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An improved high performance liquid chromatography–photodiode array detection–atmospheric pressure chemical ionization–mass spectrometry method for determination of chlorophylls and their derivatives in freeze-dried and hot-air-dried *Rhinacanthus nasutus* (L.) Kurz

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ABSTRACT

Rhinacanthus nasutus (L.) Kurz, a traditional Chinese herb possessing antioxidant and anti-cancer activities, has been reported to contain functional components like carotenoids and chlorophylls. However, the variety and amount of chlorophylls remain uncertain. The objectives of this study were to develop a high performance liquid chromatography–photodiode array detection–atmospheric pressure chemical ionization–mass spectrometry (HPLC–DAD–APCI–MS) method for determination of chlorophylls and their derivatives in hot-air-dried and freeze-dried *R. nasutus*. An Agilent Eclipse XDB-C18 column and a gradient mobile phase composed of methanol/N,N-dimethylformamide (97:3, v/v), acetonitrile and acetone were employed to separate internal standard zinc-phthalocyanine plus 12 cholorophylls and their derivatives within 21 min, including chlorophyll a, chlorophyll a′, hydroxychlorophyll a, 15-OH-lactone chlorophyll a, chlorophyll b, chlorophyll b′, hydroxychlorophyll b, pheophytin a, pheophytin a′, hydroxypheophytin a, hydroxypheophytin a′ and pheophytin b in hot-air-dried *R. nasutus* with flow rate at 1 mL/min and detection at 660 nm. But, in freeze-dried *R. nasutus*, only 4 chlorophylls and their derivatives, including chlorophyll a, chlorophyll a′, chlorophyll b and pheophytin a were detected. Zinc-phthalocyanine was found to be an appropriate internal standard to quantify all the chlorophyll compounds. After quantification by HPLC–DAD, both chlorophyll a and pheophytin a were the most abundant in hot-air-dried *R. nasutus,* while in freeze-dried *R. nasutus*, chlorophyll a and chlorophyll b dominated.

1. Introduction

Rhinacanthus nasutus (L.) Kurz, a Chinese herb widely grown in Taiwan and China, has been reported to possess several biological activities including anti-cancer, anti-mutagenicity, anti-bacteria and regulation of blood pressure and triglyceride [1–3]. However, the bioactive compounds responsible for prevention or treatment of chronic disease remain uncertain. In an early study Yang [4] reported that *R. nasutus* contained sterol derivatives (stigmasterol and β-sitosterol), sterone derivatives (stigmasterone, β-sitosterone, dihydrostigmasterone, β-dihydrositosterone, 6-hydroxystigmast-4-en-3-one and 6-hydroxy-β-sitost-4-en-3-ome), steroid derivatives (stigmasterol-β-D-glucose and β-sitosterol-β-D-glucose) and polyphenols such as 2-methoxy-4-propinylphenol, vanillic acid and syringic acid. Additionally, some other functional components including quinol, triterpenoids, benzenoids, coumarin, anthraquinone, quinine and chlorophyll were shown to be present in *R. nasutus* [5]. But, the variety and amount of chlorophylls in *R. nasutus* remain unknown.

Chlorophyll, an important biological pigment responsible for photosynthesis, can be synthesized through light-dependent or light-independent biosynthesis pathway [6]. Chlorophyll is composed of 4 pyrroles linked by four methyne bridges to form a porphyrin ring, chelated with a magnesium ion in the center, with a phytol group esterified with propionate at C7, keto group at C9 and carbomethoxy group at C10. Both chlorophyll a and chlorophyll b are the most abundant chlorophylls in green plants, with the former containing a methyl group at the C3 while the latter containing a formaldehyde group at the same carbon [7]. Chlorophylls can be susceptible to color change during heating or processing. For instance, during blanching, pheophytins a and b can be formed

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from chlorophylls a and b, respectively, through replacement of magnesium ion with organic acids liberated from green vegetables, accompanied by color change from bright green to olive green [8]. Also, the phytol group can be removed by chlorophyllase to induce generation of chlorophyllide a or b, which in turn leads to formation of pheophorbide a or b depending on heating condition [9]. Alternatively, pyropheophytin a or b can be formed through removal of carbomethoxy group at C13 during canning of vegetables, resulting in a color change to olive brown [9].

Many studies have shown chlorophylls may exhibit several biological activities, including anti-inflammation, anti-mutagenicity, scavenging of free radicals and inhibition of oxalate crystallization [7]. For example, chlorophylls were effective in scavenging DPPH free radicals to minimize the detrimental effect of free radicals to cells [10]. In another study Chiu et al. [11] reported that chlorophyllin, a sodium- or copper-containing chlorophyll, was efficient in inhibiting growth of leukemia cell lines HL-60 and K-562. Likewise, with photodynamic therapy, chlorophyllin showed a pronounced antiproliferation effect on hepatoma cell Hep3B [12]. In a review report, Ferruzzi and Blakeslee [7] concluded that the anti-cancer or anti-mutagenicity activity of chlorophyll or chlorophyllin observed in vitro and in vivo might be caused by conjugation between chlorophyll or chlorophyllin and chemical-induced mutagen or its metabolites.

