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Method of Test for Pheophorbide in Edible Algae

1. Scope

This method is applicable for the determination of pheophorbide, including existing pheophorbide and total pheophorbide, in green algae and blue algae.

2. Method

After extraction, pheophorbide is determined by spectrophotometry.

2.1. Equipment

2.1.1. Spectrophotometer: with the wavelength of visible light at 667 nm.

2.1.2. Centrifuge.

2.1.3. Water bath.

2.2. Chemicals

Refined sea sand, reagent grade;

Acetone, reagent grade;

Diethyl ether, reagent grade;

Sodium sulfate, reagent grade;

Sodium sulfate anhydrous, reagent grade;

Hydrochloric acid, reagent grade;

Disodium hydrogen phosphate (Na_2HPO_4), reagent grade;

Potassium dihydrogen phosphate (KH_2PO_4), reagent grade;

Deionized water, resistivity ≥ 18 MΩ·cm (at 25°C);

2.3. Apparatus

2.3.1. Volumetric flask: 20 mL and 100 mL.

2.3.2. Separatory funnel: 250 mL.

2.3.3. Centrifuge tube: 50 mL, PP.

2.4. Reagents

2.4.1. 85% acetone

Mix acetone and deionized water at the ratio of 85: 15 (v/v).

2.4.2. 5% sodium sulfate

Dissolve and dilute 5 g of sodium sulfate with deionized water to 100 mL.

2.4.3. 17% hydrochloric acid

Take 45.3 mL of hydrochloric acid, slowly add to 50 mL of deionized water, and dilute with deionized water to 100 mL.

2.4.4. Saturated sodium sulfate solution

Take 400 g of sodium sulfate, and add 1000 mL of deionized water. After

stirring moderately, take the upper layer for later use.

2.4.5. M/15 potassium dihydrogen phosphate

Dissolve and dilute 9.073 g of potassium dihydrogen phosphate with deionized water to 1000 mL.

2.4.6. M/15 disodium hydrogen phosphate

Dissolve and dilute 9.464 g of disodium hydrogen phosphate with deionized water to 1000 mL.

2.4.7. Phosphate buffer solution

Mix M/15 potassium dihydrogen phosphate and M/15 disodium hydrogen phosphate at the ratio of 5: 95 (v/v), and adjust pH to 8.0.

2.4.8. Phosphate buffer: acetone (7:3, v/v)

Mix phosphate buffer solution with acetone at the ratio of 7: 3 (v/v).

2.4.9. 0.5 N hydrochloric acid

Take 4.16 mL of hydrochloric acid, slowly add to 75 mL of deionized water, and dilute with deionized water to 100 mL.

2.5. Sample solution preparation

2.5.1. Existing pheophorbide

Transfer about 100 mg of the homogenized sample accurately weighed in a mortar, add about 0.5 g of refined sea sand and 20 mL of 85% acetone, grind quickly, and stand still. Transfer the supernatant into a centrifuge tube, add 10 mL of 85% acetone to the residue, and repeat the extraction procedure twice. Combine the supernatants, and centrifuge at 2000 ×g for 5 min. Transfer the supernatant into a separatory funnel filled with 30 mL of diethyl ether(note), add 50 mL of 5% sodium sulfate, and shake slowly. Allow the layers to separate, discard the lower layer, add 50 mL of 5% sodium sulfate to the upper layer, and repeat the washing procedure described above twice. Dry the ether extract with sodium sulfate anhydrous, then put into a separatory funnel, add 20 mL of 17% hydrochloric acid, and shake for extraction. Collect the lower layer, add 10 mL of 17% hydrochloric acid to the upper layer, and shake again. Combine the lower layers, transfer to a separatory funnel filled with 150 mL of saturated sodium sulfate solution and 20 mL of diethyl ether, and shake well. Take the diethyl ether layer, and dilute with diethyl ether to 20 mL as the sample solution.

Note: Redistilled diethyl ether should be used to avoid shifting the absorption wavelength towards 670-680 nm due to excess peroxide in diethyl ether.

2.5.2. Total pheophorbide

Transfer about 100 mg of the homogenized sample accurately weighed into a centrifuge tube, add 10 mL of phosphate buffer: acetone (7:3, v/v), and react for 3 hr in a water bath at 37°C. Adjust pH to 4.5-5.5 with 0.5 N hydrochloric acid, and put into a mortar. Add about 0.5 g of refined sea sand and 20 mL of 85% acetone, grind quickly, and stand still. Perform the same procedure described in section 2.5.1 as the sample solution.

2.6. Determination

Take the sample solution, and measure its absorbance with a spectrophotometer at the wavelength of 667 nm. Calculate the amount of existing pheophorbide or total pheophorbide in the sample by the following formula:

The amount of existing pheophorbide or total pheophorbide in the sample

$$(\text{mg}/100\ \text{g}) = \frac{\text{Abs}}{E} \times \frac{20}{M \times (1 - W/100)} \times 100$$

Where,

Abs: the absorbance of the sample solution at the wavelength of 667 nm

E: the absorbance of the 0.1% pheophorbide in a 1 cm cuvette at the wavelength of 667 nm. The E value is 70.2.

M: the weight of the sample (g)

W: the moisture content of the sample (%)

Remark

1. This method is applied to green algae and blue algae whose moisture content is less than 7%. Fresh products, red algae and brown algae are not applied.
2. Further validation should be performed when interfering compounds appear in the samples.