Method of Test for Cu-pyropheophytin A in Edible Oils

1. Scope

This method is applicable to the determination of Cu-pyropheophytin a, the major copper chlorophyll, in edible oils.

2. Method

After extraction and purification, Cu-pyropheophytin a is determined by high-performance liquid chromatography (HPLC) and confirmed by liquid chromatography/high resolution tandem mass spectrometry ($LC/HRMS^2$) or liquid chromatography/ tandem mass spectrometry (LC/MS/MS).

2.1. Equipments

2.1.1. High-performance liquid chromatograph

2.1.1.1. Detector: photodiode array detector.

2.1.1.2. Column: GL Sciences InertSustain C18, 2-μm, 2.1-mm i.d. × 10-cm, or an equivalent product.

2.1.2. Liquid chromatograph/high resolution tandem mass spectrometer

2.1.1.1. Ion source: negative ion atmospheric pressure chemical ionization, $APCI^-$.

2.1.1.2. Column: HALO C18, 2.7-μm, 4.6-mm i.d. × 10-cm, or an equivalent product.

2.1.3. Liquid chromatograph/tandem mass spectrometer

2.1.1.1. Ion source: negative ion atmospheric pressure chemical ionization, $APCI^-$.

2.1.1.2. Column: HALO C18, 2.7-μm, 4.6-mm i.d. × 7.5-cm, or an equivalent product.

2.1.4. Solid phase extraction vacuum manifolds.

2.1.5. Nitrogen evaporator.

2.2. Chemicals

Petroleum ether, HPLC grade;

Ethyl ether, HPLC grade;

Acetone, HPLC grade;

Methanol, HPLC grade;

Anhydrous ethanol, HPLC grade;

Ammonium acetate, GR grade;

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

Cu-pyropheophytin a, reference standard.

2.3. Apparatus

2.3.1. Centrifuge tube: 15-mL, PP.

2.3.2. Membrane filter: 0.22-μm, Nylon.

2.3.3. Solid phase extraction cartridge: Sep-Pak® silica, 1-g, 6-mL, or an equivalent product.

2.4. Reagents

2.4.1. 1 M ammonium acetate solution

Dissolve 77 g of ammonium acetate in deionized water and dilute with deionized water to 1000 mL.

2.4.2. Mobile phase for HPLC

Solvent A: methanol: 1 M ammonium acetate (8:2, v/v).

Solvent B: acetone.

Solvent C: methanol.

Solvent D: deionized water.

2.4.3. Mobile phase for LC/HRMS[2]

Solvent A: acetone.

Solvent B: methanol.

2.4.4. Mobile phase for LC/MS/MS

Solvent A: methanol.

Solvent B: anhydrous ethanol.

2.5. Standard solution preparation

Transfer about 10 mg of Cu-pyropheophytin a reference standard accurately weighed into 10-mL volumetric flask, dissolve and dilute to volume with acetone as a stock solution. When to use, dilute the stock solution with acetone to 0.05 - 5.0 μg/mL as standard solutions.

2.6. Sample solution preparation

Transfer about 1 g of the sample accurately weighed into a centrifuge tube and transfer the sample from the tube into a Sep-Pak® silica cartridge by a plastic dropper. Wash the centrifuge tube with 3 mL of petroleum ether and transfer the washing solution into the cartridge. Repeat the washing step twice. Discard the eluents. Wash the cartridge with 9 mL of petroleum ether: ethyl ether (9:1, v/v) and discard the eluent. Add 6 mL of acetone to the cartridge, collect the eluent and evaporate to dryness by gently flushing with a stream of nitrogen at 25°C. Dissolve the residue with 1 mL of acetone, then filter the solution with a membrane filter, and use it as the sample solution.

2.7. Identification and quantification

Separately inject about 20 μL of the sample solution and the standard solutions into

the HPLC and perform HPLC analysis. Identify Cu-pyropheophytin a by retention times and absorption spectra. Calculate the amount of Cu-pyropheophytin a in the sample by the following formula:

$$\text{The amount of Cu-pyropheophytin a in the sample (ppm)} = \frac{C \times V}{M}$$

where

C: the concentration of Cu-pyropheophytin a in the sample solution calculated by the standard curve (μg/mL)

V: the make up volume of sample (mL)

M: the weight of sample (g)

HPLC operating conditions:

Photodiode array detector: 430 nm.

