Spectrophotometric determination of chlorophylls in different solvents related to the leaf traits of the main tree species in Northeast China

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Abstract. The accurate detection of the leaf chlorophyll (*Chl*) is of substantial importance for the immediate assessment of forest conditions to manage and conserve forest ecosystems. We compared 80% acetone, 95% ethanol, and dimethyl sulfoxide (DMSO) over a range of incubation times (2, 4, 6, 8, 18, 26, and 32 h) to determine the *Chl* contents of 12 tree species in northeast China. The results showed that to obtain the maximum *Chl* (*a*+*b*) contents for most tree species extracted by 80% acetone and 95% ethanol required a minimum of 18 h, while the incubation periods by DMSO were 2-6 h and 18-32 h to extract 90% of the *Chl* from the broadleaved and coniferous tree species, respectively. We observed that the amount of *Chl* extracted with DMSO was significantly higher than that extraction with 80% acetone and 95% ethanol, particularly for conifer species with the exception of *Phellodendron amurense*, *Fraxinus mandshurica*, and *Tilia amurensis*, in which the maximum amount of *Chl* was extracted with acetone. The DMSO extracted *Chl* in exhibited the lowest degree of variation among the three solvents. The leaf mass area (LMA), leaf thickness, and diameter of the primary leaf vein were significantly negatively correlated with the *Chl a*, *Chl b*, and *Chl* (*a*+*b*) content for the 12 tree species. There were non-significant different slopes or intercepts between the curves for LMA and *Chl a*, *Chl b*, or *Chl* (*a*+*b*) at the different incubation times for the same solvent or the different solvents at the certain incubation time (P>0.05).

1. Introduction

Chlorophyll (*Chl*) is one of the most fundamental and important physiological parameters in forest ecology. The accurate measurement of the *Chl* content is of substantial significance for the management and protection of forest ecosystem function. The traditional process for the determination of the foliar *Chl* content (*Chl a*, *Chl b*, and *Chl* (*a*+*b*)), which are the most widely distributed two forms of *Chl* that occur naturally in the trees, has been measured by the extraction of leaf tissue obtained with acetone, methanol, ethanol, or dimethyl sulfoxide (DMSO), followed by spectrophotometric measurements. Researchers have found that solvents can vary in their ability to extract *Chl* from different plants. It is practical to determine the most effective solvent for a particular set of samples.

Acetone has been the most widely used solvent for extracting *Chl* from a variety of plant tissues at room temperature or 4 ºC for its substantial benefit of providing very sharp *Chl* absorption peaks. The drawbacks of using acetone are that the sample preparation is laborious, and the extraction is time consuming, which raises questions about loss of *Chl*, *Chl* stability, and *Chl* may not be completely extracted from many vascular plants even after physical treatments. This lack of complete extraction is a particular problem for cold acetone. Jinasena *et al*. (2016) showed that the *Chl* extraction from the leaves of *Alternanthera sessilis* using 80% acetone improved with the increase in temperature up to 50 ºC, and the *Chl* extraction at 60 ºC was slightly lower than that at 50 ºC, but the *Chl* contents remained stable through the extraction time from 1 to 5 h [5]. At the same time, hot ethanol (60-80 ºC) is often a more efficient solvent to extract *Chl* in organisms that are recalcitrant for the extraction process [8, 16]. Additionally, ethanol has relatively non-toxic, practical, and economic advantages, thus making it a more desirable solvent for *Chl* extraction in the laboratory [12, 13, 16].

DMSO has been widely used for *Chl* extraction from higher plants [1, 4, 29], lichens [1, 18] and mosses, and these methods have also been optimized for specific applications [20], since Shoaf & Lium (1976) reported that DMSO was superior to acetone in the *Chl* extraction from a wide range of algal species [22]. This procedure is relatively quick and does not require grinding or centrifugation of the sample, and a large number of samples may be quickly prepared and analyzed.

