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Determination of chlorophylls and their derivatives in *Gynostemma pentaphyllum* Makino by liquid chromatography–mass spectrometry

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ABSTRACT

The objectives of this study were to develop a high performance liquid chromatography–mass spectrometry (HPLC–MS) method for determination of chlorophylls and their derivatives in *Gynostemma pentaphyllum* Makino, a traditional Chinese herb possessing vital biological activities. Chlorophylls were extracted with a quaternary solvent system of hexane–acetone–ethanol–toluene (10:7:6:7, v/v/v/v), followed by separation of a total of 15 chlorophylls and their derivatives within 32 min using a gradient mobile phase of acetone, acetonitrile and methanol and a HyPURITY C18 column, with detection at 660 nm and flow rate at 1 mL/min. Identification was carried out on the basis of retention behavior, absorption spectra and mass spectra using atmospheric pressure chemical ionization (APCI) in positive ion mode for detection. Of the 15 analytes, chlorophyll a, chlorophyll b, pheophytin a and pheophytin b were quantified by using standard calibration curves, with the other 11 being quantified with an internal standard Fast Green FCF. Chlorophyll extracts in *G. pentaphyllum* were found to contain pheophytin a (2508.3 μg/g), pheophytin a′ (111.2 μg/g), chlorophyll a (113.8 μg/g), chlorophyll a′ (11.0 μg/g), hydroxypheophytin a (88.6 μg/g), hydroxypheophytin a′ (66.5 μg/g), pyropheophytin a (76.0 μg/g), hydroxychlorophyll a (23.8 μg/g), pheophytin b (319.6 μg/g), pheophytin b′ (13.2 μg/g), chlorophyll b (287.9 μg/g), chlorophyll b′ (11.1 μg/g), hydroxychlorophyll b (15.0 μg/g), hydroxypheophytin b (11.2 μg/g) and hydroxypheophytin b′ (8.5 μg/g).

1. Introduction

Chlorophyll, a photosynthetic pigment that is widely distributed in nature, possesses a basic skeleton structure of porphyrine with a magnesium ion in the center and a long phytol group in the tail [1]. The major chlorophylls in plants include chlorophyll a and chlorophyll b, which are usually present at a ratio of 3:1 [2]. In addition to chlorophyll a and chlorophyll b, there are several chlorophyll derivatives such as pheophytin a, pheophytin b, pyropheophytin a, pyropheophytin b, pheophorbide a, pheophorbide b, chlorophyllide a and chlorophyllide b present in plants [1]. All these derivatives could be formed through heat or acidic treatment or enzymatic degradation [2]. For instance, pheophytin a and pheophytin b could be formed from chlorophyll a and chlorophyll b during cooking of green vegetables, respectively, whereas chlorophyllide a and chlorophyllide b could be formed from chlorophyll a and chlorophyll b in the presence of chlorophyllase [3,4].

Chlorophylls and their derivatives have been extensively studied for their biological activities. Lanfer-Marquez et al. [5] reported that pheophorbide b and pheophytin b were the strongest natural antioxidant compounds, revealing the importance of the aldehyde group for antioxidant activity. In another study dealing with the effect of chlorophyllin on radiation-induced immunosuppression and modulation of immune responses, Sharma et al. [6] indicated that chlorophyllin could inhibit the in vitro lymphocyte proliferation induced by concanvalin A in a dose-dependent manner, and the expression of antiapoptoic genes bcl-2 and bcl-x_L was up-regulated in spleen cells. In addition, the antigenotoxic activity against the DNA damage by chlorophyll a, chlorophyll b and chlorophyllin, as well as the inhibition of skin tumor formation in ICR mouse by pheophorbide a have been demonstrated [7,8]. Nevertheless, several studies have shown that both chlorophyll and chlorophyllin may result in genotoxic and carcinogenic effects [9,10]. Therefore, the impact of chlorophylls and their derivatives on human health cannot be ignored.

Gynostemma pentaphyllum (Thunb.) Makino, a traditional Chinese herb that is frequently used in the treatment of chronic disease by oriental people, has been shown to possess several vital biological activities like anti-cancer and anti-inflammation as well

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as liver protection [11,12]. The presence of functional components in *G. pentaphyllum* such as flavonoids, saponins, carotenoids and chlorophylls are believed to be responsible for this beneficial effect. However, the amount and variety of chlorophylls in *G. pentaphyllum* still remain unknown. The objectives of this study were to develop a high performance liquid chromatography–mass spectrometry (HPLC–MS) method for determination of chlorophylls and their derivatives in *G. pentaphyllum*.

