Article

Determination of Chlorophylls in *Taraxacum formosanum* by High-Performance Liquid Chromatography−Diode Array Detection−Mass Spectrometry and Preparation by Column Chromatography

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ABSTRACT: *Taraxacum formosanum*, a well-known Chinese herb shown to be protective against hepatic cancer as well as liver and lung damage, may be attributed to the presence of abundant carotenoids and chlorophylls. However, the variety and content of chlorophylls remain uncertain. The objectives of this study were to develop an high-performance liquid chromatography−diode array detection−mass spectrometry method for determination of chlorophylls in *T. formosanum* and preparation by column chromatography. An HyPURITY C18 column and a gradient mobile phase of water (A), methanol (B), acetonitrile (C), and acetone (D) could resolve 10 chlorophylls and an internal standard Fast Green FCF within 30 min with a flow rate at 1 mL/min and detection at 660 nm. Both chlorophylls a and a′ were present in the largest amount (1389.6 μg/g), followed by chlorophylls b and b′ (561.2 μg/g), pheophytins a and a′ (31.7 μg/g), hydroxychlorophyll b (26.5 μg/g), hydroxychlorophylls a and a′ (9.8 μg/g), and chlorophyllides a and a′ (0.35 μg/g). A glass column containing 52 g of magnesium oxide−diatomaceous earth (1:3, w/w) could elute chlorophylls with 800 mL of acetone containing 50% ethanol at a flow rate of 10 mL/min. Some new chlorophyll derivatives including chlorophyllide b, pyropheophorbide b, hydroxypheophytin a, and hydroxypheophytin a′ were generated during column chromatography but accompanied by a 63% loss in total chlorophylls. Thus, the possibility of chlorophyll fraction prepared from *T. formosanum* as a raw material for future production of functional food needs further investigation.

KEYWORDS: *Taraxacum formosanum, chlorophylls, HPLC-DAD-MS, column chromatography*

■ INTRODUCTION

Taraxacum officinale, commonly known as dandelion, is a popular Chinese medicinal herb often consumed as a vegetable and widely used throughout the world.[1] In Europe, dried *T. officinale* is ground into powder and used as a substitute for coffee by those who are allergic to caffeine, while in Taiwan and China, it is used as a detoxifying agent as well as for the preparation of vegetable salad.[1] From a taxonomic point of view, dandelion can be divided into many species. For instance, *T. officinale* is the most abundant one in North America, whereas in Taiwan and China, *Taraxacum formosanum* and *Taraxacum mongolicum* dominate, respectively.[2] More importantly, *T. officinale* has been shown to contain many functional components, such as taraxacerin, taraxicin, taraxasterol, stigmasterol, phenolic acid, saponin, carotenoid, and chlorophyll,[3,4] all of which are believed to be important in the protection against chronic disease like hepatic cancer.[5]

Accordingly, both chlorophyll a and chlorophyll b are the major chlorophylls present in green plants with the content of the former being about 3-fold higher than the latter.[6,7] In addition, several chlorophyll derivatives like pheophytin, pyropheophytin, and chlorophyllide can be formed during the processing of green plants.[7] For example, during the blanching of green vegetables, chlorophyll a and chlorophyll b were converted to pheophytin a and pheophytin b, respectively, through replacement of magnesium ions with organic acid liberated from vegetables, resulting in a color change from deep green to olive green.[7,8] Whereas during canning, both pyropheophytin a and pyropheophytin b were formed from chlorophyll a and chlorophyll b, respectively, through the removal of magnesium ions as well as both carbonyl and methoxy groups at C-10.[7] Similarly, with mild heating conditions, chlorophyll a and chlorophyll b in green vegetables can be degraded to form chlorophyllide a and chlorophyllide b, respectively, through the activation of chlorophyllase and removal of phytol, both of which upon activation of dechelatase can be further converted to pheophorbide a and pheophorbide b.[7] In several published reports, Simonich et al.[9] found that chlorophyllin was protective against hepatic cancer, as was sodium copper chlorophyllin against colon cancer.[10] Likewise, the water-soluble pheophorbide a was shown to be effective against hepatic cancer,[11] while chlorophyll a isolated from *Ludwigia octovalvis* could inhibit apoptosis of adipose cells.[12]

The extraction of chlorophylls and their derivatives from plant tissue is often carried out by nonpolar solvents like petroleum ether or medium-polar solvents such as acetone or a combination of both.[7,13] After the extraction of chlorophylls, they are frequently subjected to thin-layer or column chromatography for further purification and separation of

chlorophylls from carotenoids, followed by high-performance liquid chromatography (HPLC) for identification and quantitation.[14,15] It has been well established that a C18 column can provide a high resolution power in separating chlorophylls and their derivatives.[14,15] In several previous studies, Almela et al.[16] reported that a total of eight chlorophylls and their derivatives in *Annona cherimola* could be separated within 30 min by using a Spherisorb ODS-2 column (250 mm × 4.6 mm, 5 μm particle size) and a gradient mobile phase of methanolic solution containing ammonium acetate (A) and acetone (B) with a flow rate at 1 mL/min and detection at 660 nm. Similarly, a total of nine chlorophylls and their derivatives were separated in spinach within 30 min by employing a LichroCART 250-4 RP-C18 column (250 mm × 2 mm i.d., 4 μm particle size) and a gradient mobile phase of methanol (A), acetone (B), and dimethyl formamide (C) with a flow rate at 0.28 mL/min and fluorescence detection.[13] However, the resolution of chlorophylls and their derivatives remains inadequate when both carotenoids and chlorophylls are separated simultaneously, apparently caused by interference of the former. For instance, only three chlorophylls and their derivatives were separated in grapes by a Nova-Pak C18 column (300 mm × 3.9 mm i.d., 4 μm particle size) and a gradient mobile phase of ethyl acetate (A) and acetonitrile (B) with flow rate at 1 mL/min and detection at 447 nm,[17] as were four chlorophylls and their derivatives in green leaf lettuce by a Zorbax ODS column (250 mm × 4.6 mm i.d., 5 μm particle size) and an isocratic mobile phase of ethyl acetate−methanol−water with a flow rate at 1 mL/min and detection at 430 nm.[18] Gandul-Rojas et al.[19] simultaneously separated 12 carotenoid and 16 chlorophyll compounds; however, several peaks overlapped, making the purity questionable. In addition, the detection was carried out sequentially by four different wavelengths with the mobile phase system containing an ion-pair reagent, all of which should make the method more complicated. Thus, it is necessary to develop a better HPLC method to separate more chlorophylls and their derivatives within a shorter length of time.