The *Chl* extraction efficiency of the terrestrial plants was compared using different solvents [1, 4, 25]. However, most of the materials were limited in herbs or crops, and the woody plants were less involved. In addition, the DMSO method, originally applied to the determination of the *Chl* content in algae, was successfully applied to gymnosperms and angiosperms [4], and then was widely used in different plants [1, 18, 23, 29]. The application of hot ethanol, acetone, and DMSO to terrestrial woody plants to compare the extraction efficiencies of the *Chl* content for 11 tree species was only attempted by Minocha *et al*. (2009) [9]. Since *Chl* extraction methods may provide variable results, studies using different solvents should be compared cautiously unless the specific methods have been calibrated.

The extraction efficiency of *Chl* is closely related to the type of organic solvent [2, 12] due to the differences in the leaf tissue [21, 25] and anatomic characteristics [10]. For example, Shinano *et al*. (1996) found that the different morphological and anatomical characteristics of graminoid leaf vascular were among the factors that affected the extraction efficiency using DMSO at 65 ºC that was incubated for at least 1 h [21]. Tait & Hik (2003) also certified that the *Chl* extraction from the thick, highly cutinized leaves of the graminoid *C. citrates* using DMSO was generally lower than that 80% acetone [25]. Nikolopoulos *et al*. (2008) observed that the specific leaf mass, leaf density, and leaf thickness did not strongly affect the extraction efficiency of DMSO for 19 species with different anatomical characteristics [10]. However, heterobaric leaves, which are characterized by the occurrence of bundle sheath extensions in the mesophyll, showed lower extraction efficiency in DMSO compared with homobaric leaves and conifer needles, while the extraction efficiencies of the latter in DMSO were comparable to those obtained using the acetone extraction. Bundle sheath extensions within heterobaric leaves behave as anatomical barriers that prevent the diffusion of DMSO, even after prolonged incubation with the solvent.

In this study, we compared the foliar DMSO extraction efficiency to determine the extraction capabilities of *Chl a*, *Chl b*, and *Chl* (*a*+*b*) with 80% acetone and 95% ethanol under the same conditions to determine the optimal extraction time for the whole leaf tissue pigments extraction in a variety of tree species in northeast China. We hypothesized that the foliar *Chl* extraction efficiency without grinding the plant material, a procedural step normally required during acetone extraction, in different solvents would be influenced by changes in the extraction periods. Particularly, we expected that hot acetone would be unsuccessful at measuring the leaf *Chl* for long incubation periods. A second objective of our study was to investigate the extraction efficiency using DMSO, acetone, and ethanol versus specific leaf traits parameters to identify which character is responsible for the low solvent efficiency in the different tree species.

2. Materials and methods

2.1. Site description and plant material

The experiment was conducted at the Demonstration Base of Urban Forestry in the northeast Forestry University, Harbin, Heilongjiang Province, northeast China (45°43′N, 126°37′E). The demonstration base area is 43.95 hm^2. The regional climate is described as a temperate monsoon, which is characterized by warm summers, cold winters, a short growing season, and abundant precipitation with the annual average temperature and annual precipitation of 3.5 ºC and 569.1 mm primarily occurring from June to September, respectively. The base was farmland before 1949, and its original vegetation was valley meadow steppe. We investigated the main tree species in northeast China—*Betula platyphylla* (*Bp*), *Tilia amurensis* (*Ta*), *Quercus mongolica* (*Qm*), *Ulmus davidiana* var. *japonica* (*Ud*), *Acer mono* (*Am*), *Phellodendron amurense* (*Pa*), *Fraxinus mandshurica* (*Fm*), *Juglans mandshurica* (*Jm*), *Pinus koraiensis* (*Pks*), *Picea koraiensis* (*Pkn*), *Larix gmelinii* (*Lg*), and *Pinus sylvestris* var. *mongolica* (*Ps*) with a range of species whose leaf tissues were quite different.