2. Materials and methods

2.1. Materials

Both chlorophyll a and chlorophyll b standards were purchased from Sigma (St. Louis, MO, USA). Internal standard Fast Green FCF was procured from Fluka (Buchs, Switzerland). Pheophytin a and pheophytin b were prepared from chlorophyll a and chlorophyll b, respectively, using a method as described by Teng and Chen [13]. Pyropheophytin a was prepared from pheophytin a based on a procedure by Pennington et al. [14]. Briefly, 1-mL of pheophytin a in acetone was evaporated to dryness under nitrogen, followed by dissolving in 1-mL of pyridine and heating in an oil bath at 45 °C. A portion of pheophytin a solution was collected every 10 min after heating and subjected to HPLC analysis. The HPLC-grade solvents including methanol, ethanol, isopropanol, *n*-butanol, acetone, hexane, acetonitrile and diethyl ether were obtained from J.T. Baker (Phillipsburg, NJ, USA). Deionized water was made using a Milli-Q purification system from Millipore (Bedford, MA, USA). A HyPURITY C18 column (150 mm × 4.6 mm i.d., particle size 5 μm) used for separation of chlorophylls and their derivatives was from Thermo Hypersil-keystone (Bellefonte, PA, USA).

2.2. Instrumentation

The HPLC instrument is from Agilent Technologies (1100 series), composed of a column temperature controller (G1316A), a degasser (G1379A), a quaternary pump (G1311A), a binary pump (G1312A), a photodiode array detector (G1315B), and a quadrupole mass spectrometer (6130) with multi-mode ion source (ESI and APCI). The spectrophotometer (DU 640) was from Beckman (Fullerton, CA, USA). The rotary evaporator (N-1) was from Eyela (Tokyo, Japan). The high-speed centrifuge (Sorvall RC5C) was from Du Pont (Wilmington, DE, USA).

2.3. Analysis of chlorophylls and their derivatives in *G. pentaphyllum*

2.3.1. Extraction

A method based on Chen and Chen [2] was modified to extract chlorophylls and their derivatives from *G. pentaphyllum*. A 0.5-g powder sample of *G. pentaphyllum* was mixed with 30-mL of hexane–acetone–ethanol–toluene (10:7:6:7, v/v/v/v) in a flask, and the mixture was shaken at room temperature for 20 min and then centrifuged at 4000 rpm for 30 min. The supernatant was collected and the residue was extracted with the same solvent and centrifuged using the same procedure. Both supernatants were combined and mixed with 20 mL of 10% anhydrous sodium sulfate solution, after which the solution was shaken for 1 min and the upper pigment layer was collected. Then hexane (15 mL) was added to the extract and the upper chlorophyll layer was also collected. This procedure was repeated twice and the chlorophyll extracts were pooled, evaporated to dryness under vacuum, dissolved in 5-mL acetone, filtered through a 0.22-μm membrane filter and 20 μL was injected for HPLC analysis.

2.3.2. Separation

For separation of chlorophylls and their derivatives by HPLC, various binary and ternary solvent systems in isocratic or gradient mode were compared. Solvents including methanol, acetonitrile, tetrahydrofuran, water and acetone in different combinations were tried to evaluate the separation efficiency. After numerous studies, a ternary solvent system of acetone (A), acetonitrile (B) and methanol (C) in gradient mode was developed: 2% A, 93% B and 5% C in the beginning, changed to 2% A, 71% B and 27% C in 0.3 min, 2% A, 64% B and 34% C in 6 min, 2% A, 45% B and 53% C in 9 min, 2% A, 39% B and 59% C in 21 min, 2% A, 24% B and 74% C in 24 min, 20% A and 80% C in 26 min, 40% A and 60% C in 28 min, 50% A and 50% C in 30 min and returned to 2% A, 93% B and 5% C in 35 min. A total of 15 chlorophylls and their derivatives, including hydroxychlorophyll b, chlorophyll b, chlorophyll b′, hydroxychlorophyll a, chlorophyll a, chlorophyll a′, hydroxypheophytin b, hydroxypheophytin b′, pheophytin b, pheophytin b′, hydroxypheophytin a, hydroxypheophytin a′, pheophytin a, pheophytin a′ and pyropheophytin a were resolved within 32 min with detection at 660 nm and flow rate at 1 mL/min. The purity of each peak was automatically determined using a photodiode-array detector. The separation efficiency was evaluated based on retention factor (k) and separation factor (α).

2.3.3. Identification

The various chlorophylls and their derivatives were identified by comparing retention times and absorption spectra of unknown peaks with reference standards and cochromatography with added standards. In addition, a quadrupole MS with APCI in positive mode was used for detection. The scanning range of MW was 500–1000, with drying gas flow at 5 L/min, nebulizer pressure at 20 psi, dry gas temperature at 350 °C, vaporizer temperature at 250 °C, capillary voltage at 2000 V, charging voltage at 2000 V, corona current at 4 μA and fragmentor voltage at 100 V.