In light of the impact of chlorophylls on human health, it would be beneficial to the health food industry if a large amount of chlorophylls can be isolated from *T. officinale* by column chromatography and used as raw materials for the future production of health food. The objectives of this study were to develop an HPLC method for separation, identification, and quantitation of chlorophylls and their derivatives from *T. formosanum*, followed by development of a column chromatographic method for large-scale preparation.

■ MATERIALS AND METHODS

Materials. *T. formosanum* was procured from a local Chinese herb medicine store and subjected to cleaning, freeze-drying, grinding into powder, vacuum packaging, and storing at −20 °C prior to use. Both chlorophyll a and chlorophyll b standards were obtained from Sigma (St. Louis, MO). The internal standard Fast Green FCF was from Fluka Co. (Buchs, Switzerland). Both pheophytin a and pheophytin b standards were prepared by dissolving 1 mg of chlorophyll a and chlorophyll b each in 1 mL of acetone, adding a few drops of 0.1 M HCl in methanol, and shaking. The HPLC-grade solvents including acetonitrile, methanol, acetone, toluene, methylene chloride, hexane, ethyl acetate, and ethanol were from Lab-Scan Co. (Gliwice, Poland). Deionized water was made using a Milli-Q water purification system from Millipore Co. (Bedford, MA). The adsorbent diatomaceous earth was from J. T. Baker Co. (Phillipsburg, NJ), while magnesium oxide

was from Sigma. Anhydrous sodium sulfate was from Nacalai Tesque Co. (Kyoto, Japan).

Instrumentation. The HPLC system (Agilent 1100 series) is composed of G1311A quaternary pump, G1312A binary pump, G1315B photodiode array detector, G1379A degasser, G1316A column temperature controller, and 6130 quadrupole mass spectrometer with multimode ion source [electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)]. A HyPURITY C18 column (150 mm × 4.6 mm i.d., particle size 5 μm) was from Thermo Hypersil-Keystone Co. (Bellefonte, PA). The Eyela rotary evaporator (N−I) was from Tokyo, Japan. The low-temperature circulating water bath (Firstek B402L) was from Taoyuan, Taiwan. The sonicator (2210R-DTH) was from Branson Co. (Danbury, CT). The freeze-dryer (FD-24) was from Gin-Ming Co. (Taipei, Taiwan). The low-temperature incubator (TL 520R) was from Sheng-Long Co. (Taipei, Taiwan). The spectrophotometer (DU 640) was from Beckman Co. (Fullerton, CA).

Extraction of Chlorophylls. A method based on Huang et al.[14] was modified to extract chlorophylls from *T. formosanum*. A 0.8 g dried sample of *T. formosanum* was mixed with 30 mL of hexane/ethanol/acetone/toluene (10:6:7:7, v/v/v/v) in a flask, after which the mixture was shaken at room temperature for 1 h, followed by adding 15 mL of hexane, shaking for 10 min, and adding 15 mL of 10% anhydrous sodium sulfate solution for partition. The upper phase containing chlorophylls was collected, and the lower phase was repeatedly extracted with 15 mL of hexane for four times until the supernatant became colorless. Then, all of the supernatants were pooled, evaporated to dryness, and dissolved in 5 mL of acetone to obtain crude chlorophyll extract. After it was filtered through a 0.2 μm membrane filter, 20 μL of the filtrate was injected into HPLC for analysis. It should be pointed out that the extraction procedure was carried out under dimmed light, and nitrogen gas was flushed into a flask or vial whenever necessary during extraction.

Preparation of Chlorophylls by Column Chromatography. A 10 g dried sample of *T. formosanum* was mixed with 80 mL of hexane/ethanol/acetone/toluene (10:6:7:7, v/v/v/v), and the solution was shaken at room temperature for 1 h. Then, 80 mL of hexane was added and shaken for 10 min, after which 30 mL of 10% sodium sulfate solution was added for partition, and the upper layer was collected. Next, the lower layer was repeatedly extracted four times until the supernatant became colorless. All of the supernatants were combined, evaporated to dryness, and dissolved in 10 mL of hexane to obtain crude chlorophyll extract for storage at −70 °C until use. Likewise, the extraction procedure was performed under dimmed light with nitrogen gas being flushed simultaneously. As the crude chlorophyll extract was found to contain carotenoid as well, the carotenoid fraction should be removed first during column chromatography. A glass column (400 mm × 42 mm i.d.) containing 52 g of magnesium oxide−diatomaceous earth (1:3, w/w) was prepared and preactivated with 500 mL of hexane. Then, anhydrous sodium sulfate was added to form about a 1 cm layer above the adsorbent, followed by pouring 3 mL of crude chlorophyll extract into the column and eluting the carotenoid fraction with 300 mL of 100% ethyl acetate at a flow rate of 10 mL/min. The remaining chlorophyll fraction was next eluted with 800 mL of acetone containing 50% ethanol with the same flow rate. Then, the chlorophyll eluate was evaporated to dryness, dissolved in 5 mL of acetone, and filtered through a 0.2 μm membrane filter, and 20 μL was injected for HPLC-DAD-MS analysis.