Column: GL Sciences InertSustain C18, 2 μm, 2.1-mm i.d. × 10-cm.

Column temperature: 30 °C.

Mobile phase: gradient.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)
0.0 → 3.0	30 → 30	0 → 0	0 → 0	70 → 70
3.0 → 7.0	30 → 100	0 → 0	0 → 0	70 → 0
7.0 → 9.0	100 → 100	0 → 0	0 → 0	0 → 0
9.0 → 11.0	100 → 50	0 → 25	0 → 25	0 → 0
11.0 → 13.0	50 → 50	25 → 25	25 → 25	0 → 0
13.0 → 15.0	50 → 0	25 → 50	25 → 50	0 → 0
15.0 → 25.0	0 → 0	50 → 50	50 → 50	0 → 0
25.0 → 26.0	0 → 0	50 → 70	50 → 30	0 → 0
26.0 → 35.0	0 → 0	70 → 90	30 → 10	0 → 0
35.0 → 35.1	0 → 30	90 → 0	10 → 0	0 → 70
35.1 → 40.0	30 → 30	0 → 0	0 → 0	70 → 70

Injection volume: 20 μL.

Flow rate: 0.25 mL/min.

2.8. Confirmation

2.8.1. LC/HRMS^2

Separately inject about 40 μL of the HPLC positive sample solution and the standard solution into the LC/HRMS^2 and operate according to the following LC/HRMS^2 conditions. Identify Cu-pyropheophytin a based on retention times,

mass accuracy (< 5 ppm) and relative ion intensities.

LC/HRMS^2 operating conditions:

Column: HALO C18, 2.7-μm, 4.6-mm i.d. × 10-cm.

Column temperature: 30 °C.

Mobile phase: gradient.

Time (min)	Solvent A (%)	Solvent B (%)
0.0 → 10.0	5 → 80	95 → 20
10.0 → 11.0	80 → 100	20 → 0
11.0 → 14.0	100 → 100	0 → 0
14.0 → 14.1	100 → 5	0 → 0
14.1 → 21.0	5 → 95	95 → 5

Injection volume: 40 μL.

Flow rate: 1 mL/min.

Ion source: negative ion atmospheric pressure chemical ionization, APCI$^-$.

Collision energy: 20 eV.

Detection mode: product ion scan.

Resolution: 70000.

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)
Cu-pyropheophytin a	873.4749	522.1468 550.1799 594.1697

2.8.2. LC/MS/MS

Separately inject about 40 μL of the HPLC positive sample solution and the standard solution into the LC/MS/MS and operate according to the following LC/MS/MS conditions. Identify Cu-pyropheophytin a based on retention times and relative ion intensities.

LC/MS/MS operating conditions:

Column: HALO C18, 2.7-μm, 4.6-mm i.d. × 7.5-cm.

Column temperature: 30 °C.

Mobile phase: gradient.

Time (min)	Solvent A (%)	Solvent B (%)
0.0 → 1.0	100 → 70	0 → 30
1.0 → 6.0	70 → 40	30 → 60
6.0 → 14.0	40 → 0	60 → 100
14.0 → 15.5	0 → 0	100 → 100
15.5 → 16.0	0 → 100	100 → 0

16.0 → 20.0	100 → 100	0 → 0

Injection volume: 40 µL.

Flow rate: 1 mL/min.

Ion source: negative ion atmospheric pressure chemical ionization, $APCI^-$.

Curtain gas: 20 psi.

Collision gas: high.

Gas 1: 55 psi.

Gas 2: 0 psi.

Temperature: 400 °C.

Detection mode: multiple reaction monitoring (MRM).

Analyte	Precursor ion (*m/z*) > Product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)
Cu-pyropheophytin a	873.5 > 522	-68	-50
	873.5 > 535	-68	-57
	873.5 > 550	-68	-50
	873.5 > 594	-68	-38

Notes:

1. All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.
2. Maximum permitted tolerances for relative ion intensities by $LC/HRMS^2$ or LC/MS/MS are as follows:

Relative ion intensity (% of base peak)	Tolerance (%)
> 50	± 20
> 20~50	± 25
> 10~20	± 30
≤ 10	± 50

3. Further validation is necessary when interference compounds appear in samples.