2.3.4. Quantitation

Internal standard Fast Green FCF was dissolved in 80% methanolic solution for a concentration of 1000 μg/mL. Four standards of chlorophyll a, chlorophyll b, pheophytin a and pheophytin b were each dissolved in 1-mL acetone. Then five concentrations of 1, 2, 5, 10 and 20 μg/mL were each prepared for chlorophyll a, chlorophyll b and pheophytin b, while 25, 50, 80, 100 and 150 μg/mL were prepared for pheophytin a, with each concentration containing 150 μg/mL Fast Green FCF. Twenty microlitres of each standard solution was then injected into HPLC and four calibration curves were prepared by plotting concentration ratio (chlorophyll or pheophytin standard vs. internal standard) against its area ratio. The linear regression equations and correlation coefficient (R^2) were calculated from the standard curves. The regression equations for chlorophyll a, chlorophyll b, pheophytin a and pheophytin b were $y = 0.4411x - 0.0003$, $y = 0.1373x - 0.0002$, $y = 0.2974x + 0.0001$ and $y = 0.1834x - 0.0008$, respectively, with R^2 being all higher than 0.99. Chlorophylls and their derivatives were quantified using the following formula:

$$W(\mu g/g) = \left\{ \left[\left(\frac{A_s}{A_i} \right) a + b \right] \times C_i \times V \times \text{dilution factor} \times \left(\frac{1}{\text{recovery}} \right) \times \left(\frac{1}{\text{weight of sample}} \right) \right\}$$

where W: amount of sample; A_s: peak area of sample (660 nm); A_i: peak area of internal standard (626 nm); a: slope of regression equation; b: intercept of regression equation; C_i: concentration of internal standard; V: final volume of sample extract.

2.4. Detection limit (DL) and quantitation limit (QL)

Three concentrations of 50, 100 and 150 ng/mL for each chlorophyll and pheophytin standard were prepared, and 20 μL was injected three times, with the linear regression curve obtained by plotting concentration against peak height and then the slope (s) and maximum noise height (N_{p-p}) were determined. Both DL and QL were calculated using the following formula [15]:

$$\delta = \frac{N_{p-p}}{5}$$

$$DL = 3.3\frac{\delta}{s}$$

$$QL = 3DL$$

2.5. Recovery

For recovery determination, a concentration of 647.5 μg/mL chlorophyll a, 342.4 μg/mL chlorophyll b, 537.7 μg/mL pheophytin a and 314.3 μg/mL pheophytin b were prepared. Two volumes of 62 μL (40.1 μg) and 155 μL (100.4 μg) chlorophyll a, 117 μL (40.1 μg) and 292 μL (100.0 μg) chlorophyll b, 74 μL (39.8 μg) and 186 μL (100.0 μg) pheophytin a, as well as 127 μL (39.9 μg) and 318 μL (99.9 μg) pheophytin b were each collected and added to 0.5 g of powder sample of *G. pentaphyllum* for extraction and HPLC analysis. The recovery of each standard was determined based on the ratio of the amount of each standard after HPLC (spiked amount minus original amount) and before HPLC (spiked amount). Because of unavailability of commercial standards of pheophytin a′, chlorophyll a′, hydroxypheophytin a, hydroxypheophytin a′, pyropheophytin a, hydroxychlorophyll a, pheophytin b′, chlorophyll b′, hydroxychlorophyll b, hydroxypheophytin b and hydroxypheophytin b′, they were quantified by measuring peak area ratio of each to internal standard and multiplying concentration of internal standard.

2.6. Reproducibility test

The reproducibility of chlorophylls and their derivatives were determined based on a procedure described by International Conference on Harmonization [15]. The intra-day variability was measured by three determinations each in the morning, afternoon and evening and a total of nine analyses were carried out. Likewise, the inter-day variability was calculated by three analyses each day for a total of nine determinations for 3 days.

2.7. Statistical analysis

Triplicate analyses were performed for extraction and HPLC. Both α and k values as well as retention time were also determined in triplicate. All the data were subjected to analysis of variance and mean comparison for significance ($\alpha = 0.05$) by using Duncan's multiple range test using statistical analysis system [16].

