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Extraction and determination of chlorophylls from moso bamboo (*Phyllostachys pubescens*) culm

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Abstract—The purpose of this study is to establish a simple and reliable method for bamboo chlorophyll extraction. Chlorophylls in moso bamboo (*Phyllostachys pubescens*) epidermis were extracted with acetone, DMF (N,N-dimethylformamide) and DMSO (dimethyl sulfoxide) using three methods, including ultrasonics, centrifugation and grinding. Ultraviolet-visible spectrometry was then used to evaluate the efficiency of these extraction methods. It was also used to quantitatively analyze the extracted chlorophylls. The results revealed that the extraction efficiency of epidermis chlorophylls is related to the size of bamboo culm meal, solvent types, and the method of extraction and filtration. A fast and reliable extraction method was developed. It extracts chlorophylls from bamboo culm meal (<0.7 mm) using an acetone bath in ultrasonics for 3 min and followed by centrifugal filtration. This extraction procedure has been proven to be easy to use and also highly reproducible. Chlorophylls in acetone showed the best stability, followed by DMF and then DMSO. In a dark environment kept at 4 °C, chlorophylls can be preserved for up to 8 days in DMF and 30 days in acetone. On the other hand, acetone extracts higher content of chlorophylls. In 80% acetone, DMF and DMSO extracts, the total chlorophylls contents are 4.80, 4.18, and 3.78 mg per gram of epidermis meal, respectively.

Key words: Bamboo; chlorophyll; ultrasonics; UV spectrometry; quantitative analysis.

INTRODUCTION

Bamboo, a perennial lignified plant in the *Bambusoideae* family, is an important forest resource and grows more rapidly than any other woody plants on earth [1]. There are many genera of bamboos cultivated in Taiwan. Among them, moso bamboo (*Phyllostachys pubescens* Mazel) is one of the most popular and valuable species because of its rapid growth rate, excellent flexibility, and easy machinability. It is widely used as an industrial material in pulping, furniture, and construction.

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Bamboos also exhibit a fascinating greenish skin, thanks to the abundant chlorophylls in the epidermis. After drying, aging, or some manufacturing processes, however, the chlorophyll is easily degraded and the greenish color fades resulting in loss of the attractive appearance and reduction of the economic value. To overcome this problem, we successfully developed treatments that protect the green color of bamboo culm as described in our previous papers [2–6]. However, the mechanism of why these treatment methods work has never been discussed. To establish how these treatments work, it is necessary to have an accurate and efficient method to extract and measure chlorophyll from the epidermis of bamboo.

Several solvents, such as acetone, N,N-dimethylformamide (DMF), and dimethyl sulfoxide (DMSO), have been used to extract chlorophyll from plant tissues as described in a number of studies [7–11]. However, extraction of epidermis chlorophyll of bamboo culm has never been mentioned thoroughly in any literature. In practice, it is not easy to extract chlorophyll from bamboo. There are many capes of silica cells in the cuticular layer of bamboo culm. These capes are rich in SiO_2 that strengthen the outer epidermis of bamboo and prevent external invasions [12]. As a result, it is very difficult to obtain a complete chlorophyll extraction using a conventional grinding method in preliminary work because the bamboo epidermis is very tough. In our previous short, rapid communication that applied ultrasonics in chlorophyll extraction from bamboo culm, it was revealed that ultrasonics might be a feasible method to assist bamboo chlorophyll extraction [13]. In the present study, detailed evaluation of applying ultrasonics in the chlorophyll extraction of bamboo culm is conducted. The results are also compared to the traditional methods in terms of extraction efficiency. Finally, the stability of pigment extracts in different solvents was investigated, and chlorophyll *a* and chlorophyll *b* were also quantified.