In view of the impact of chlorophylls on human health, the variety and amount of chlorophylls and their derivatives in *R. nasutus* need to be further investigated. The objectives of this study were to develop a HPLC–DAD–APCI–MS method to determine various chlorophylls and their derivatives in *R. nasutus* as affected by freeze-drying and hot-air-drying.

2. Materials and methods

2.1. Materials and instrumentation

2.1.1. Materials

R. nasutus was procured from a local drug store in Taipei, Taiwan. Chlorophyll a and chlorophyll b standards as well as internal standard zinc-phthalocyanine were purchased from Sigma (St. Louis, MO, USA). The HPLC-grade solvents including methanol, acetonitrile, acetone and N,N-dimethylformamide (DMF) were from LAB-SCAN Co. (Dublin, Ireland) and Mallinckrodt Co. (Phillipsburg, NJ, USA). The analytical-grade solvents including hexane and toluene were from Grand Chemical Co. (Taipei, Taiwan). 95% ethanol was from Taiwan Tobacco and Wine Co. (Tainan, Taiwan). Anhydrous sodium sulfate was from Nacalai Tesque Co. (Kyoto, Japan). Deionized water was made from a Milli-Q water purification system from Millipore Co. (Bedford, MA, USA). Three columns evaluated in this study were (1) Vydac 201TP54 C18 (250 mm × 4.6 mm I.D., particle size 5 μm), (2) Agilent Eclipse XDB-C18 (150 mm × 4.6 mm I.D., particle size 5 μm) and (3) Thermo Hypersil-Keystone HyPURITY C18 (150 mm × 4.6 mm I.D., particle size 5 μm).

2.1.2. Instrumentation

The HPLC–MS system was from Agilent Co. (Palo Alto, CA, USA), which is composed of G1311A pump, G1316A column temperature controller, G1315B photodiode-array detector and 6130 quadrupole mass spectrometer with multiple ion source. The Eyela N-1 rotary evaporator was from Tokyo (Japan). The freeze dryer (FD24) was from Gin-Ming Co. (Taipei, Taiwan), while the hot-air dryer (KVEO-1) was from Taiwan Tong-Yuan Electric Co. (Taipei, Taiwan). The Sorvall RC5C high-speed centrifuge was from Du Pont Co. (Wilmington, Delaware, USA).

2.2. Experimental

2.2.1. Extraction of chlorophylls from R. nasutus

A total amount of 6 kg of *R. nasutus* was divided into two portions with 3 kg each, both of which were subjected to hot-air-drying at 60 °C for 4 h and freeze-drying at −40 °C (60 mTorr) for 48 h separately with the final moisture content being lower than 10%, followed by pouring each into plastic bags, sealing under vacuum and storing at −20 °C for use. A method similar to that used by Huang et al. [13] was modified to extract chlorophylls from *R. nasutus*. Briefly, a 0.2 g powder sample of *R. nasutus* was mixed with 30 mL of hexane–ethanol–acetone–toluene (10:6:7:7, v/v/v/v) and the mixture was shaken for 1 h, after which 15 mL of hexane was added and shaken for another 10 min. Then 15 mL of 10% anhydrous sodium sulfate solution was added and shaken for 1 min and the mixture was allowed to stand in the air at room temperature for separation into two layers. The organic layer containing chlorophylls was collected and 15 mL of hexane was added to the lower layer for repeated extraction of chlorophylls. This procedure was repeated three times, and all the supernatants were combined, evaporated to dryness under vacuum at 25 °C, followed by dissolving in 5 mL of acetone, filtering through a 0.22 μm membrane filter and injecting 20 μL onto HPLC–MS.

2.2.2. Preparation of 15-OH-lactone chlorophyll a

A method based on Minguez-Mosquera et al. [14] was modified to prepare 15-OH-lactone chlorophyll a. Briefly, a 10 ppm of chlorophyll a standard in acetone was prepared and a mixture containing 1 mL of diethyl ether and 2 mL of saturated NaCl solution was added for separation into two layers. Then the ether layer was collected, evaporated to dryness under nitrogen at 25 °C, dissolved in acetone and subjected to HPLC–MS analysis for further identification.

2.2.3. Preparation of pheophytin a and pheophytin b

Both pheophytins a and b were prepared based on a method as described by Teng and Chen [8]. One mg of chlorophyll a and chlorophyll b was dissolved in 1 mL of acetone separately, followed by adding a few drops of 0.1 N HCl. After shaking homogeneously for a few minutes, both pheophytins a and b were prepared and then subjected to HPLC–MS analysis for further identification.