3. Results and discussion

3.1. HPLC separation of chlorophylls and their derivatives

In the beginning several solvent systems in isocratic or gradient mode reported in the literature were used for separation of chlorophylls and their derivatives in *G. pentaphyllum* [17–21]. However, these methods fail to resolve chlorophylls and their derivatives in *G. pentaphyllum*, probably caused by the presence of different varieties of chlorophyll derivatives. Thus a new solvent system has to be developed. By calculating the polarity index (solvent strength) of each solvent system, a mobile phase in gradient mode with an optimum polarity index was developed. Different modifiers such as acetone or tetrahydrofuran were added to the mobile phase as well for comparison of separation efficiency. The most appropriate mobile phase is composed of a solvent mixture of 2% acetone (A), 93% acetonitrile (B) and 5% methanol (C) initially, changed to 2% A, 71% B and 27% C in 0.3 min, 2% A, 64% B and 34% C in 6 min, 2% A, 45% B and 53% C in 9 min, 2% A, 39% B and 59% C in 21 min, 2% A, 24% B and 74% C in 24 min, 20% A and 80% C in 26 min, 40% A and 60% C in 28 min, 50% A and 50% C in 30 min and returned to 2% A, 93% B and 5% C in 35 min. A total of 15 chlorophylls and their derivatives were adequately resolved within 32 min with detection at 660 nm and flow rate at 1 mL/min (Fig. 1). Fast Green FCF was shown to be an appropriate internal standard as it was first eluted within 4 min and did not interfere with separation of the other chlorophyll pigments (Fig. 1). Table 1 shows the retention time, retention factor (k), separation factor (α) and peak purity of chlorophyll extract from *G. pentaphyllum*. The k value ranged from 2.83 to 15.68, indicating a proper solvent strength of the mobile phase was controlled, while the α value was 1.08–1.20, revealing an optimum selectivity of the mobile phase to sample components was attained for most peaks. Nevertheless, resolution should be more important than selectivity in this case as Fig. 1 clearly shows that baseline separation was hard to achieve for peaks 2/3. A high peak purity of chlorophylls and their derivatives was also shown, which ranged from 92.6 to 99.4%. Chen and Chen [2] developed a quaternary solvent system to separate a total of 17 chlorophylls and carotenoids in sweet potato leaves within 30 min by employing a C18 column with detection at 440 nm and flow rate at 1 mL/min. However, the resolution remained inadequate as a partial peak overlapping occurred. In another study Almela et al. [19] developed a gradient binary mobile phase to separate eight chlorophylls and their derivatives in *Annona cherimola* within 30 min with fluorescence detection at 440 nm (excitation) and 660 nm (emission) and flow rate at 1 mL/min. A complete resolution was attained, however, the number of chlorophyll derivatives separated is limited. Likewise, a total of 12 chlorophylls and carotenoids in phytoplankton samples were separated within 29 min by using a gradient ternary solvent system with detection at 436 nm and flow rate at 1 mL/min [20], but the major drawback is that a partial peak overlapping still occurred. In a recent study Roca et al. [21] used a gradient binary solvent system containing a paired-ion reagent tetrabutylammonium to resolve 11 chlorophylls and their derivatives in *Olea europaea* fruit within 30 min with detection at 666 nm and flow rate

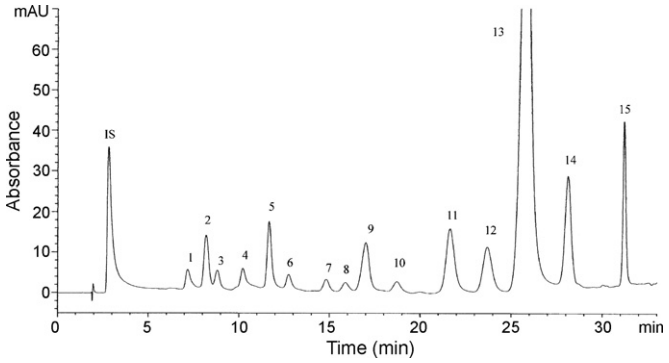


Fig. 1. HPLC chromatogram of chlorophyll extract from *Gynostemma pentaphyllum* (column: C_{18}, mobile phase—A: acetone, B: ACN, C: MeOH, flow rate: 1 mL/min, detection wavelength: 660 nm). Peak identification shown in Table 1.

Table 1
Retention time, retention factor (k), separation factor (α) and peak purity of chlorophyll extract from *Gynostemma pentaphyllum*

Peak no.	Compound	t_R (min)	Retention factor (k)	Separation factor (α)	Peak purity (%)
1	Hydroxychlorophyll b	7.17	2.83	1.19 (1, 2)[a]	93.4
2	Chlorophyll b	8.19	3.38	1.19 (1, 2)[a]	97.5
3	Chlorophyll b′	8.80	3.71	1.10 (2, 3)[a]	98.3
4	Hydroxychlorophyll a	10.21	4.46	1.20 (3, 4)[a]	96.2
5	Chlorophyll a	11.66	5.24	1.17 (4, 5)[a]	98.0
6	Chlorophyll a′	12.74	5.81	1.11 (5, 6)[a]	94.7
7	Hydroxypheophytin b	14.82	6.93	1.19 (6, 7)[a]	96.7
8	Hydroxypheophytin b′	15.82	7.46	1.08 (7, 8)[a]	96.8
9	Pheophytin b	17.00	8.09	1.08 (8, 9)[a]	98.5
10	Pheophytin b′	18.71	9.00	1.11 (9, 10)[a]	92.6
11	Hydroxypheophytin a	21.64	10.57	1.17 (10, 11)[a]	99.4
12	Hydroxypheophytin a′	23.68	11.66	1.10 (11, 12)[a]	99.0
13	Pheophytin a	25.80	12.80	1.10 (12, 13)[a]	99.2
14	Pheophytin a′	28.11	14.03	1.10 (13, 14)[a]	98.8
15	Pyropheophytin a	31.19	15.68	1.12 (14, 15)[a]	99.2

[a] Numbers in parentheses represent values between two neighboring peaks.

at 2 mL/min. However, this method is more complex and several peaks are partially overlapped. Comparatively, the HPLC method developed in our study provided a better resolution and a larger number of chlorophylls and their derivatives separated within a reasonable period of time.

