuvets.

XANTHOPHYLLS — continued

REAGENTS

1. Acetone, Anhydrous: Alcohol-free. Distill over granular zinc, about 10 mesh

2. Ethyl Alcohol, Absolute

3. Hexane: Reagent grade

4. Toluene: Reagent grade

5. Methanol, Anhydrous: Reagent grade

6. Methanolic Potassium Hydroxide, 40%: Dissolve 40 g of potassium hydroxide (KOH) in anhydrous methanol, cool and dilute to 100 mL with methanol.

7. Extraction Solvent: Mix 100 mL hexane, 70 mL acetone, 60 mL absolute ethanol and 70 mL toluene in a 500 mL glass stoppered bottle.

8. Carotenes Eluent: Mix 384 mL hexane and 16 mL acetone in a glass stoppered bottle.

9. Xanthophylls Eluent: Mix 320 mL hexane, 40 mL acetone and 40 mL methanol in a 500 mL glass stoppered bottle.

10. Sodium Sulfate, Anhydrous: Reagent grade, granular

11. Chromatographic Adsorbent: Combine 1 lb activated magnesia (SeaSorb 43, Fisher Scientific Co.) with 1 lb diatomaceous earth (Hyflo Super-Cel) and mix in a suitable mechanical blender for 2 hours.

12. 1-(Phenylazo)-2-Naphthol, (C.I. Solvent Yellow 14, or Sudan 1): Available from Aldrich Chemical Co., or MC/B Manufacturing Chemists. Recrystallize material from hot absolute ethanol. Dry crystals to constant weight at 70° C in a vacuum oven.

XANTHOPHYLLS — continued

Stock Solution: 1.0 millimolar (mM): Dissolve 0.1241 g in 500 mL acetone-isopropyl alcohol (1+1) mixture.

Working Standard, 0.04 mM: Dilute 20 mL stock solution to 500 mL with acetone-isopropyl alcohol (1+1) mixture.

PROCEDURE

Pigment Extraction: Grind Sample completely through a laboratory cutting mill to 40 mesh or finer, taking precautions to prevent significant loss of moisture, and mix thoroughly (Note 1).

Weigh accurately a sample containing about 400 µg xanthophylls (Note 2) into a 100 mL volumetric flask. Pipette 30.0 mL extraction solvent into flask and add 1.0 mL deionized water per 2.0 g sample. Stopper and swirl 1 minute.

Pipette 2.0 mL 40% methanolic potassium hydroxide solution into flask (4.0 mL when analyzing gluten feed), swirl 1 minute and place flask in 56 °C water bath for 20 minutes. Attach air condenser to flask while heating to prevent loss of solvent. Cool sample and let stand in dark 1 hour. Pipette 30.0 mL hexane into flask, swirl 1 minute, dilute to volume with 10% sodium sulfate solution, and shake vigorously 1 minute. Let stand in dark 1 hour before chromatography. Upper phase volume is assumed to be 50 mL.

Spectrophotometer Calibration: In matching 1 cm cuvets, with the blank cuvet filled with acetone-isopropyl alcohol (1 + 1) mixture, measure absorbance of the 1-(phenylazo)-2-naphthol working standard solution at 1 nm intervals between 469 and 479 nm. If maximum absorbance is not at 474 nm, recalibrate instrument. When instrument shows maximum absorbance at 474 nm and the slit width is 0.03, absorbance values for the working standard solution should be 0.561 at 474 nm and 0.460 at 436 nm. If these are not observed, use the following instrument deviation factors to calculate the carotenes and xanthophylls contents (Note 3).

XANTHOPHYLLS — continued

$$f_{436\,nm} = \frac{0.460}{A_{436\,nm\,(obs)}}$$

$$f_{474\,nm} = \frac{0.561}{A_{474\,nm\,(obs)}}$$

Chromatography: Attach column to Filtrator, place absorbent cotton or glass wool plug in bottom and add about 12 cm layer of chromatographic adsorbent. Apply full vacuum and add more adsorbent to give a 7 cm layer. Use a flat instrument, such as an inverted cork on a glass rod, to press and flatten the adsorbent layer surface. Place a 2 cm layer of anhydrous sodium sulfate on top of the adsorbent and press firmly (Note 4).

Place a 50 mL flask in Filtrator below column to receive eluent, apply vacuum to column and pipette 25 mL upper phase onto column. Adjust vacuum to give flow of 2 to 3 drops of eluent per second (needle valve in vacuum line helps control flow rate). As soon as solvent enters the sodium sulfate layer, release vacuum carefully and replace flask with 50 mL volumetric flask to receive effluent. Apply vacuum, add carotenes eluent to upper column, adjust flow rate as before, and continue adding carotenes eluent as the discrete carotenes band moves down the column and passes into the 50 mL receiver. When carotenes have passed completely into the receiver, discontinue addition of eluent, and allow flow to continue until remaining eluent just enters the sodium sulfate layer.

Release vacuum carefully, remove carotenes-containing flask and place in dark until it reaches room temperature. Dilute to volume (50 mL) with carotenes eluent, invert flask several times to mix thoroughly, and determine absorbance immediately with a calibrated spectrophotometer.

Replace carotenes-containing flask with clean 50 mL volumetric flask to receive the xanthophylls. Apply vacuum, add xanthophylls eluent, adjust flow rate as before, and continue adding xanthophylls eluent until all pigment has passed through the column into the receiver. Discontinue addition of xanthophylls eluent, release vacuum carefully, remove xanthophylls-containing flask and place in dark until it reaches room temperature. Dilute to volume (50 mL) with xanthophylls

XANTHOPHYLLS — continued

eluent, invert flask several times to mix thoroughly, and determine absorbance immediately with a calibrated spectrophotometer.

Analysis: Measure absorbance (A) of the carotenes (436 nm) and xanthophylls (474 nm) eluent solutions promptly to minimize isomerization and autoxidation losses.

CALCULATION

$$\text{Xanthophylls, mg/lb (as is)} = \frac{A_{474} \times 454 \times f_{474}}{236 \times b \times d}$$

$$\text{Carotenes, mg/lb (as is)} = \frac{A_{436} \times 454 \times f_{436}}{196 \times b \times d}$$

where:

236 = Xanthophylls specific absorbance

196 = Carotenes specific absorbance

f_{474} = Spectrophotometer correction at 474 nm

f_{436} = Spectrophotometer correction at 436 nm

b = Cuvet path length, cm

d = Dilution factor = $\frac{\text{Sample Wt. (g) x mL Extract on Column}}{\text{50 mL Upper Phase x mL Final Dilution}}$

NOTES AND PRECAUTIONS

1. To prevent moisture loss when grinding a series of samples, allow the mill to cool between samples.

2. Recommended sample sizes for products from corn are: purified corn gluten - 1.0 g; corn gluten meal - 2.0 g; corn gluten feed - 5.0 g; corn – 5.0 g.

XANTHOPHYLLS — continued

3. If the spectrophotometer lacks a controllable slit, establish concentrations by assuming that the working standard solution of dye has the same absorbance as 2.35 mg carotenes per liter at 436 nm and 2.38 mg xanthophylls per liter at 474 nm.

4. Packing a chromatographic column is an art which one learns by experience. The procedure described generally gives a satisfactory column. However, column performance may sometimes be improved by pressing the top of the completed column firmly with a flat instrument such as a cork attached to a glass rod.

REFERENCES

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METHOD HISTORY

Feedstuffs, Xanthophylls (G-40), Date of Acceptance 11-16-1964, Revised 10-09-2009.