2.2.4. HPLC–DAD–APCI–MS analysis

A method as described by Huang et al. [13] was modified to separate various chlorophylls and their derivatives in *R. nasutus*. An Eclipse XDB-C18 column and a gradient mobile phase of methanol/DMF (97:3, v/v) (A), acetonitrile (B) and acetone (C) was employed: 65% A, 30% B and 5% C initially, changed to 60% A, 30% B and 10% C in 8 min, 90% A and 10% C in 13 min, 60% A and 40% C in 15 min, maintained for 7 min, and returned to original ratio in 25 min. A total of 12 chlorophylls and their derivatives plus internal standard zinc-phthalocyanine were resolved within 21 min with flow rate at 1 mL/min and detection at 660 nm. The mass spectrum of each chlorophyll was detected using APCI with positive mode with the following condition: MW 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. The peak purity was determined automatically based on the extent of spectra overlapping within a peak by using an Agilent G2180A spectral evaluation software data management system. Identification of chlorophylls and their derivatives in *R. nasutus* was performed by comparing retention time, absorption spectra and mass spectra of unknown peaks with reference standards and those in the literature. Also, the addition of various chlorophyll standards to sample for co-chromatography was employed for positive identification.

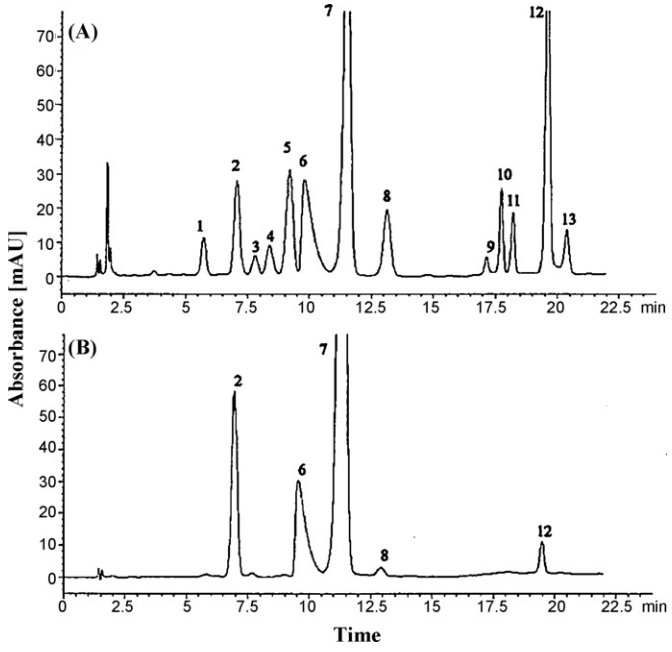


Fig. 1. HPLC chromatograms of chlorophylls and their derivatives extracted from hot-air-dried (A) and freeze-dried (B) *R. nasutus*. Chromatographic condition described in text. Peak identification: (1) hydroxychlorophyll b, (2) chlorophyll b, (3) chlorophyll b′, (4) 15-OH-lactone chlorophyll a, (5) hydroxychlorophyll a, (6) zinc-phthalocyanine (IS), (7) chlorophyll a, (8) chlorophyll a′, (9) pheophytin b, (10) hydroxypheophytin a, (11) hydroxypheophytin a′, (12) pheophytin a, (13) pheophytin a ′.

2.2.5. Precision study

The intra-day variability was carried out by injecting chlorophyll extract containing 5 μg/mL of internal standard zinc-phthalocyanine into HPLC in the morning, afternoon and evening three times each for a total of 9 injections. Similarly, the inter-day variability was performed by injecting into HPLC in the first, second and third day, with three injections in each day for a total of 9 injections.

2.2.6. Detection and quantitation limits

Concentrations of 0.1, 0.2 and 0.3 μg/mL were prepared for chlorophyll a, chlorophyll b, pheophytin a and pheophytin b standards, separately, followed by injecting into HPLC three times for each concentration. The detection limit was calculated based on S/N ≥ 3, whereas the quantitation limit based on S/N ≥ 10.

2.2.7. Recovery

Both chlorophyll a (20 μg) and chlorophyll b (70 μg), as well as pheophytin a (7 μg) and pheophytin b (30 μg) were mixed with 0.2 g sample of *R. nasutus* separately, after which chlorophylls and their derivatives were extracted using the same procedure as described in the preceding section. After HPLC analysis, the recovery of each chlorophyll was determined based on the ratio of the amount of measured analyte (spiked amount minus original amount) relative to that before HPLC (spiked amount). The recovery of chlorophyll a was also used to quantify chlorophyll a′, hydroxychlorophyll a and hydroxychlorophyll a′ because of commercial unavailability of these standards. Likewise, the recovery of chlorophyll b was employed to quantify chlorophyll b′ and hydroxychlorophyll b, whereas pheophytin a used to quantify pheophytin a′, hydroxypheophytin a and hydroxypheophytin a′.