3.2. Identification of chlorophylls and their derivatives

As mentioned in the preceding section, pheophytins a and b were prepared from chlorophylls a and b, respectively, using a method described by Teng and Chen [13]. Similarly, pyropheophytin a was prepared from pheophytin a according to a procedure of Pennington et al. [14]. Fig. 2 shows conversion of pheophytin a to its derivatives as affected by heating time. Originally pheophytin a was present at 2.1×10^{-2} μmol in unheated solution, and a level of 1.1×10^{-2} μmol of pyropheophytin a was formed after 10- or 20-min heating, accompanied by complete degradation of pheophytin a. However, the content of pyropheophytin a decreased to 4.7×10^{-3} μmol 30 min after heating, indicating a partial degradation of pyropheophytin a occurred. Thus, a heating time of 20 min was selected to convert pheophytin a to its corresponding pyropheophytin a. On the basis of comparison of retention time of unknown peaks with reference standards, only five chlorophyll pigments including chlorophyll b (peak 2), chlorophyll a (peak 5), pheophytin b (peak 9), pheophytin a (peak 13) and pyropheophytin a (peak 15) were identified. These pigments were further confirmed by LC–MS, so were the other unknown peaks. Due to the low-polarity nature of chlorophylls, the APCI detection mode was used to facilitate ionization producing $[M+H]^+$ ions.

Table 2 shows UV and MS spectral data of chlorophylls and their derivatives in *G. pentaphyllum*. Peak 1 was identified as hydroxychlorophyll b based on the absorption wavelength of [460,646] nm and $[M+H]^+$ of 923, which was similar to that reported by Hyvärinen and Hynninen [22]. Peak 3 was identified as chlorophyll b′ based on the absorption wavelength of [462,648] nm and $[M+H]^+$ of 907 as well as retention behavior, which was identical to that shown by Chen and Chen [2] and Gauthier-Jaques et al. [3]. Peak 4 was identified as hydroxychlorophyll a as the absorption wavelength of [422,660] nm and $[M+H]^+$ of 909 were similar to that reported by Gauthier-Jaques et al. [3]. Peak 6 was identified as chlorophyll a′ since the absorption wavelength of [430,664] nm and $[M+H]^+$ of 893 as well as the elution order were the same as that shown by Chen and Chen [2] and Gautheir-Jaques et al. [3]. Peak 7 was identified as hydroxypheophytin b on the basis of the absorption wavelength of [434,652] nm and $[M+H]^+$ of 901, which was 16 higher than $[M+H]^+$ of pheophytin b (885), revealing that the hydrogen at C-10 position was replaced by hydroxyl group. Peak 8 was identified as hydroxypheophytin b′ based on the absorption wavelength of [436,652] nm and $[M+H]^+$ of 901, as well as retention behavior, which was eluted after hydroxypheophytin b. Peak 10 was identified as pheophytin b′ as the absorption wavelength of [436,656] nm and $[M+H]^+$ of 885 were the same as pheophytin b, which was also eluted before pheophytin b′. Peak 11 was identified as hydroxypheophytin a based on the absorption wavelength of [406,666]

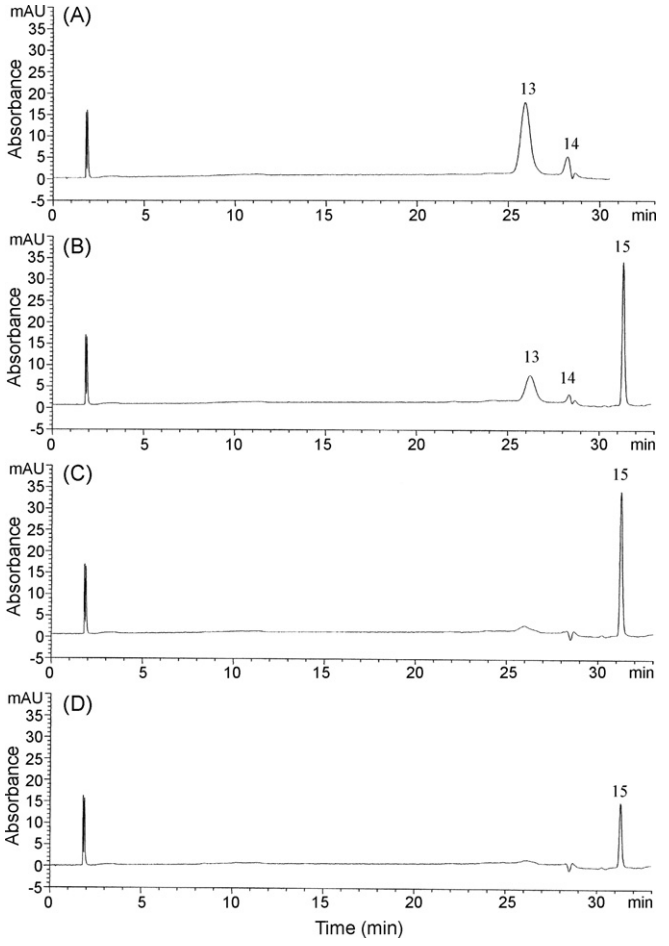


Fig. 2. Conversion of chlorophylls to their derivatives as affected by heating time: A, 0 min; B, 10 min; C, 20 min; D, 30 min. Peaks: 13, pheophytin a; 14, pheophytin a′; 15, pyropheophytin a.