HPLC Analysis of Chlorophylls. A method based on Huang et al.[14] was modified to separate the various chlorophylls in *T. formosanum*. As compared to an earlier method,[14] a quaternary solvent system with different gradient conditions was used instead of a ternary solvent system by including water as the fourth solvent. A HyPURITY C18 column (150 mm × 4.6 mm i.d., 5 μm particle size) and a quaternary mobile phase composed of (A), methanol (B), acetonitrile (C), and acetone (D) with the following gradient condition was developed: 70% A and 30% B initially; changed to 45% A and 55% B in 0.3 min; 100% B in 4 min; 38% B and 62% C in 6 min; 50% B and 50% C in 10 min; 0.2% A, 50.8% B, and 49% C in 15
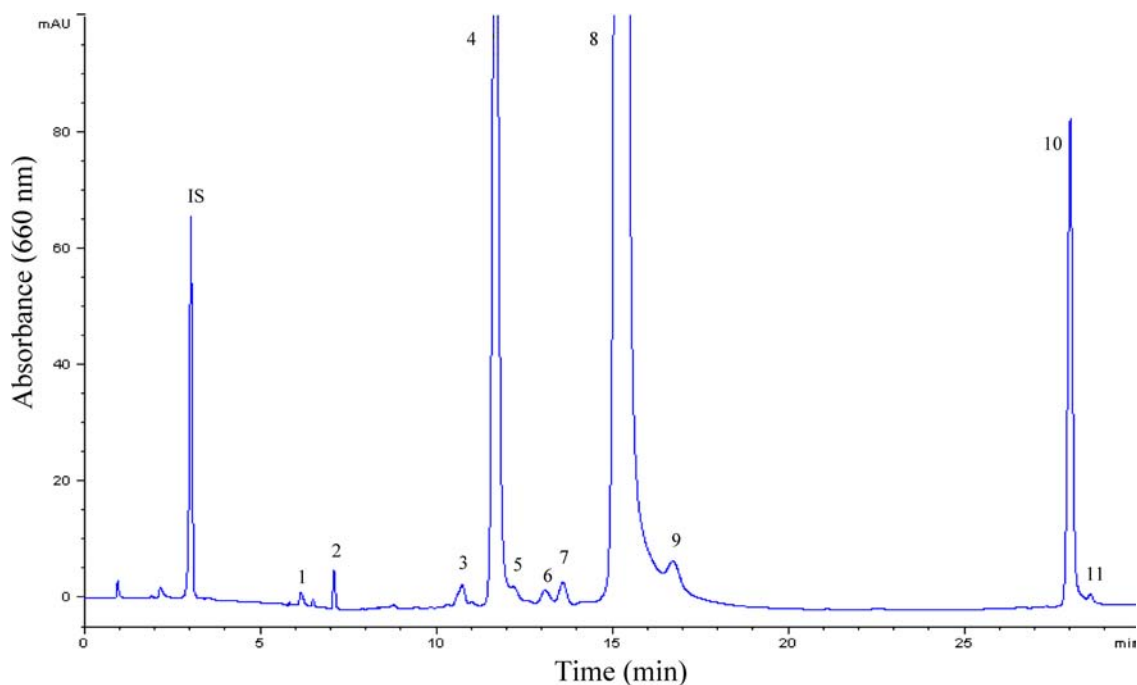
Figure 1. HPLC chromatogram of chlorophylls extracted from *T. formosanum*. Column: C_{18}; mobile phase: (A) H_2O, (B) MeOH, (C) ACN, and (D) acetone; flow rate: 1 mL/min; and detection wavelength: 660 nm. Peak identification is shown in Table 1.

min; 57% B and 43% C in 22 min; 60% B and 40% D in 26 min; 45% B and 55% D in 30 min; and returned to original ratio in 35 min. The flow rate was 1 mL/min with detection at 660 nm and the column temperature at 25 °C. The separation efficiency was evaluated based on k and α values, with the former denoting the retention factor and the latter denoting the separation factor (selectivity). Identification was performed by comparing retention time, absorption spectra, and mass spectra of unknown peaks with authentic standards. In addition, both absorption and mass spectra of chlorophylls reported in the literature were used for comparison. The mass spectra of various chlorophylls were determined with APCI mode using the following conditions: scanning range, 500−1000; drying gas flow, 5 L/min; nebulizer pressure, 20 psi; dry gas temperature, 350 °C; vaporizer temperature, 250 °C; capillary voltage, 2000 V; charging voltage, 2000 V; corona current, 4 μA; and fragmentor voltage, 100 V. Also, the selected ion monitoring (SIM) mode was used to enhance sensitivity during MS detection. The peak purity of each chlorophyll was determined by a photodiode array detector automatically based on the Agilent G2180A Spectral Evaluation Software through measurement of degree in overlapping of absorption spectra within each peak or between unknown peaks and reference standards.