2.2. Chlorophyll extraction

For each species, six fully developed, outermost healthy fresh green leaves from the top third of the south-oriented crown per tree of three sample trees were randomly chosen to measure the *Chl* contents at approximately 9 a.m. on sunny days. The leaves were placed in labeled plastic bags in coolers with ice and immediately transported to the laboratory for *Chl* extraction.

We determined the *Chl* based on the same procedures and conditions for sampling, pigments extraction, and measured by the same spectrophotometer to reduce the potential of a large amount of error into the results (Linder, 1974). Briefly, the leaf area discs for broadleaves and needles were cut into pieces approximately 2 mm in length, and the fresh mass (FM) of the leaf was determined by an analytical balance (Sartorius BT224S, Sartorius Scientific Instruments Co., Ltd., China). Six replicates of each species were placed in 10 ml 80% acetone, 95% ethanol, and DMSO, which were incubated in a water bath maintained at 65 ºC for 32 h in the dark. The absorbance of the solution was measured at 664 nm and 647 nm for 80% acetone, 664 nm and 649 nm for 95% ethanol, 665 nm and 649 nm for DMSO for *Chl a* and *Chl b* at 2, 4, 6, 8, 18, 26, and 32 h for the broadleaved species, and at 4, 8, 18, 26, 32 h for the conifer trees by a UV-visible spectrophotometer (WFJ-2100, INESA Analytical Instrument Co. LTD., Shanghai, China). The *Chl* contents ($mg \cdot g^{-1}$) were determined by the specific published equations by applying the absorbance values to the equations reported by Lichtenthaler (1987) [6] for the acetone and ethanol, and Wellburn (1994) [29] for the DMSO. *Chl* (*a*+*b*) was calculated as the sum of *Chl a* and *Chl b*. All the procedures were performed under diffused light to eliminate the exposure of the leaf materials to direct, bright or sun light.

2.3. Leaf traits measurement

An additional 30 leaf samples per three trees of each species were collected to determine the leaf traits, including the leaf thickness (LT), primary leaf vein diameter (LVDa), leaf mass area (LMA) and leaf water content (LWC). Calipers were placed on a leaf at a representative point of the midrib, closed until the calipers had securely grasped the leaf, and the calipers were slowly opened until the leaf would slide out when gently pulled. This distance was considered to be the leaf thickness. The fresh mass of each leaf in which the petioles were cut was determined by an analytical balance, and the leaves were then scanned (Model T210, Founder Technology Instrument Co. Ltd., Beijing, China) to obtain high-resolution images to measure the leaf area, and leaf vein diameter using Image J software (NIH, Bethesda, MD, USA). Finally, the leaf samples were dried at 85 ° C for at least 26 h, and the dry mass was recorded. The LMA was calculated as the ratio of the leaf dry mass to the leaf area. The LWC was calculated as the ratio of the difference between the fresh mass and the dry mass to the fresh mass.

2.4. Statistical analysis

We analyzed the species, solvents, and temporal effects on the Chl contents using repeated measures ANOVA. The species was treated as a fixed factor; the extraction time was treated as the fixed repeated factor, and the individual tree was treated as a random factor. The mean *Chl* content among all the leaves within an individual tree and measurement period was used in the analysis (*i.e.*, $n = 3$). The treatment means were compared using Fisher's Least Significant Difference test to determine the extraction time and solvent. The ratio of the maximum to minimum was used to describe the variation of the *Chl* content with the extraction time. All the analyses were performed using a mixed model procedure (PROC MIXED) of SAS Version 9.3 (SAS, Inc., Cary, NC, USA) with $\alpha = 0.05$.

We explored the relationships between the *Chl a*, *Chl b*, and *Chl* (*a*+*b*) contents and leaf traits through correlation procedures and fit the relationships between the *Chls* contents and LMA through regression analysis using the curve-fitting procedure and chose the highest R^2 of SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). The ordinary least squares regression techniques were performed to test the incubation time and different solvents on the LMA versus the *Chls* relationships [27].