2.2.8. Quantitation of chlorophylls

Eight concentrations of 0.5, 1, 5, 10, 30, 50, 70 and 100 μg/mL of chlorophyll a and pheophytin a were prepared in acetone,

Table 1
Retention time (t_R), retention factor (k), resolution factor (R), peak purity, UV and MS spectral data of chlorophylls and their derivatives extracted from hot-air-dried *R. nasutus*.

Peak no.	Compound	t_R (min)	Retention factor (k)	Resolution (R)		Peak purity (%)	λ_{max} (on-line)	λ_{max} (reported)	$[M+H]^+$ (on-line)	$[M+H]^+$ (reported)
1	Hydroxychlorophyll b	5.8	2.87	5.9	(1, 2)[b]	91.5	462, 598, 648	460, 598, 646[d]	923	923[d]
2	Chlorophyll b	7.2	3.80	2.4	(2, 3)[b]	92.2	464, 600, 648	462, 600, 648[e]	907	907[d]
3	Chlorophyll b′	7.8	4.20	3.1	(3, 4)[b]	83.5	462, 602, 650	462, 600, 648[e]	907	907[d]
4	15-OH-lactone chlorophyll a	8.6	4.73	2.4	(4, 5)[b]	88.2	418, 614, 654	420, 614, 656[f]	925	_[c]
5	Hydroxychlorophyll a	9.3	5.20	1.7	(5, 6)[b]	97.2	430, 618, 664	422, 614, 660[d]	909	909[d]
6	Zinc-phthalocyanine (IS)[a]	9.9	5.60	4.5	(6, 7)[b]	99.9	666	666[g]	579	_[c]
7	Chlorophyll a	11.5	6.67	5.4	(7, 8)[b]	95.7	430, 618, 664	430, 618, 664[e]	893	893[d]
8	Chlorophyll a′	13.2	7.80	16.3	(8, 9)[b]	94.9	430, 618, 664	430, 618, 664[e]	893	893[d]
9	Pheophytin b	17.2	10.47	3.7	(9, 10)[b]	99.8	436, 526, 598, 654	436, 528, 598, 652[e]	885	885[d]
10	Hydroxypheophytin a	17.8	10.87	2.5	(10, 11)[b]	99.8	408, 502, 532, 610, 666	406, 502, 532, 610, 666[d]	887	887[d]
11	Hydroxypheophytin a′	18.2	11.13	7.5	(11, 12)[b]	98.1	408, 504, 534, 610, 666	408, 504, 534, 610, 666[d]	887	887[d]
12	Pheophytin a	19.6	12.07	3.1	(12, 13)[b]	97.1	408, 506, 536, 608, 666	408, 506, 536, 608, 666[e]	871	871[d]
13	Pheophytin a′	20.3	12.53	5.6	(12, 13)[b]	98.6	408, 506, 538, 610, 666	408, 506, 536, 610, 666[d]	871	871[d]

[a] IS = internal standard.
[b] Numbers in parentheses represent values between two neighboring peaks.
[c] "–": Data not available.
[d] Based on a reference by Huang et al. [13].
[e] Based on a reference by Kamffer et al. [27].
[f] Based on a reference by Roca et al. [23].
[g] Based on a reference by Zhang et al. [28].

Table 2
Retention time (t_R), retention factor (k), resolution factor (R), peak purity, UV and MS spectral data of chlorophylls and their derivatives extracted from freeze-dried *R. nasutus*.

Peak no.	Compound	t_R (min)	Retention factor (k)	Resolution (R)	Peak purity (%)	λ_{max} (on-line)	λ_{max} (reported)	$[M+H]^+$ (on-line)	$[M+H]^+$ (reported)
2	Chlorophyll b	7.3	3.87	7.9 (2, 3)[b]	97.1	464, 600, 648	462, 600, 648[d]	907	907[f]
6	Zinc-Phthalocyanine (IS)[a]	10.0	5.67	4.5 (IS, 6)[b]	99.6	666	666[e]	579	–[c]
7	Chlorophyll a	11.7	6.80	4.5 (6, 7)[b]	96.4	430, 618, 664	430, 618, 664[d]	893	893[f]
8	Chlorophyll a'	13.4	7.90	19.7 (7, 11)[b]	99.8	434, 620, 664	430, 618, 664[d]	893	893[f]
12	Pheophytin a	19.8	12.20	53.3 (7, 11)[b]	99.9	408, 506, 536, 608, 666	408, 506, 536, 608, 666[d]	871	871[f]