The intraday variability was determined in the morning, afternoon, and evening with three injections each for a total of nine replicates, whereas the interday variability was measured in the first, second, and third day with three injections each for a total of nine replicates. The recovery was determined by adding 200 and 500 μL each of chlorophyll a standard (1000 μg/mL) or 100 and 200 μL each of chlorophyll b standard (1000 μg/mL) or 189 and 755 μL each of pheophytin a standard (53 μg/mL) to 0.8 g of *T. formosanum* sample for extraction and subsequent HPLC-DAD analysis. The recovery was obtained based on the ratio of the amount of chlorophyll standard after HPLC relative to that before HPLC (spiked amount). Both the limit of detection (LOD) and the limit of quantitation (LOQ) were determined by preparing three concentrations of standards: 0.2, 0.5, and 1 μg/mL each for chlorophyll a and chlorophyll b; 0.05, 0.2, and 0.5 μg/mL for pheophytin a. Then, each concentration was analyzed three times, and the standard curve was obtained by plotting the concentration against peak height. The slope (s) and largest noise height ($N\rho-\rho$) were determined and used for calculation of LOD and LOQ based on the following formula:

$$\delta = N\rho - \rho/5$$

$$LOD = 3.3 \times \delta/s$$

$$LOQ = 3 \times LOD$$

For quantitation, five concentrations each of 1, 5, 10, 20, and 50 μg/mL were prepared for chlorophyll a and chlorophyll b separately in acetone, while 0.2, 1, 2, 5, and 10 μg/mL were prepared for pheophytin a. Additionally, five more concentrations of 100, 150, 200, 250, and 300 μg/mL were prepared for chlorophyll a. Each standard solution was then mixed with 20 μg/mL of Fast Green FCF (internal standard) each and injected into HPLC twice. Each standard curve was prepared by plotting the concentration ratio (chlorophyll standard vs internal standard) against its area ratio, with the linear regression equation and correlation coefficient (R^2) being obtained automatically based on an EXCEL software system. The various chlorophylls and their derivatives were quantified using a formula as described in a previous study.[15]

Statistical Analysis. Duplicate experiments were performed with each sample being analyzed three times, and the mean data were subjected to analysis of variance and Duncan's multiple range test for statistical significance by using SAS,[20] whereas triplicate analyses were done for quality control studies including recovery and intraday and interday variability.

RESULTS AND DISCUSSION

HPLC Analysis. It has been well established that the employment of a C18 column is appropriate for the separation of chlorophylls and their derivatives.[14] Many types of C18 columns with difference in column length, internal diameter, and particle size have been used. In this study, we compared a HyPURITY C18 column (150 mm × 4.6 mm i.d., particle size 5 μm), Nucleosil ODS 100 column (250 mm × 4 mm i.d., particle size 5 μm), Spherisorb ODS-2 column (250 mm × 4 mm i.d., particle size 5 μm), and Alltima C18 column (250 mm × 4.6 mm i.d., particle size 10 μm).[14,21−23] Among them, the HyPURITY C18 column provided a better resolution of 11 chlorophylls and their derivatives under the condition of a

Table 1. Retention Time (t_R), Retention Factor (k), Separation Factor (α), Peak Purity, MS Data, λ_{max}, and Contents (μg/g) of Chlorophylls in *T. formosanum*

peak no.	compd	t_R (min)	retention factor (k)[b]	separation factor (α)[c]	peak purity (%)	MS data (m/z) online	MS data (m/z) reported	λ_{max} (nm) online	λ_{max} (nm) reported	content (μg/g)[i]
1	chlorophyllide a	6.14	1.80	1.25 (1, 2)[d]	90.1		614.3 [M][e]	430, 620, 666	427, 616, 668[h]	0.17 ± 0.08
2	chlorophyllide a'	7.12	2.25	1.73 (2, 3)[d]	83.6			436, 618, 664		0.18 ± 0.09
3	hydroxychlorophyll b	10.74	3.90	1.11 (3, 4)[d]	98.7	923.5 [M + H]+	923 [M + H]+f	462, 600, 646	460, 598, 646f	26.53 ± 1.01
4	chlorophyll b[a]	11.66	4.32	1.05 (4, 5)[d]	97.2	907.4 [M + H]+, 629.3 [M + H − 278]+	907.4 [M + H]+, 629.3 [M + H − 278]+g	462, 600, 648	462, 600, 648f	533.74 ± 10.40
5	chlorophyll b'	12.16	4.55	1.10 (5, 6)[d]	96.8	907.4 [M + H]+, 629.3 [M + H − 278]+	907.4 [M + H]+, 629.3 [M + H − 278]+g	462, 602, 650	462, 600, 648f	27.49 ± 0.73
6	hydroxychlorophyll a	13.17	5.01	1.03 (6, 7)[d]	95.7	909.5 [M + H]+, 891.5 [M + H − 18]+	909.5 [M + H]+, 891.5 [M + H − 18]+g	422, 618, 662	422, 614, 660f	0.71 ± 0.61
7	hydroxychlorophyll a'	13.54	5.18	1.15 (7, 8)[d]	99.3	909.5 [M + H]+, 891.5 [M + H − 18]+		428, 618, 662		9.09 ± 1.52
8	chlorophyll a[a]	15.19	5.94	1.09 (8, 9)[d]	99.7	893.5 [M + H]+, 615.3 [M + H − 278]+	893 [M + H]+g	430, 618, 664	430, 618, 664f	1335.92 ± 32.87
9	chlorophyll a'	16.39	6.48	1.80 (9, 10)[d]	97.8	893.5 [M + H]+	893 [M + H]+g	430, 618, 664	430, 618, 664f	53.71 ± 1.09
10	pheophytin a[a]	27.73	11.66	1.03 (10, 11)[d]	99.9	871.6 [M + H]+	871 [M + H]+f	408, 506, 536, 610, 666	408, 506, 536, 608, 666f	30.68 ± 1.03
11	pheophytin a'	28.40	11.97	1.03 (10, 11)[d]	99.9	871.6 [M + H]+	871 [M + H]+f	408, 506, 536, 608, 666	408, 506, 536, 610, 666f	1.07 ± 0.04