3. Results

The *Chl a*, *Chl b*, and *Chl* (*a*+*b*) extracted by 80% acetone increased with the extraction extension, reaching the highest values on 18, 26, and 32 h, and there were no significant differences in the three time periods ($P>0.05$) with the exception that *Chl a*, *Chl b*, and *Chl* (*a*+*b*) for *Lg*, *Ud* and *Am* peaked at 4 and 6 h with a pattern for the former of a concave curve, while the latter was a single peak curve (Figure 1). The *Chl a*, *Chl b*, and *Chl* (*a*+*b*) for *Fm* increased sharply at 18, 26, and 32 h, and the ratio of the maximum to minimum was as high as 4. The extreme value ratio of the three indices for the rest of species were 1.1- 2.2 with the exception of the *Chl b* of *Ta*.

The *Chl a*, *Chl b*, and *Chl* (*a*+*b*) extracted by 95% ethanol also increased with the extraction extension. The highest values occurred at 18, 26, and 32 h, and most of them were non-significant differences during the above time periods ($P>0.05$) with the exception that *Chl a*, *Chl b*, and *Chl* (*a*+*b*) for *Pa* and *Jm* peaked at 2 and 4 h and then decreased slightly (Figure 1). The extreme value ratio of *Chl a*, *Chl b*, and *Chl* (*a*+*b*) for *Ps*, *Pkn* and *Pks* were the highest, followed by *Fm*, and the rest of the species were the lowest. The values were 2.5-3.6, 1.6-2.5, and 1.0-1.5, respectively.

The *Chl a*, *Chl b* and *Chl* (*a*+*b*) extracted by DMSO for *Ps*, *Pkn* and *Pks* increased with the extraction time and reached the highest at 26, 32 and 18 h, respectively, which was significantly higher than those of the other time periods ($P<0.05$) (Figure 1). The extreme value ratios were 1.3-1.9. The *Chl a* and *Chl b* values for the other nine species decreased or increased with the extension time, and the amplitude varied between species. For example, the *Chl a* contents for *Jm* and *Fm* were the highest at 2-8 h ($P>0.05$). The extreme value ratios were 1.2 and 1.6. However, the *Chl b* readings were the highest at 18-26-32 h, and the extreme value ratios were 1.6 and 3.3. The extreme value ratios of *Chl a* and *Chl b* for the remaining seven tree species were in the range of 1.1-1.4. The extreme value ratios of *Chl* (*a*+*b*) for the eight tree species were in the range of 1.0-1.1 with the exception of *Fm*.