MATERIALS AND METHODS

Materials

Samples of three-year-old moso bamboo (*Phyllostachys pubescens* Mazel) were obtained from the Experimental Forest of National Taiwan University in Nan-Tou County. To study the effects of sample particle size on the extraction of chlorophyll, the green epidermis of moso bamboo culm was ground to powder using a Wig-L-Bug grinder (Cresent Co., USA). The samples were divided into three groups based on particle diameters: <0.7, $0.7-3.0$, and >3.0 mm, respectively.

Chlorophyll extraction of bamboo culm

The procedures for extracting chlorophyll from bamboo culm are as follows. Add 40 mg of green bamboo epidermis powder to a closed sample vial containing 25 ml of solvent (acetone, DMF, or DMSO). Perform the extraction using one of the three methods: grind the sample in a cold mortar for 3 min, use an ultrasonic oscillator

(Ney 104X, USA; power 355 watts; output frequency 43–47 kHz — an ultrasonic oscillator is the same as an ultrasonic bath) for 3 min, or apply centrifugation at 8000 × g for 10 or 30 min, respectively. To clarify the chlorophyll solution, the extracted bamboo powders were filtered using a glass filter (G4) or centrifugation. The final volume of each filtrate (or supernatant) was then adjusted to 25 ml by adding the same solvent as used in the extraction before spectral analysis.

Determination of chlorophyll content

The absorption spectrum of chlorophyll was recorded over the range between 350 and 750 nm at a scan speed of 400 nm/min using an ultraviolet-visible (UV-VIS) spectrophotometer (Jasco V-550, Japan). Each experiment was performed in triplicate and the chlorophyll content was then calculated using the specific equations by Barnes *et al*. [11] for acetone and DMSO respectively, and by Inskeep and Bloom [10] for DMF.

Stability of extracts

The chlorophyll extracts were stored in a dark environment under ambient condition or at 4 ◦C. To evaluate the stability of chlorophyll extracts, spectroscopic characteristics of the extracts were monitored using a UV-VIS spectrophotometer (Jasco V-550, Japan).

Color measurement

The color of bamboo epidermis powder was measured using a color and color difference meter (Dr. Lange Co., Germany) based on a D_{65} light source. The tristimulus values X, Y, and Z of all specimens were obtained directly from the colorimeter. From these data, the CIE LAB color parameters L^* ($L^* = 116(Y/100)^{1/3} - 16$), a^* ($a^* = 500[(X/94.81)^{1/3} - (Y/100)^{1/3}]$), and b^* ($b^* = 200[(Y/100)^{1/3} - (Z/107.34)^{1/3}]$) were calculated as established by the Commission Internationale de Enluminure (CIE) in 1976 [4]. Accordingly, the L^*, a^* and b^* values are color coordinates on the white-black, red-green, and blue-yellow axes, respectively. Positive a^* values represent red color, whereas negative a^* values represent green color.

RESULTS AND DISCUSSION

Effects of extraction methods on the extraction of chlorophylls a and b from bamboo culm

Traditional methods of chlorophyll extraction involve grinding the plant tissue in water-miscible solvents, followed by centrifugation to remove solid plant material [14, 15]. These procedures became noticeably time-consuming when the number of samples is large. In this study we tried to extract the chlorophylls of bamboo

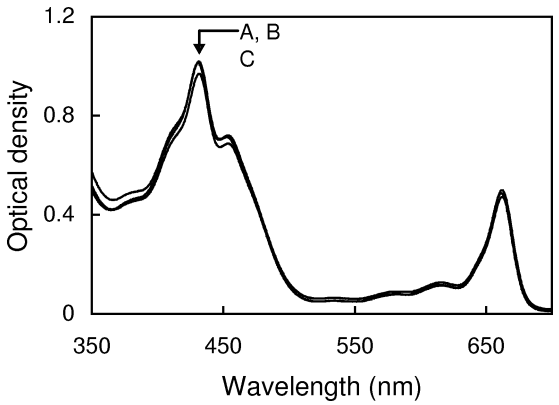

Figure 1. UV-VIS absorption spectra of chlorophylls of moso bamboo culm obtained by using ultrasonics for 3 min (A); centrifugation at 8000 × *g* for 10 min (B); and grinding (C).