[a]Compound conclusively identified by comparison with authentic standard, while others were tentatively identified by comparison of absorption and MS spectra with that reported in the literature. [b]The retention factor (k) is defined as $k = (t_R − t_0)/t_0$ where t_0 denotes the retention time of the solvent peak and t_R is the retention time of the sample peak. [c]The separation factor (α) is defined as $\alpha = (t_{R2} − t_0)/(t_{R1} − t_0)$, where t_0 denotes the retention time of the solvent peak and t_{R1} and t_{R2} are the retention times of the two neighboring peaks. [d]Numbers in parentheses represent values between two neighboring peaks. [e]On the basis of a reference by van Breemen et al.[25]. [f]On the basis of a reference by Huang et al.[14]. [g]On the basis of a reference by Gauthier-Jaques et al.[26]. [h]On the basis of a reference by Almela et al.[16]. [i]Average of duplicate analyses ± standard deviation based on sample weight.

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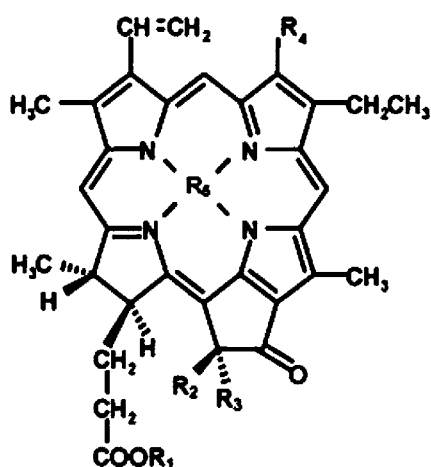
Compound	R_1	R_2	R_3	R_4	R_5
Chlorophyll a	Phytol	H	$COOCH_3$	CH_3	Mg
Chlorophyll b	Phytol	H	$COOCH_3$	CHO	Mg
Chlorophyll a'	Phytol	$COOCH_3$	H	CH_3	Mg
Chlorophyll b'	Phytol	$COOCH_3$	H	CHO	Mg
Hydroxychlorophyll a	Phytol	OH	$COOCH_3$	CH_3	Mg
Hydroxychlorophyll b	Phytol	OH	$COOCH_3$	CHO	Mg
Hydroxychlorophyll a'	Phytol	$COOCH_3$	OH	CH_3	Mg
Pheophytin a	Phytol	H	$COOCH_3$	CH_3	2H
Pheophytin a'	Phytol	$COOCH_3$	H	CH_3	2H
Hydroxypheophytin a	Phytol	OH	$COOCH_3$	CH_3	2H
Hydroxypheophytin a'	Phytol	$COOCH_3$	OH	CH_3	2H
Chlorophyllide a	H	H	$COOCH_3$	CH_3	Mg
Chlorophyllide a'	H	$COOCH_3$	H	CH_3	Mg
Chlorophyllide b	H	H	$COOCH_3$	CHO	Mg
Pyropheophorbide a	H	H	H	CH_3	2H

Figure 2. Chemical structures of chlorophylls and their derivatives in *T. formosanum*.

gradient mobile phase of methanol, acetonitrile, and acetone, as developed by Huang et al.[14] However, with the same column and mobile phase, several chlorophyll peaks were overlapped in the HPLC chromatogram (figure not shown), which should be caused by a difference in solvent strength and sample variety. Thus, the HPLC condition used for separation of chlorophylls and their derivatives in *T. formosanum* has to be modified. After various studies, a quaternary mobile phase of water (A), methanol (B), acetonitrile (C), and acetone (D) as described in the preceding section was developed to optimize the solvent strength for good resolution. This solvent system could separate 11 chlorophylls and their derivatives in *T. formosanum* within 30 min with a flow rate at 1 mL/min and detection at 660 nm (Figure 1). As compared to an earlier method,[14] the inclusion of a fourth solvent water with different gradient conditions facilitated separation of high-polar dephytylated chlorophyll derivatives chlorophyllide a, chlorophyllide a', and chlorophyllide b. The retention time, retention factor (*k*), and separation factor (*α*) of various chlorophylls and their derivatives are shown in Table 1, which ranged from 6.14 to 28.4 min, from 1.8 to 11.97, and from 1.03 to 1.80, respectively, implying that there was a proper solvent strength and that selectivity was controlled. With the exception of chlorophyllide a' (peak 2), the purities of the other peaks were higher than 90%. In several other published reports, both the number in chlorophyll separation and the resolution remained inadequate. For example, only four chlorophylls in green leaf lettuce were resolved within 30 min,[24] whereas eight chlorophylls in spinach were separated within 30 min.[13] Likewise, only three chlorophylls in grape were separated within 32 min.[17] Comparatively, the HPLC condition developed in this study provided a better resolution in chlorophylls and their derivatives in *T. formosanum* within a reasonable length of time. As demonstrated by Huang et al.,[14] Fast Green FCF was found to be a suitable internal standard as it did not interfere with the separation of the other compounds and was completely eluted from the column (Figure 1).