[a] IS = internal standard.
[b] Numbers in parentheses represent values between two neighboring peaks.
[c] "–": Data not available.
[d] Based on a reference by Kamffer et al. [27].
[e] Based on a reference by Zhang et al. [28].
[f] Based on a reference by Huang et al. [13].

separately while 7 concentrations of 0.5, 1, 5, 10, 20, 30 and 50 μg/mL of chlorophyll b and pheophytin b prepared in the same solvent. Then each solution was mixed with internal standard zinc-phthalocyanine with the final concentration being fixed at 5 μg/mL, after which each solution containing internal standard was injected into HPLC twice, and the standard curve was obtained by plotting concentration ratio against its area ratio, with the regression equation and correlation coefficient (r^2) being calculated automatically. With the exception of 15-OH-lactone chlorophyll a, the contents of various chlorophylls and their derivatives were quantified using the following formula:

$$\text{Concentrations of chlorophylls or their derivatives}(\mu g/g) = \frac{[(\text{As}/\text{Ai}) \times a + b] \times \text{Ci} \times V \times \text{dilution factor}/\text{recovery}}{\text{Ws}}$$

where RRF: relative response factor = $(A/\text{Ai})/(C/\text{Ci})$

As: peak area of chlorophylls or their derivatives.
Ai: peak area of IS.
a: slope of calibration curve.
b: intercept of calibration curve.
Ci: concentration of IS.
V: volume of extract.
Ws: weight of sample (g).

Because of unavailability of extinction coefficient, 15-OH-lactone chlorophyll a was quantified based on its area ratio relative to internal standard.

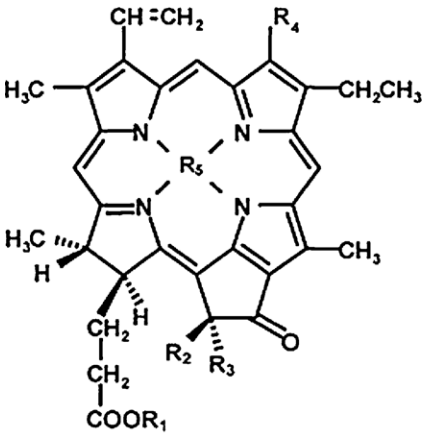
2.2.9. Statistical analysis

Triplicate extractions were carried out for each sample and the data were analyzed statistically using ANOVA and Duncan's multiple range tests for significance in mean difference ($\alpha < 0.05$) based on SAS software system [15].

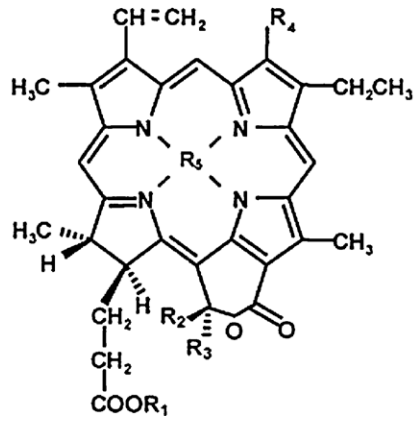
3. Results and discussion

3.1. Development of HPLC method

Many published reports have employed a C18 column for separation of chlorophylls and their derivatives [13,16], however, the separation efficiency can be varied with porosity, carbon load, end capping and degree of polymerization of packing material. Thus, in our study three columns as described in the preceding section were compared. By adopting a gradient mobile phase composed of methanol, acetonitrile and acetone as reported by Huang et al. [13], the retention time was reduced substantially by a Vydac C18 column, but overlapping of several peaks occurred. Similarly, with a HyPURITY C18 column, the separation efficiency was improved, yet several peaks partially overlapped (Figure not shown). Instead, with the Eclipse XDB-C18 column, the resolution was enhanced greatly, but the separation time was slightly longer. Therefore, the Eclipse XDB-C18 column was chosen for subsequent experiments and for improvement of separation efficiency through adjustment of gradient mobile phases based on solvent strength and selectivity. Initially several solvents including water, methanol, acetonitrile, acetone, ethyl acetate, tert-butyl methyl ether (TBME) and N,N-dimethylformamide in different combinations were tested [17–19]. After numerous studies, we found that with DMF as modifier in the mobile phase, the resolution of chlorophylls and their derivatives in *R. nasutus* could be improved pronouncedly, and thereby the most appropriate gradient solvent system containing methanol/DMF (97:3, v/v), acetonitrile (B) and acetone (C) was obtained: 65% A, 30% B and 5% C in the beginning, changed to 60% A, 30% B and 10% C in 8 min, 90% A and 10% C in 13 min, 60% A and 40% C in 15 min,