culm by either ultrasonics or centrifugation and avoided the conventional grinding method. Figure 1 shows the UV-VIS absorption spectra of chlorophyll extracted from bamboo epidermis powders (particle diameter <0.7 mm) in acetone by ultrasonics (3 min), centrifugation (30 min), and grinding (3 min) (used as a reference). The characteristic absorption peaks for chlorophyll *a* (662 and 431 nm) and chlorophyll *b* (642 and 452 nm) are readily recognized in those derivative spectra (not shown). Optical density (OD) comparison of the absorption peaks at 662 and 431 nm as shown in Fig. 1 revealed that the OD values of those peaks of ultrasonics method ($OD_{662} = 0.504$; $OD_{431} = 1.027$) were almost equal to that of the centrifugation method ($OD_{662} = 0.499$; $OD_{431} = 1.019$). In contrast, the traditional grinding method had the smallest OD among the three extraction methods. The OD_{662} and OD_{431} values of extracts were only 0.448 and 0.933, respectively when grinding was used.

On the other hand, the CIE LAB color parameter a^* value has been used as the main physical parameter for characterizing the extent of the green color degradation in peas [16]. Therefore, the extraction efficiency of chlorophyll can be easily evaluated by looking at the a^* value. As shown in Table 1, post-ultrasonic extraction L^*, a^*, and b^* values of bamboo epidermis meal were changed from 48.8, −7.4, and 23.1 initially to 72.3, 0.4, and 24.2, respectively. This result indicated that the green pigments in bamboo epidermis, including chlorophyll, could be effectively extracted using ultrasonics.

Comparison of the extraction efficiency for acetone, DMSO, and DMF

Since the discovery of chlorophylls by Pelletier and Caventou in 1818, several methods have been developed to extract chlorophylls. In general, a good extraction procedure should bring all chloroplast into the solution with little or no alteration to the chlorophyll [17]. To investigate the extraction efficiency of various organic solvents, three commonly used solvents — acetone, DMF and DMSO — were se-

Table 1.
Changes in the color parameters of bamboo epidermis meal of moso bamboo after chlorophyll extraction in acetone by ultrasonics for 3 min

Specimens	CIE LAB		
	L^*	a^*	b^*
Fresh epidermis meal	48.8 ± 0.5	-7.4 ± 0.5	23.1 ± 0.6
Extracted epidermis meal	72.3 ± 0.5	0.4 ± 0.3	24.2 ± 0.2

Notes: The results are mean $\pm$ SD ($n = 3$).

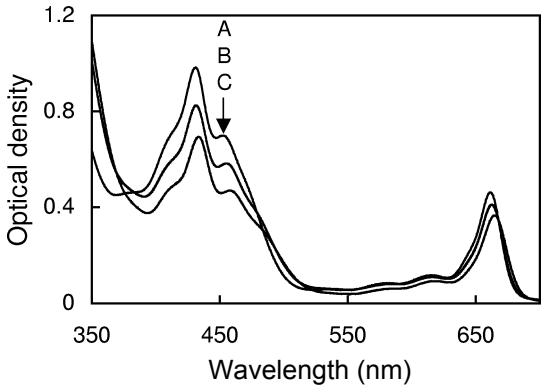

Figure 2. UV-VIS absorption spectra of chlorophylls of moso bamboo culm extracted by ultrasonics in acetone (A), DMF (B), and DMSO (C).