Table 2
UV and MS spectral data of chlorophylls and their derivatives in *G. pentaphyllum*

Peak no.	Compound	$[M+H]^+$ (on-line)	$[M+H]^+$ (reported)	λ_{max} (on-line)	λ_{max} (reported)	Content[f] (μg/g)
1	Hydroxychlorophyll b	923	923[a]	460, 598, 646	451, 641[a]	15.0 ± 0.4
2	Chlorophyll b	907	907.4[b]	462, 600, 648	462, 651[c]	287.9 ± 8.6
3	Chlorophyll b′	907	907.4[b]	462, 600, 648	458, 648[e]	11.1 ± 0.3
4	Hydroxychlorophyll a	909	909.9[b]	422, 614, 660	–	23.8 ± 0.6
5	Chlorophyll a	893	893.5[b]	430, 618, 664	428, 663[d]	113.8 ± 3.9
6	Chlorophyll a′	893	893.5[b]	430, 618, 664	430, 663[d]	11.0 ± 0.3
7	Hydroxypheophytin b	901	–	434, 522, 598, 652	–	11.2 ± 0.4
8	Hydroxypheophytin b′	901	–	436, 528, 600, 652	–	8.5 ± 0.2
9	Pheophytin b	885	885.4[b]	436, 528, 598, 652	432, 654[c]	319.6 ± 8.1
10	Pheophytin b′	885	–	436, 524, 600, 656	–	13.2 ± 0.5
11	Hydroxypheophytin a	887	887.4[b]	406, 502, 532, 610, 666	–	88.6 ± 1.9
12	Hydroxypheophytin a′	887	–	408, 504, 534, 610, 666	–	66.5 ± 1.6
13	Pheophytin a	871	871.5[b]	408, 506, 536, 608, 666	410, 669[c]	2508.3 ± 52.0
14	Pheophytin a′	871	871.5[b]	408, 506, 536, 610, 666	–	111.2 ± 2.2
15	Pyropheophytin a	813	813.8[b]	410, 508, 538, 610, 666	410, 506, 536, 608, 666[e]	76.0 ± 1.4

Underlined values represent major absorption wavelength.
[a] Based on a reference by Hyvärinen and Hynninen [22].
[b] Based on a reference by Gauthier-Jaques et al. [3].
[c] Based on a reference by Lanfer-Marquez et al. [5].
[d] Based on a reference by Chen and Chen [2].
[e] Based on a reference by Hornero-Méndez et al. [23].
[f] Peaks 2, 5, 9 and 13 were quantified based on standard calibration curves, with the other 11 being quantified with an internal standard Fast Green FCF.

nm and $[M+H]^+$ of 887, which was also identical to that shown by Gauthier-Jaques et al. [3]. Peak 12 was identified as hydroxypheophytin a′ since the absorption wavelength of [408,666] nm and $[M+H]^+$ of 887 were the same as hydroxypheophytin a, which was eluted before hydroxypheophytin a′ as well. Peak 14 was identified as pheophytin a′ as the absorption wavelength of [408,666] nm and $[M+H]^+$ of 871 were identical to that of pheophytin a, which was also eluted before pheophytin a′. The same outcome was observed by Gauthier-Jaques et al. [3]. Peak 15 was identified as pyropheophytin a based on the absorption wavelength of [410,666] nm and $[M+H]^+$ of 813, which was the same as pyropheophytin a standard and that reported by Gauthier-Jaques et al. [3] and Hornero-Méndez et al. [23]. Fig. 3 shows structural formulas and nomenclature of chlorophylls and their derivatives in *G. pentaphyllum* as well as internal standard Fast Green FCF.

The presence of chlorophyll and pheophytin isomers such as chlorophyll a′, chlorophyll b′, pheophytin a′ and pheophytin b′ in *G. pentaphyllum* were probably formed during processing. It has been well documented that chlorophylls and their derivatives are susceptible to epimerization at C-10 position during cooking or storage of green vegetables [3,24]. *G. pentaphyllum*, a dried product and a kind of green plants rich in chlorophylls, should be prone to undergo epimerization as well during drying. In addition to chlorophyll isomers, there are several hydroxyl-containing derivatives like hydroxychlorophyll a, hydroxychlorophyll b, hydroxypheophytin a and hydroxypheophytin b present. Several studies have also demonstrated the presence of these hydroxyl-containing derivatives in Mandarin fruit, broccoli, olive, red capsicum fruit and banana, with the amount increased following a rise in maturity [25–29]. The formation of hydroxychlorophylls is probably caused by chlorophyll oxidation at C-10 in the presence of chlorophyll oxidase [30]. In addition, the presence of peroxidase in chloroplast may play a significant role in chlorophyll oxidation [4,31].