Compound	R1	R2	R3	R4	R5
Chlorophyll a	Phytol	H	$COOCH_3$	CH_3	Mg
Chlorophyll a'	Phytol	$COOCH_3$	H	CH_3	Mg
Chlorophyll b	Phytol	H	$COOCH_3$	CHO	Mg
Chlorophyll b'	Phytol	$COOCH_3$	H	CHO	Mg
Pheophytin a	Phytol	H	$COOCH_3$	CH_3	2H
Pheophytin b	Phytol	H	$COOCH_3$	CHO	2H
Hydroxychlorophyll a	Phytol	OH	$COOCH_3$	CH_3	Mg
Hydroxychlorophyll b	Phytol	OH	$COOCH_3$	CHO	Mg
Hydroxypheophytin a	Phytol	OH	$COOCH_3$	CH_3	2H
Hydroxypheophytin a'	Phytol	$COOCH_3$	OH	CH_3	2H



Compound	R1	R2	R3	R4	R5
15-OH-lactone chlorophyll a	Phytol	OH	$COOCH_3$	CH_3	Mg

Fig. 2. The chemical structures of chlorophylls and their derivatives.

maintained for 7 min and returned to original ratio in 25 min. A total of 12 chlorophylls and their derivatives plus internal standard zinc-phthalocyanine in hot-air-dried *R. nasutus* were separated within 21 min with detection at 660 nm and flow rate at 1 mL/min (Fig. 1A). Table 1 shows the retention time, retention factor (k), resolution (R) and peak purity of chlorophylls and their derivatives in hot-air-dried *R. nasutus*, which were ranged from 5.8–20.3 min, 2.87–12.53, 1.7–16.3 and 83.5–99.9%, respectively, indicating a proper solvent strength and selectivity of mobile phase to sample components were attained. Following the same approach, a total of 4 chlorophylls and their derivatives, namely, chlorophyll b, chlorophyll a, chlorophyll a′ and pheophytin a were separated in freeze-dried *R. nasutus*, with the retention time, k, R and peak purity ranging from 7.3–19.8 min, 3.87–12.20, 4.5–53.3 and 96.4–99.9%, respectively (Fig. 1B; Table 2). Fig. 2 depicts the chemical structures of chlorophylls and their derivatives.

In several previous studies Wong and Wong [20] developed a ternary mobile phase of (A) methanol/0.5 M ammonium acetate solution (pH 7.2) (80:20, v/v) (B) acetonitrile/water (90:10, v/v) and (C) ethyl acetate to separate 12 carotenoids and chlorophylls in marine phytoplankton within 29 min with flow rate at 1 mL/min and detection at 436 nm by an Eclipse XDB C18 column (250 mm × 4.6 mm I.D., 5 μm particle size). However, the number of chlorophylls derivatives in the separation is limited. Similarly, Bohn and Walczyk [18] employed a ternary solvent system of methanol (A), acetone (B) and DMF (C) to resolve 8 chlorophylls and their derivatives in spinach by a LiChroCART 250-2 C18 column (250 mm × 2 mm I.D., 4 μm particle size) with flow rate at 0.28 mL/min and detection by fluorescence detector. Yet, the number of chlorophylls derivatives in the separation is inadequate and separation time is lengthy. More recently, a total of 15 chlorophylls and their derivatives were separated in *Gynostemma pentaphyllum*, a popular Chinese herb widely consumed in Asian countries within 32 min by a HyPURITY C18 column (150 × 4.6 mm I.D., 5 μm particle size) and a ternary gradient mobile phase of acetone (A), acetonitrile (B) and methanol (C) with flow rate at 1 mL/min and detection at 660 nm [13]. Although the number of chlorophylls derivatives in the separation is increased, the separation time is still lengthy. By comparison, in our experiment the separation time of chlorophylls and their derivatives in *R. nasutus* was shortened to 21 min with an adequate resolution being maintained.

Moreover, zinc-phthalocyanine was found to be an appropriate internal standard and added after extraction for quantitation of chlorophylls and their derivatives. Compared to the internal standard fast green FCF as used by Huang et al. [13], zinc-phthalocyanine should be more accurate in quantitation as the λ_{max} was 626 nm for the former and 660 nm for the latter. Additionally, the separation of various chlorophyll compounds was not interfered after incorporation of zinc-phthalocyanine.

3.2. Quality control data

The reproducibility data of chlorophylls and their derivatives in hot-air-dried and freeze-dried *R. nasutus* are shown in Table 3, with the RSD% of the intra-day variability being ranged from 1.45–5.08% and 1.27–2.44%, respectively, whereas the inter-day variability from 1.95–7.21% and 2.37–3.85%. This outcome demonstrated that a high reproducibility was achieved by using our

Table 3
Reproducibility data and contents of chlorophylls and their derivatives in hot-air-dried and freeze-dried *R. nasutus*.