lected to extract epidermis chlorophyll from bamboo culm by ultrasonics. Figure 2 shows the UV-VIS absorption spectra of epidermis pigments in those solvents. At around 662 nm, the characteristic absorption peak for chlorophyll *a*, the OD values of extracts in the three solvents, in decreasing order, are as follows: acetone (0.462) > DMF (0.411) > DMSO (0.366). Similarly, the OD at around 452 nm, the characteristic absorption peak for chlorophyll *b*, showed the same pattern as with OD_{662}. The OD_{452} values of chlorophyll extracts in acetone, DMF, and DMSO are 0.699, 0.583, and 0.470, respectively. According to the equations by Barnes *et al*. [11] as well as Inskeep and Bloom [10], the total chlorophyll contents calculated are 4.76, 4.24, and 3.84 mg/g for acetone, DMF, and DMSO, respectively. With this evidence, it was obvious that the content of epidermis chlorophyll extracted with acetone is higher than that using the other two solvents. Furthermore, when compared with the absorption position of extracts in acetone, a bathochromic shift was observed at the absorption peaks for both chlorophylls *a* and *b* in DMF or DMSO. The wavelength-shift-difference ($\Delta\lambda$) values of the characteristic absorption peaks for chlorophylls *a* (red λ_{max}, blue λ_{max}) and *b* (red λ_{max}, blue λ_{max}) in DMF were +1, +1 nm and +1, +4 nm, respectively. As for the DMSO extract, the bathochromic shift was more than that of DMF, the $\Delta\lambda$ values of chlorophylls *a*

(red λ_{max}, blue λ_{max}) and *b* (red λ_{max}, blue λ_{max}) were +3, +3 nm and +2, +6 nm, respectively.

Effects of particle size of bamboo epidermis and filtration methods on the extraction of chlorophylls

Figure 3 shows the UV-VIS absorption spectra of chlorophyll extracted from a variety of particle sizes of bamboo epidermis in acetone with ultrasonics. The results showed that the extraction efficiency of chlorophyll is a function of the sample particle size. Among the three particle sizes of bamboo epidermis, the acetone extract of the finest sample (particle diameter < 0.7 mm) had the highest OD for both chlorophylls *a* and *b*. It was followed by the mid-sized sample (0.7−3.0 mm) and then the coarse sample (>3.0 mm). To find out whether the low OD in the coarse sample results from incomplete extraction, the residue of extracted coarse bamboo epidermis was ground again using a Wig-L-Bug grinder to fine meal epidermis (particle diameter less than 0.7 mm). After extracting this sample with ultrasonics for the second time, the UV-VIS absorption spectrum of the acetone extract still exhibited significant characteristic peaks for chlorophyll, as shown in Fig. 3D. The OD values at 662 and 431 nm were 0.072 and 0.145, respectively. This result demonstrated that the residue of extracted coarse bamboo epidermis still retained a portion of the original chlorophyll in it. Incomplete extraction of bamboo epidermis with particle sizes greater than 3.0 mm is thus confirmed.

On the other hand, it is known that, after extraction, the filtration method is another factor that affects the extraction efficiency of chlorophyll. In addition to conventional filtration using a glass filter, centrifugation was also employed to remove solid epidermis material from the filtrate. After ultrasonic extraction, the acetone extracts of fine bamboo epidermis were centrifuged at $8000 \times g$ for 10 min.

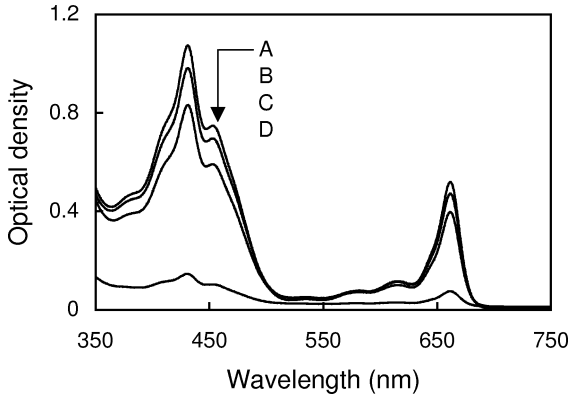

Figure 3. UV-VIS absorption spectra of chlorophylls extracted from different sizes of bamboo epidermis in acetone by ultrasonics. (A) particle diameter < 0.7 mm; (B) particle diameter between 0.7 and 3.0 mm; (C) particle diameter > 3.0 mm; (D) extracted coarse bamboo epidermis was ground again to fine meal epidermis (particle diameter < 0.7 mm).