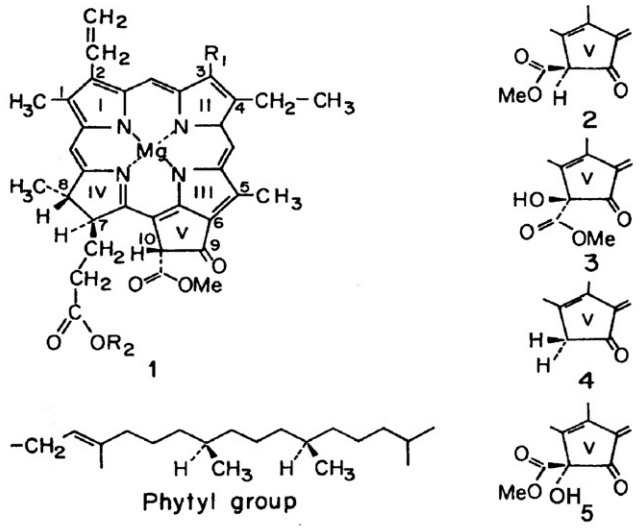
3.3. Quality control

Table 3 shows the quality control data of chlorophylls and their derivatives by HPLC analysis. The intra-day variability (R.S.D.%) of chlorophylls and their derivatives ranged from 1.8 to 3.8%, whereas the inter-day variability was from 2.1 to 6.5%. The detection limits for chlorophyll a, chlorophyll b, pheophytin a and pheophytin b were 3.81, 9.00, 11.0 and 16.5 ng/mL, respectively, while the

Table 3
Quality control data of chlorophylls and their derivatives by HPLC-DAD

Peak no.	Chlorophylls	Intra-day variability[a]		Inter-day variability[a]	
		Mean ± S.D. (μg/g)	R.S.D. (%)	Mean ± S.D. (μg/g)	R.S.D. (%)
1	Hydroxychlorophyll b	15.0 ± 0.4	2.6	14.9 ± 0.4	2.4
2	Chlorophyll b	287.9 ± 8.6	3.0	287.6 ± 9.4	3.3
3	Chlorophyll b′	11.1 ± 0.3	3.1	11.2 ± 0.5	4.1
4	Hydroxychlorophyll a	23.8 ± 0.6	2.6	23.6 ± 1.1	4.7
5	Chlorophyll a	113.8 ± 3.9	3.5	116.2 ± 4.9	4.2
6	Chlorophyll a′	11.0 ± 0.3	3.2	11.1 ± 0.7	6.5
7	Hydroxypheophytin b	11.2 ± 0.4	3.8	11.0 ± 0.4	3.8
8	Hydroxypheophytin b′	8.5 ± 0.2	2.1	8.6 ± 0.5	5.7
9	Pheophytin b	319.6 ± 8.1	2.5	318.9 ± 11.9	3.7
10	Pheophytin b′	13.2 ± 0.5	3.7	13.1 ± 0.5	3.7
11	Hydroxypheophytin a	88.6 ± 1.9	2.2	87.5 ± 2.8	3.2
12	Hydroxypheophytin a′	66.5 ± 1.6	2.4	66.7 ± 2.8	4.2
13	Pheophytin a	2508.3 ± 52.0	2.1	2494.9 ± 64.4	2.6
14	Pheophytin a′	111.2 ± 2.2	2.0	109.9 ± 2.7	2.5
15	Pyropheophytin a	76.0 ± 1.4	1.8	75.6 ± 1.6	2.1

[a] Mean of triplicate analyses ± standard deviation.



Chlorophylls and their derivatives

Compound	Mg*	R1	R2	Isocyclic Ring (V)
Chlorophyll a	+	CH_3	Phytyl	1
Chlorophyll b	+	CHO	Phytyl	1
Chlorophyll a'	+	CH_3	Phytyl	2
Chlorophyll b'	+	CHO	Phytyl	2
Hydroxypheophytin a	–	CH_3	Phytyl	3
Hydroxypheophytin a'	–	CH_3	Phytyl	5
Pheophytin a	–	CH_3	Phytyl	1
Pheophytin a'	–	CH_3	Phytyl	2
Hydroxypheophytin b	–	CHO	Phytyl	3
Hydroxypheophytin b'	–	CHO	Phytyl	5
Pheophytin b	–	CHO	Phytyl	1
Pheophytin b'	–	CHO	Phytyl	2
Hydroxychlorophyll a	+	CH_3	Phytyl	3
Hydroxychlorophyll b	+	CHO	Phytyl	3
Pyropheophytin a	–	CH_3	Phytyl	4

* Mg is replaced by 2H in pheophytins.