Peak no.	Chlorophyll	Intra-day variability[a]				Inter-day variability[a]			
		Hot-air-drying		Freeze-drying		Hot-air-drying		Freeze-drying	
		Mean ± SD	RSD (%)[b]	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
		(μg/g)		(μg/g)		(μg/g)		(μg/g)	
1	Hydroxychlorophyll b	108.6 ± 1.58	1.46	ND[c]		108.35 ± 2.45	2.26	ND	
2	Chlorophyll b	324.7 ± 8.83	2.72	1280 ± 17	1.36	322.04 ± 9.84	3.06	1284.12 ± 36.61	2.85
3	Chlorophyll b'	67.08 ± 1.31	1.95	ND		67.08 ± 1.31	1.95	ND	
4	15-OH-lactone chlorophyll a	9.25 ± 0.45	4.89	ND		8.99 ± 0.64	7.21	ND	
5	Hydroxychlorophyll a	206.4 ± 3.44	1.67	ND		205.48 ± 4.22	2.05	ND	
7	Chlorophyll a	814.1 ± 11.82	1.45	4707 ± 59	1.27	813.51 ± 16.01	1.97	4721.83 ± 122.03	2.58
8	Chlorophyll a'	131.2 ± 2.10	1.60	53.47 ± 1.30	2.44	132.20 ± 3.03	2.29	54.72 ± 2.11	3.85
9	Pheophytin b	39.65 ± 2.01	5.08	ND		38.96 ± 1.99	5.11	ND	
10	Hydroxypheophytin a	88.29 ± 2.42	2.74	ND		88.31 ± 2.20	2.49	ND	
11	Hydroxypheophytin a'	69.69 ± 2.70	3.87	ND		69.62 ± 2.40	3.44	ND	
12	Pheophytin a	440.2 ± 7.02	1.59	84.07 ± 1.73	2.06	437.48 ± 9.74	2.23	83.86 ± 1.99	2.37
13	Pheophytin a'	69.68 ± 1.15	1.65	ND		69.97 ± 2.01	2.87	ND	

[a] Mean of triplicate analyses ± standard deviation.
[b] RSD% = (SD/mean) × 100%.
[c] ND: not detected.

developed HPLC method. The detection limits for chlorophyll a, pheophytin a, chlorophyll b and pheophytin b were 0.3, 0.2, 0.3 and 0.2 μg/mL, respectively, while the quantitation limits were 0.9, 0.6, 0.9 and 0.6 μg/mL, with the recoveries being 92.4%, 93.8%, 95.5% and 98.2%, respectively (Table 4). The recoveries were substantially higher than that reported by Huang et al. [13], which were 84.8%, 97.6%, 88.6% and 96.4% for chlorophyll a, pheophytin a, chlorophyll b and pheophytin b in *G. pentaphyllum*, respectively. In some other published reports the recoveries of pheophytin a and pheophytin b in olive oil were 99.56–100.46% and 94.44–105.88%, respectively [21]. Likewise, the recoveries of chlorophyll a and chlorophyll b in grape leaves were 112.9% and 115.1%, respectively, but were 119.8% and 93.9% in grape pulps [19].

The regression equations and correlation coefficients (r^2) are also shown in Table 4, with the latter being all higher than 0.99. As several chlorophyll derivatives such as chlorophyll a′ and hydroxychlorophyll a are not commercially available, they were quantified based on the standard curve of chlorophyll a. Likewise, pheophytin a, pheophytin a′, hydroxypheophytin a and hydroxypheophytin a′ were quantified by using the standard curve of pheophytin a; chlorophyll b, chlorophyll b′ and hydroxychlorophyll b by the standard curve of chlorophyll b, and pheophytin b by the standard curve of pheophytin b. Only 15-OH-lactone chlorophyll was quantified based on the internal standard as mentioned above.

3.3. Chlorophylls and their derivatives in R. nasutus as affected by hot-air-drying and freeze-drying

As shown in Table 1, the hot-air-dried *R. nasutus* was found to contain chlorophyll a, chlorophyll a′, hydroxychlorophyll a, 15-OH-lactone chlorophyll a, chlorophyll b, chlorophyll b′, hydroxychlorophyll b, pheophytin a, pheophytin a′, hydroxypheophytin a, hydroxypheophytin a′ and pheophytin b. However, in dried *G. pentaphyllum*, some more chlorophyll derivatives including hydroxypheophytin b, hydroxypheophytin b′, pheophytin b′ and pyropheophytin a were detected by Huang et al. [13], which should be due to the difference in Chinese herb variety and processing method as the processing of *G. pentaphyllum* involved blanching, rolling and baking. Nonetheless, one more chlorophyll derivative, 15-OH-lactone chlorophyll a was detected in hot-air-dried *R. nasutus* in our experiment, which may be attributed to oxidation of chlorophyll by peroxidase [22]. In another study Roca et al. [23] further pointed out that both hydroxychlorophyll and 15-OH-lactone chlorophyll were oxidation products of chlorophylls, with the latter being generated only under stronger oxidizing condition when compared to the former.