Table 2.
The optical density (OD) of chlorophylls *a* and *b* extracted by ultrasonics with different filtration manners

Filtration manners	Chlorophyll *a*		Chlorophyll *b*	
	OD_{662}	OD_{431}	OD_{642}	OD_{452}
Glass filter (G4)	0.507	1.053	0.213	0.748
Centrifugation (8000 × *g*, 10 min)	0.512	1.054	0.207	0.740
Centrifugation alone[a]	0.504	1.041	0.191	0.720

[a] The acetone extract was obtained directly from centrifugation at 8000 × *g* for 30 min, i.e. no extraction by ultrasonics before centrifuging.

The ODs of the supernatant for chlorophylls *a* and *b*, as shown in Table 2, were 0.512 (OD_{662}), 1.054 (OD_{431}), 0.207 (OD_{642}), and 0.740 (OD_{452}), respectively. The ODs of filtrate obtained using a glass filter were also measured and found to have similar values to that when a centrifuge was used. In general, most of the experimental errors are human errors. Simplifying the treatment process steps should decrease the error. The 'ultrasonics plus centrifugation' procedure had the least process steps, because both extraction and filtration were finished in the same sample vial, a centrifugal tube. This combination is therefore superior to that using 'ultrasonics plus glass filter filtration'. In the third method, in which the sample solution was centrifuged at 8000 × *g* for 30 min without extraction by ultrasonics first, the ODs of the supernatant for chlorophylls *a* and *b* were 0.504 (OD_{662}), 1.041 (OD_{431}), 0.191 (OD_{642}), and 0.720 (OD_{452}), respectively. This result showed that centrifugation alone also had produced good chlorophyll extraction. However, it is about 3-fold more time-consuming than that of the other two ultrasonics methods.

Stabilities of acetone, DMSO, and DMF extracts

It has been well known that many plants store organic acids in their vacuoles or contain a high proportion of phenolic acid derivatives. These substances are acidic enough to cause chlorophyll degradation to pheophytin during pigment extraction [11, 18]. Furthermore, numerous studies have reported that light, oxygen [19–21], and enzymes [22] also play important roles that cause chlorophyll degradation, both *in vitro* and *in vivo*. To understand the stabilities of acetone, DMSO, and DMF extracts under the ambient conditions and 4 °C dark environment, the absorption spectra were monitored as a function of time. It was observed that, for all solvents, pigment extracts changed rarely within 3 h under the ambient conditions (not shown). However, for long-term storage, the extracts prepared in acetone were considerably more stable than those in DMF or DMSO under the same conditions (Fig. 4). After storing for 192 h, the acetone extract (Fig. 4A′) retained more than 50% of the original chlorophylls in it. In contrast, under the same conditions, the characteristic chlorophyll absorption peaks of both DMF (Fig. 4B′) and DMSO (Fig. 4C′) extracts almost disappeared. Those OD values

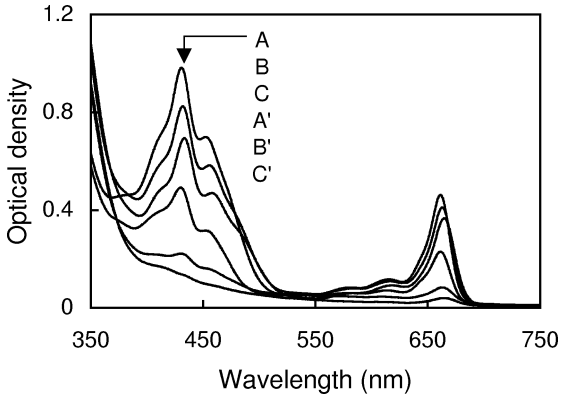

Figure 4. Comparison of the absorption spectra of chlorophylls before (A to C) and after (A′ to C′) storing for 192 h under the ambient conditions in acetone (A and A′), DMF (B and B′), and DMSO (C and C′).