Identification of Chlorophylls and Their Derivatives. As described in the preceding section, the retention time, absorption spectra (λ_{max}), and mass spectra were used for

identification of chlorophylls and their derivatives in *T. formosanum* (Table 1). Peaks 4, 8, and 10 were conclusively identified as chlorophyll b, chlorophyll a, and pheophytin a, respectively, by comparison with authentic standards, while peaks 1 and 2 were tentatively identified as chlorophyllides a and a', respectively, based on retention behavior and absorption spectra, as well as comparison with that by Almela et al.[16] and van Breeman et al.[25] However, the *m/z* ion of chlorophyllides a and a' remained undetected, probably caused by interference of carotenoid peaks at the same retention time. Peak 3 was tentatively identified as hydroxychlorophyll b according to elution order, absorption spectra, and mass spectra as well as comparison with that reported by Huang et al.,[14] while peak 5 was identified as chlorophyll b', which possessed the same absorption spectra and mass spectra as chlorophyll b. Following the same approach, peaks 6 and 7 were identified as hydroxychlorophylls a and a', respectively, as both absorption spectra and mass spectra were the same as that reported by Gauthier-Jaques et al.[26] and Huang et al.[14] Similarly, peaks 9 and 11 were identified as chlorophyll a' and pheophytin a', respectively. Figure 2 shows the chemical structures of chlorophylls and their derivatives in *T. formosanum*.

As most chlorophylls in nature are mainly present as chlorophyll a and chlorophyll b, the formation of chlorophyll isomers such as chlorophyll a', chlorophyll b', and pheophytin a' in *T. formosanum* are probably due to drying or extraction.[14] Additionally, several hydroxylated chlorophyll derivatives including hydroxychlorophyll a, hydroxychlorophyll b, and hydrochlorophyll a' were generated in *T. formosanum* as well, which may be attributed to the presence of peroxidase in *T. formosanum*. It was postulated that peroxidase may catalyze oxidation of chlorophyll a to produce hydroxychlorophyll a through the intermediate phenoxy radical, formed between phenolic compounds at the para-position like *p*-coumaric acid and peroxide in the presence of peroxidase.[27] Nevertheless, the chlorophyll oxidation may also occur through several other mechanistic pathways as well, as elaborated by Hynninen et al.[28] Also, the presence of both chlorophyllide a and chlorophyllide a' in *T. formosanum* should be due to hydrolysis of chlorophyll a by chlorophyllase.[29] By comparison, some

Table 2. Quality Control Data of Chlorophylls and Their Derivatives in *T. formosanum* by HPLC-DAD

peak no.	chlorophylls	intraday variability[a]		interday variability[a]	
		mean ± SD (μg/g)	RSD (%)	mean ± SD (μg/g)	RSD (%)
1	chlorophyllide a	0.17 ± 0.08	3.5	0.17 ± 0.14	4.7
2	chlorophyllide a'	0.18 ± 0.09	2.7	0.16 ± 0.11	4.4
3	hydroxychlorophyll b	26.53 ± 1.01	3.4	26.21 ± 1.31	5.5
4	chlorophyll b	533.74 ± 10.40	2.6	528.65 ± 11.29	2.9
5	chlorophyll b'	27.49 ± 0.73	3.2	27.77 ± 1.02	4.5
6	hydroxychlorophyll a	0.71 ± 0.61	3.0	0.75 ± 0.31	5.8
7	hydroxychlorophyll a'	9.09 ± 1.52	2.6	9.71 ± 2.04	5.9
8	chlorophyll a	1335.92 ± 32.87	3.2	1355.48 ± 39.24	4.0
9	chlorophyll a'	53.71 ± 1.09	3.0	54.55 ± 1.82	5.3
10	pheophytin a	30.68 ± 1.03	2.1	31.81 ± 1.60	3.3
11	pheophytin a'	1.07 ± 0.04	2.4	1.33 ± 0.17	4.1

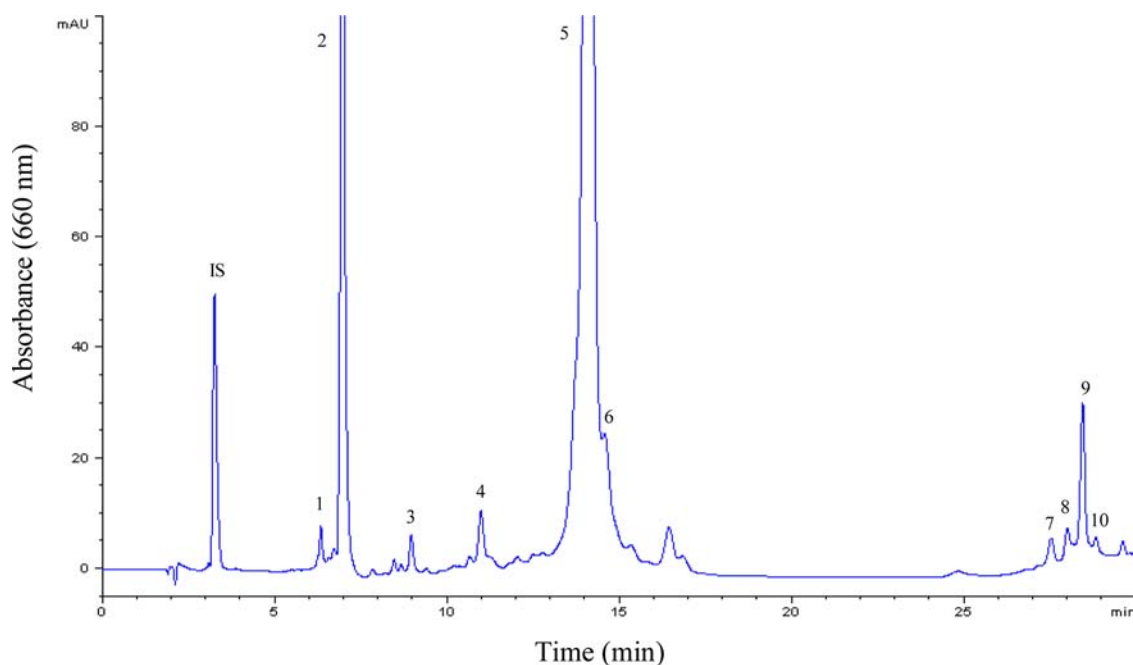[a]Mean of triplicate analyses ± standard deviation.