The contents of chlorophylls and their derivatives in hot-air-dried *R. nasutus* are shown in Table 3. Both chlorophyll a and chlorophyll b were the most abundant in freeze-dried *R. nasutus*, which equaled 4707 and 1280 μg/g, respectively, which were much higher than those in hot-air-dried *R. nasutus* by 3892.9 and 955.3 μg/g. This outcome implied that both chlorophyll a and chlorophyll b could undergo a great loss during hot-air-drying because of degradation or conversion to some other derivatives or epimers, including hydroxychlorophyll b (108.6 μg/g), chlorophyll b′ (67.08 μg/g), 15-OH-lactone chlorophyll a (9.25 μg/g), hydroxychlorophyll a (206.4 μg/g), pheophytin b (39.65 μg/g), hydroxypheophytin a (88.29 μg/g), hydroxypheophytin a′ (69.69 μg/g) and pheophytin a′ (69.68 μg/g). Similar results were observed in several previous studies [24–26]. For instance, in a study dealing with the effect of drying temperature on chlorophyll a and chlorophyll b contents in kale and spinach, Lefsrud et al. [24] reported that the highest yields of both chlorophylls a and b occurred at −25 °C (freeze-drying), followed by 0 °C,

Table 4
Quality control data of chlorophyll and pheophytin standards.

Chlorophyll	Calibration curve (Regression equations)	r^2	Test range (ppm)	LOD[a] (ppm)	LOQ[b] (ppm)	Recovery (%) (RSD %)[c]
Chlorophyll a	$y = 0.53x - 0.0213$	0.9983	0.5–50	0.3	0.9	92.4 (1.30%)
Pheophytin a	$y = 0.2662x - 0.0278$	0.9979	0.5–50	0.2	0.6	93.8 (4.32%)
Chlorophyll b	$y = 0.2415x + 0.0088$	0.9996	0.5–100	0.3	0.9	95.5 (3.73%)
Pheophytin b	$y = 0.2112x - 0.0375$	0.9955	0.5–50	0.2	0.6	98.2 (3.34%)

[a] LOD: Limit of detection based on S/N ≥ 3.
[b] LOQ: Limit of quantification based on S/N ≥ 10.
[c] RSD% = (SD/mean) × 100%.

25 °C and 50 °C. Similarly, the contents of chlorophylls in leafy vegetables were higher when dried at 30 ± 2 °C than at 65 ± 5 °C [25]. In another study Gauthier-Jaques et al. [26] pointed out that the major chlorophylls in freeze-dried spinach powder were chlorophyll a, chlorophyll a′ and pheophorbide a. In contrast, both pheophytin a and pyropheophorbide a dominated in conventionally canned bean, revealing the impact of heating on generation of these chlorophyll derivatives. It has been well established that in addition to degradation, chlorophylls can be susceptible to epimerization at C-13 for chlorophyll a′ formation [27], magnesium ion removal for pheophytin production [8], or oxidation for hydroxychlorophyll generation [13], depending on processing condition.

As the total amount of chlorophylls and their derivatives in freeze-dried and hot-air-dried *R. nasutus* were 6125 and 2369 μg/g (Table 3), respectively, this large difference revealed that most chlorophylls were more susceptible to undergoing degradation than conversion to other derivatives during hot-air-drying. Compared to freeze-drying, a greater loss was shown for chlorophyll a (82.7%) than for chlorophyll b (74.6%) during hot-air-drying, impling that the former may be degraded to a higher extent than the latter. This finding is similar to that reported by Teng and Chen [8], demonstrating that the degradation rate of chlorophyll a was faster than chlorophyll b during heating of spinach.

4. Conclusion

In conclusion, an Agilent Eclipse XDB-C18 column and a gradient mobile phase composed of (A) methanol/DMF (97:3, v/v), (B) acetonitrile and (C) acetone were developed to separate 12 chlorophylls and their derivatives in *R. nasutus* plus internal standard zinc-phthalocyanine within 21 min with flow rate at 1 mL/min and detection at 660 nm, with zinc-phthalocyanine being used for quantitation. Both chlorophyll a and chlorophyll b dominated in freeze-dried *R. nasutus*, but chlorophyll a and pheophytin a in hot-air-dried *R. nasutus*. Only 4 chlorophylls and their derivatives were detected in freeze-dried *R. nasutus*. During hot-air-drying, 8 chlorophyll derivatives were generated, including hydroxychlorophyll b, chlorophyll b′, 15-OH-lactone chlorophyll a, hydroxychlorophyll a, pheophytin b, hydroxypheophytin a, hydroxypheophytin a′ and pheophytin a′.

Conflict of interest

There is no conflict of interest to be disclosed by the authors.

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