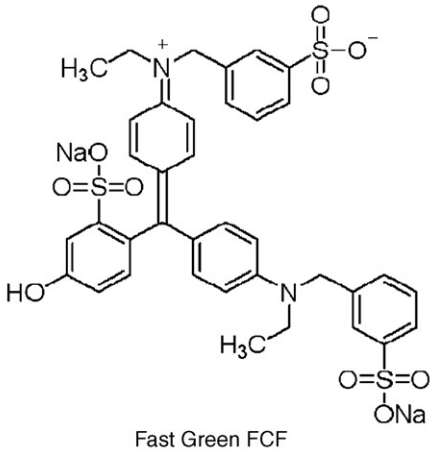


Fig. 3. Structural formulas and nomenclature of chlorophylls and their derivatives in *G. pentaphyllum* as well as internal standard Fast Green FCF.

quantitation limits were 11.43, 27.0, 33.0 and 49.5 ng/mL, with the recoveries being 84.8, 88.6, 97.6 and 96.4% (Table 4). Diaz et al. [32] determined chlorophylls in olive oil and the recoveries of chlorophyll a, chlorophyll b, pheophytin a and pheophytin b were shown to be 70–112, 71–111, 76–105 and 82–109%, respectively. In a similar report dealing with analysis of chlorophylls in olive oil, the recoveries of pheophytin a and pheophytin b were 99.56–100.46% and 94.44–105.88%, respectively. All the recoveries were higher than that of chlorophyll a and chlorophyll b observed in our study, which may be due to isomerization or degradation during extraction.

Table 4
Recovery of chlorophylls and their derivatives by HPLC-DAD

Chlorophyll	Original (μg)	Spiked (μg)	Found (μg)	Recovery (%)[a]	Mean ± S.D. (%)	R.S.D. (%)[b]
Chlorophyll a	45.9	40.1	81.6	89.0	84.8	3.6
	46.6	40.1	80.5	84.6	± 3.1	
	45.9	100.4	129.9	83.7		
	46.6	100.4	128.7	81.7		
Chlorophyll b	125.4	40.1	159.8	85.8	88.6	3.4
	124.7	40.1	159.5	86.9	± 3.0	
	125.4	100.0	214.5	89.1		
	124.7	100.0	217.2	92.6		
Pheophytin a	1223.8	39.8	1263.9	100.7	97.6	2.3
	1213.4	39.8	1251.4	95.5	± 2.3	
	1223.8	100.0	1321.5	97.7		
	1213.4	100.0	1309.9	96.5		
Pheophytin b	154.4	39.9	193.9	98.9	96.4	1.8
	151.8	39.9	190.3	96.5	± 1.8	
	154.4	99.9	249.2	94.9		
	151.8	99.9	247.2	95.5		

[a] Recovery (%) = (amount spiked − original amount)/amount spiked × 100.
[b] R.S.D. (%) = (S.D./mean) × 100.

3.4. Quantitation of chlorophylls and their derivatives

Because of unavailability of standards of most chlorophyll derivatives, the contents of chlorophyll a, chlorophyll b, pheophytin a and pheophytin b were determined using the standard curves, whereas the other 11 pigments were quantified by calculating the peak area ratio of each pigment (660 nm) to Fast Green FCF (626 nm) and multiplying the concentration of Fast Green FCF, followed by taking recovery into consideration by using a formula described in Section 2. However, we have to point out here that using Fast Green FCF for quantitation may decrease accuracy caused by difference in maximum absorption wavelength between Fast Green FCF and chlorophyll pigments. The amounts of pheophytin a, pheophytin a′, chlorophyll a, chlorophyll a′, hydroxypheophytin a, hydroxypheophytin a′, pyropheophytin a and hydroxychlorophyll a were 2508.3, 111.2, 113.8, 11.0, 88.6, 66.5, 76.0 and 23.8 μg/g, respectively, while those of pheophytin b, pheophytin b′, chlorophyll b, chlorophyll b′, hydroxychlorophyll b, hydroxypheophytin b and hydroxypheophytin b′ were 319.6, 13.2, 287.9, 11.1, 15.0, 11.2 and 8.5 μg/g (Table 2). Theoretically both chlorophyll a and chlorophyll b should be present in largest amount in green plants, but in our study pheophytin a and pheophytin b were substantially higher than the other chlorophyll pigments, which may be accounted for by partial conversion or degradation of chlorophylls in *G. pentaphyllum* during processing. Also, only pyropheophytin a was detected instead of pyropheophytin b. As pyropheophytin can only be formed under drastic condition [2], the formation of pyropheophytin a should be from pheophytin a in *G. pentaphyllum* during drying, which was also present at a much higher level than pheophytin b.

4. Conclusion

In conclusion, a gradient ternary solvent system of acetone–acetonitrile–methanol was developed to separate chlorophylls and their derivatives in *G. pentaphyllum* by HPLC, and a total of 15 pigments including pheophytin a, pheophytin a′, chlorophyll a, chlorophyll a′, hydroxypheophytin a, hydroxypheophytin a′, pyropheophytin a, hydroxychlorophyll a, pheophytin b, pheophytin b′, chlorophyll b, chlorophyll b′, hydroxychlorophyll b, hydroxypheophytin b and hydroxypheophytin b′ were resolved within 32 min with detection at 660 nm and flow rate at 1 mL/min. The identification was carried out by APCI in positive ion mode and absorption spectra as well as retention behavior of each pigment, with quantitation being accomplished using an internal standard Fast Green FCF.

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