Identification of Copper Chlorophyll in Edible Oils

1. Scope

This method is applicable to the identification of copper chlorophyll in edible oils.

2. Method

After extraction and purification, samples are screened 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.

2.1.6. TLC developing tank.

2.2. Chemicals

Petroleum ether, HPLC grade;

Ethyl ether, HPLC grade;

Acetone, HPLC grade;

Methanol, HPLC grade;

Anhydrous ethanol, HPLC grade;

n-Hexane, AR grade;

Trichloromethane, AR grade;

Ethanol, AR grade;

Ammonium acetate, GR grade;

Milli-Q water, resistivity ≧ 18 MΩ • cm (25 °C);

Copper chlorophyll complex, a food additive.

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.

2.3.4. Microcapillary pipette: 5-μL.

2.3.5. Thin-layer chromatographic plate: silica gel 60, thickness 0.2-mm, 10-cm × 10-cm.

2.4. Reagents

2.4.1. 1 M ammonium acetate solution

Dissolve 77 g of ammonium acetate in Milli-Q water and dilute with Milli-Q water to 1000 mL.

2.4.2. TLC developing solvent system

n-Hexane: trichloromethane: ethanol (10:9:1, v/v/v).

2.4.3. Mobile phase for HPLC

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

Solvent B: acetone.

Solvent C: methanol.

Solvent D: Milli-Q water.

2.4.4. Mobile phase for LC/HRMS[2]

Solvent A: acetone.

Solvent B: methanol.

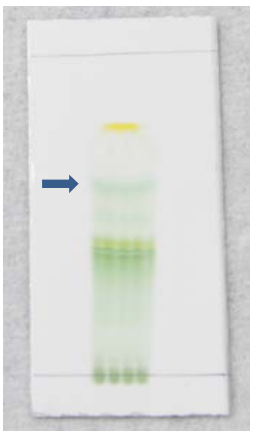
2.4.5. Mobile phase for LC/MS/MS

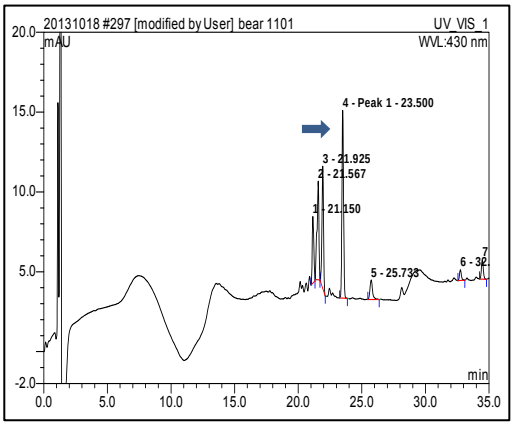
Solvent A: methanol.

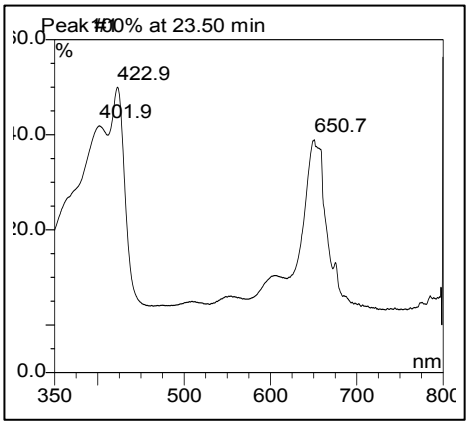
Solvent B: anhydrous ethanol.

2.5. Purification of copper chlorophyll complex

Dissolve about 0.1 g of copper chlorophyll complex in 2 mL of acetone and use it as a solution for TLC analysis. Apply the solution as a band with a diameter of about 0.5 cm onto a TLC plate at about 1 cm from the lower edge. After drying the plate, place it in a TLC developing tank with the sample spots about 0.5 cm above the level of the developing solvent. Develop the plate with the developing solvent system until the solvent ascends to a point about 7 cm above the initial spots. Remove and dry the plate, then visually examine the separated spots and identify the components of interests by their R_F values and colors. Scrape the green spot at an approximate R_F value of 0.6, then extract with 2 mL of acetone and filter with a membrane filter to obtain the purified copper chlorophyll solution. Inject about 20 μL of the solution into the HPLC and follow the HPLC conditions shown in Section 2.7. The retention time of copper chlorophyll is about 23.5 min. Examples of its TLC chromatogram, HPLC chromatogram and UV absorption spectrum are shown as follows:







TLC chromatogram HPLC chromatogram UV absorption spectrum

2.6. Sample solution preparation

Weigh about 1 g of the sample 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℃. 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. Screening

Separately inject about 20 μL of the sample solution and the purified copper chlorophyll solution into the HPLC and perform HPLC analysis. Identify copper chlorophyll by retention times and absorption spectra.

HPLC operating conditions:

Photodiode array detector: scanning wavelength, 350-800 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 purified copper chlorophyll solution into the LC/HRMS^2 and operate according to the following LC/HRMS^2 conditions. Identify copper chlorophyll 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 → 95
14.1 → 21.0	5 → 95	95 → 95

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 purified copper chlorophyll solution into the LC/MS/MS and operate according to the following LC/MS/MS conditions. Identify copper chlorophyll 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.