Figure 3. HPLC chromatogram of chlorophyll fraction isolated from *T. formosanum*. Peak identification is shown in Table 2.

more chlorophyll isomers and derivatives such as pheophytin b', hydroxypheophytin b', and pyropheophytin a were reported to be present in *Gynostemma pentaphyllum* by Huang et al.,[14] which should be caused by the differences in Chinese herb variety and processing method. More specifically, in this study, freeze-drying was used for pretreatment of *T. formosanum* sample, while in Huang's study some more steps including blanching, withering, and baking were adopted for the processing of *G. pentaphyllum*.

Quality Control. The linear regression equations used for quantitation of chlorophyll b, pheophytin a, and low and high concentrations of chlorophyll a were $y = 1.8476x - 0.0357$, $y = 10.836x + 0.2019$, $y = 3.0624x + 0.2541$, and $y = 4.5507x + 4.8318$, respectively, with R^2 being 0.9961, 0.9969, 0.9934, and 0.9954. The LODs for chlorophyll a, chlorophyll b, and pheophytin a were 0.05, 0.05, and 0.02 μg/mL, respectively, while the LOQs were 0.15, 0.15, and 0.06 μg/mL. The relative standard deviations (RSD %) for the intraday variability ranged from 2.1 to 3.5% and 2.9 to 5.9% for the interday variability (Table 2). As for recovery, an average of 89.3, 89.1, and 94.1% was shown for chlorophyll a, chlorophyll b, and pheophytin a,

respectively. This outcome was similar to that reported by Huang et al.,[14] with a recovery of 81.7−89.0%, 85.8−92.6%, and 95.5−100.7% being found for chlorophyll a, chlorophyll b, and pheophytin a, respectively, in *G. pentaphyllum*. After quantitation, as expected, both chlorophyll a and chlorophyll b dominated in *T. formosanum*, which amounted to 1335.92 and 533.74 μg/g, respectively (Table 1). However, their epimers chlorophyll a' and chlorophyll b' were present at a much smaller amount, which equaled 53.71 and 27.49 μg/g, respectively. Similarly, a low content of 30.68 μg/g was shown for pheophytin a and 1.07 μg/g for its epimer pheophytin a', as were hydroxychlorophyll a (0.71 μg/g), hydroxychlorophyll a' (9.09 μg/g), and hydroxychlorophyll b (26.53 μg/g). Comparatively, the polar chlorophyll derivatives chlorophyllide a and chlorophyllide a' were present in a minor amount of 0.17 and 0.18 μg/g, respectively, indicating that chlorophyllide was more difficult to generate than the other chlorophyll derivatives. Apparently, chlorophyllide is a derivative formed exclusively by the enzymatic action of chlorophyllase; therefore, its formation will depend on several processing variables and the level of chlorophyllase activity in a specific plant material.

Table 3. Retention Time, λ_{max}, MS Data, and Contents of Chlorophyll Fraction Prepared from *T. formosanum* by Column Chromatography

| peak no. | compd | t_R (min) | λ_{max} (nm) | | mass data (*m/z*) | | content ($\mu g/g$)[e] |
			online	reported	online	reported	
1	chlorophyllide b	6.33	456, 586, 634	458, 594, 643[a]	629 [M + H]$^+$	628 [M]^c	15.10 ± 1.82
2	chlorophyllide a	6.96	420, 530, 616, 658	427, 616, 668[a]	615 [M + H]$^+$	614 [M]^c	140.92 ± 2.21
3	pyropheophorbide a	8.99	402, 502, 538, 672	—[f]	535 [M + H]$^+$	535 [M + H]$^{+d}$	13.85 ± 1.40
4	hydroxychlorophyll b	11.04	452, 586, 634	460, 598, 646[b]	923 [M + H]$^+$	923 [M + H]$^{+b}$	26.90 ± 0.31
5	hydroxychlorophyll a	14.19	420, 528, 614, 656	422, 618, 662[b]	909 [M + H]$^+$	909 [M + H]$^{+b}$	488.14 ± 7.14
6	hydroxychlorophyll a′	14.61	422, 532, 614, 660	—	909 [M + H]$^+$	—	47.26 ± 0.59
7	hydroxypheophytin a	27.52	398, 500, 526, 606, 664	406, 502, 532, 610, 666[b]	887 [M + H]$^+$	887 [M + H]$^{+b}$	0.61 ± 0.08
8	hydroxypheophytin a′	27.98	400, 500, 528, 614, 668	408, 504, 534, 610, 666[b]	887 [M + H]$^+$	887 [M + H]$^{+b}$	1.69 ± 0.04
9	pheophytin a	28.42	402, 502, 540, 674	408, 506, 536, 608, 666[b]	871 [M + H]$^+$	871 [M + H]$^{+b}$	8.32 ± 0.07
10	pheophytin a′	28.80	402, 500, 530, 666	408, 506, 536, 610, 666[b]	871 [M + H]$^+$	871 [M + H]$^{+b}$	0.97 ± 0.17

[a]On the basis of a reference by Almela et al.[16] [b]On the basis of a reference by Huang et al.[14] [c]On the basis of a reference by van Breemen et al.[25] [d]On the basis of a reference by Gauthier-Jaques et al.[26] [e]Average of duplicate analyses ± standard deviation based on sample weight. [f]Data not available.