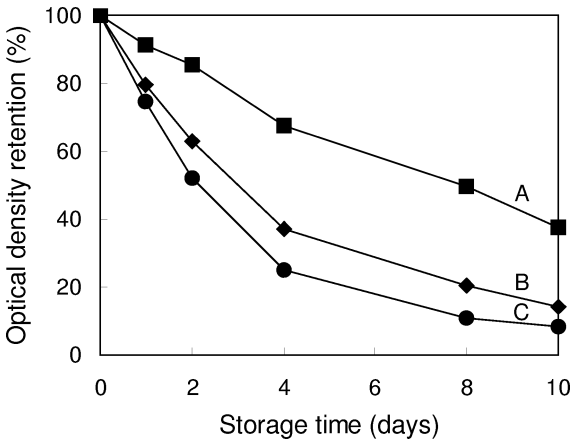

Figure 5. The optical density retention (red λ_{max}) of acetone (A), DMF (B), and DMSO (C) extracts as a function of storage time under the ambient conditions.

of red λ_{max} changed from 0.462 (DMF) and 0.411 (DMSO) down to 0.084 and 0.040, respectively. Figure 5 shows a comparison of chlorophyll retention of these three extracts during storage and it clearly reveals that the chlorophyll degradation in acetone extract is the mildest, followed by DMF and DMSO.

However, the stability of chlorophyll solution can be significantly improved if the extract is stored at 4°C in the dark. The DMF and acetone extracts could be stored in this condition for up to 8 and 30 days, respectively, with no significant changes in the optical density at 662 nm (data not shown here).

Determination of chlorophylls a and b in bamboo epidermis

There is a vast array of solvents used for the extraction and determination of the chlorophylls, but most of them require grinding and centrifuging with or without

Table 3.
Chlorophyll content of moso bamboo culm extracted by ultrasonics using different solvents

Solvents	Chlorophyll *a* (mg/g)	Chlorophyll *b* (mg/g)	Total chlorophylls (mg/g)
80% Acetone	3.23 ± 0.06	1.56 ± 0.15	4.80 ± 0.21
DMF	2.81 ± 0.05	1.38 ± 0.01	4.18 ± 0.06
DMSO	2.86 ± 0.12	0.92 ± 0.06	3.78 ± 0.08

Notes: Each experiment was performed in triplicate, and the results are mean $\pm$ SD.

heating [23]. The ultrasonics method is simple, efficient, and reliable for bamboo chlorophyll extraction, as described in the foregoing section. Therefore, to determine the content of chlorophylls *a* and *b* in moso bamboo epidermis, the ultrasonic extraction method was employed and the chlorophyll content was calculated following the equations used by Inskeep and Bloom [10] and Barnes *et al*. [11]. Table 3 shows the chlorophyll contents of moso bamboo epidermis extracted by ultrasonics using 80% acetone, DMF, and DMSO. The results demonstrated that, in acetone, the contents of chlorophylls *a*, *b*, and total chlorophylls were 3.23, 1.56, and 4.80 mg/g, respectively. In contrast, in DMF and DMSO, both chlorophylls contents were less than that in acetone. The total chlorophylls contents were only 4.18 and 3.78 mg/g for DMF and DMSO extracts, respectively.

CONCLUSIONS

Ultrasonics is a simple, efficient, and reliable method for chlorophyll extraction from the epidermis of bamboo culm. The contents of chlorophylls *a* and *b* extracted with 80% acetone were 3.23 and 1.56 mg per gram of epidermis meal, respectively. This is accomplished by applying ultrasonics for 3 min and subsequently centrifuging at $8000 \times g$ for 10 min. In addition, the extract can be cold stored (4°C) in a dark environment for up to 30 days with no significant change in the optical density at 662 nm. This effective chlorophyll extraction method has thus set the foundation for future studies to understand the roles of chlorophylls on the green color protection of bamboo culm.

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