Furthermore, enzymatic reactions are not involved in epimeric chlorophyll derivatives, while oxidized derivatives such as hydroxychlorophylls can have both chemical and enzymatic origin. The chlorophyll a content in *T. formosanum* was higher than in most of the other green plants. For instance, there is a level of 283.0 $\mu g/g$ in lettuce[17] and 113.8 $\mu g/g$ in *G. pentaphyllum*.[14] However, pheophytin a in *G. pentaphyllum* (2508.3 $\mu g/g$) was much higher than in *T. formosanum* (30.7 $\mu g/g$), which should be probably due to a difference in the drying method employed. Likewise, as compared to *T. formosanum* (2019.3 $\mu g/g$), higher total chlorophyll contents were reported to be present in *G. pentaphyllum* (3665.7 $\mu g/g$)[14] and freeze-dried *Rhinacanthus nasutus* (6124.5 $\mu g/g$).[15] However, upon hot-air drying, the total chlorophyll content in *R. nasutus* reduced drastically to 2368.8 $\mu g/g$, demonstrating that a difference in drying method can affect the content of total chlorophylls and their derivatives in plants.[15]

Preparation of Chlorophylls by Column Chromatography. Initially, various combinations of adsorbents including silica gel, magnesium oxide, and diatomaceous earth in different proportions were compared with respect to isolation efficiency of chlorophylls from *T. formosanum* by column chromatography. After numerous studies, the most appropriate condition for preparation of chlorophylls as described in the Materials and Methods was developed, and the isolated chlorophyll fraction was then subjected to HPLC-DAD-MS analysis. Figure 3 shows the HPLC chromatogram of chlorophylls and their derivatives in chlorophyll fraction isolated from *T. formosanum*. Following the same identification criteria as shown in the preceding section, a total of 10 chlorophylls and their derivatives were present in chlorophyll fraction, in which hydroxychlorophylls a and a′ were present in the largest amount (535.4 $\mu g/g$), followed by chlorophyllide a (140.92 $\mu g/g$), hydroxychlorophyll b (26.9 $\mu g/g$), chlorophyllide b (15.1 $\mu g/g$), pyropheophorbide a (13.85 $\mu g/g$), pheophytins a and a′ (9.29 $\mu g/g$), and hydroxypheophytins a and a′ (2.3 $\mu g/g$). It is worth pointing out that the mass spectrum of chlorophyllide a (*m/z* 615) could be detected after separation of chlorophylls from carotenoids by column chromatography. As compared to original chlorophyll extract in *T. formosanum* sample, several chlorophyll derivatives including chlorophyllide b, pyropheophorbide a, hydroxypheophytin a, and hydroxypheophytin a′ were generated during column chromatography but were accompanied by a loss of chlorophyll a, chlorophyll a′, chlorophyll b, chlorophyll b′, and chlorophyllide a′ (Table 3

and Figure 2). The absence of chlorophylls a and b was further confirmed by employing SIM for detection with the former being set at 893 [M + H]$^+$ and the latter at 907 [M + H]$^+$. Likewise, chlorophyll a′, chlorophyll b′, and chlorophyllide a′ remained undetected in SIM mode. As mentioned before, both chlorophyll a and chlorophyll b were present in an abundant amount in *T. formosanum* (Table 1). Thus, it may be inferred that during column chromatography, both chlorophyll a and chlorophyll b can undergo degradation to form the various chlorophyll derivatives or epimers as shown above. In addition, as compared to a freeze-dried sample, a minor difference in λ_{max} was observed for hydrochlorophylls a and a′ after column chromatography, which may be due to purity change (Table 3). A 10 times lower level of hydroxychlorophyll a over hydroxychlorophyll a′ in the freeze-dried sample implied that the epimerization could proceed fast even during extraction. The degradation of chlorophylls into hydroxychlorophylls may be probably caused by some other pro-oxidants induced oxidation, while the acidic nature of column adsorbent may be responsible for pheophytin formation, as peroxidase or chlorophyllase is unlikely to be activated in chlorophyll extract in hexane. By comparison, a 63% loss (1275.53 $\mu g/g$) of total chlorophylls occurred during column chromatography, which can be attributed to the unstable nature of chlorophylls (Table 3). Nonetheless, the chlorophyll degradation products such as pheophytin a and its derivatives may exhibit vital biological activity like anti-inflammation.[30] Thus, the possibility of a chlorophyll fraction prepared from *T. formosanum* as raw material for future production of functional food needs further investigation.

In conclusion, an HPLC gradient quaternary solvent system was developed to separate 10 chlorophylls and internal standard Fast Green FCF within 30 min by employing a HyPURITY C18 column with a flow rate at 1 mL/min and detection at 660 nm. Both chlorophylls a and a′ were present in the largest amount, followed by chlorophylls b and b′, pheophytins a and a′, hydroxychlorophyll b, hydroxychlorophylls a and a′, and chlorophyllides a and a′. Several chlorophyll derivatives including chlorophyllide b, pyropheophorbide a, and hydroxypheophytins a and a′ were generated during column chromatography with a glass column containing 52 g of magnesium oxide—diatomaceous earth (1:3, w/w) as the adsorbent.

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Notes
The authors declare no competing financial interest.

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