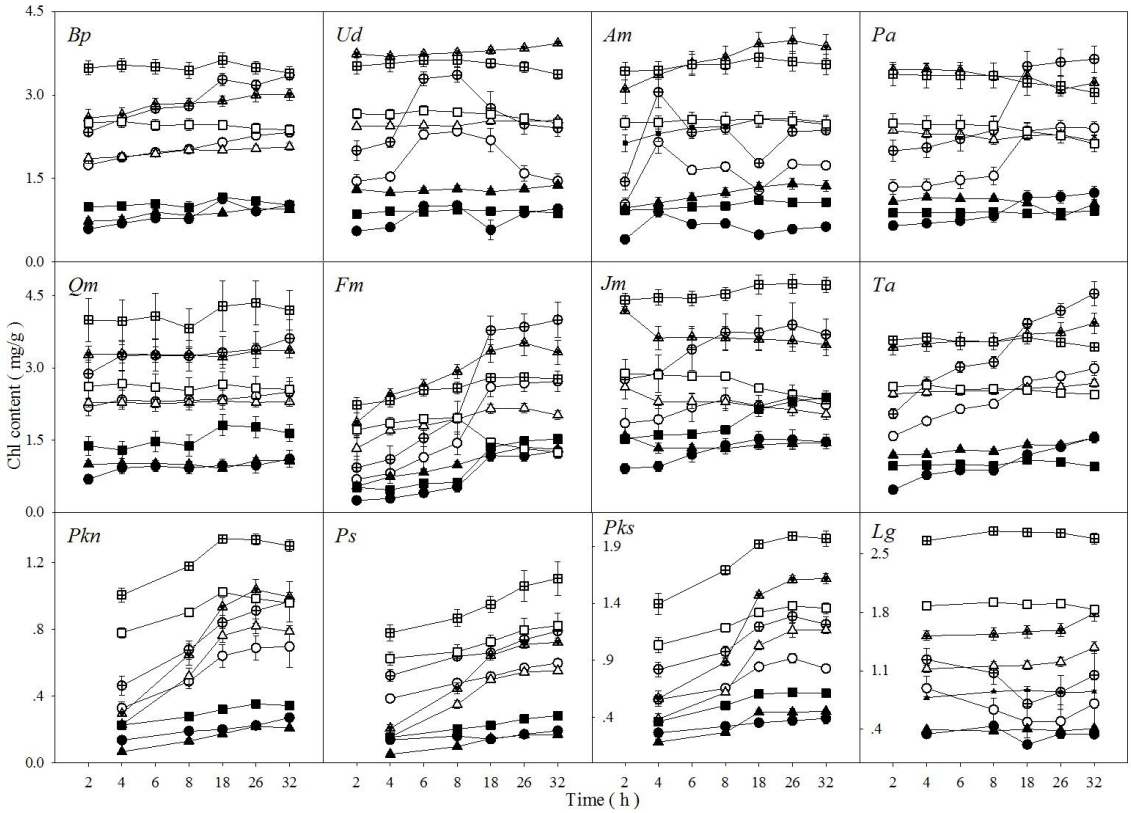

Figure 1. Diagrams relating mean ± SE chlorophylls to the extraction time for 80% acetone (open circle, *Chl a*; filled circle, *Chl b*; half-filled circle, *Chl* (*a*+*b*)), 95% ethanol (open triangle, *Chl a*; filled triangle, *Chl b*; half-filled triangle, *Chl* (*a*+*b*)), and DMSO (open square, *Chl a*; filled square, *Chl b*; half-filled square, *Chl* (*a*+*b*)) for 12 tree species.

DMSO-extracted *Chl* (*a*+*b*) for the coniferous tree species were significantly higher than those of 80% acetone and 95% ethanol ($P<0.05$) during the same period. The DMSO extraction of *Chl* (*a*+*b*) for *Ps*, *Pkn*, *Pks* and *Lg* was 1.4-1.7, 1.3-2.2, and 2.2-3.9 fold greater than that of 80% acetone, respectively. The *Chl* (*a*+*b*) extracted by 95% ethanol for *Lg* were 1.4-2.2 fold greater than that of 80% acetone ($P<0.05$) whereas the *Chl* (*a*+*b*) extracted by 95% ethanol for *Ps*, *Pkn* and *Pks* was lower than that of 80% acetone. Specifically, the former was 0.4-0.7 times that of the latter from 2 to 8 h ($P<0.05$).

The *Chl* (*a*+*b*) extraction from the broad-leaved trees could be divided into three groups. First, DMSO extracted the highest *Chl* content from *Bp*, *Jm*, and *Qm*. The extraction amount of DMSO was 1.1-1.6-fold that of 95% ethanol and 80% acetone, respectively. The ratio between 95% ethanol and 80% acetone was 0.9-1.1. Second, the extraction efficiency of *Chl* (*a*+*b*) by DMSO and 95% ethanol for *Ud* and *Am* was similar and was 1.1-2.4 fold that of 80% acetone. Third, the extraction of *Chl* (*a*+*b*) by DMSO and 95% ethanol for *Pa*, *Fm* and *Ta* was similar at 2-8 h and was 1.3-2.4 fold that of the 80% acetone. However, at 18-32 h, the extraction amount from high to low was 80% acetone, 95% ethanol and DMSO. The extraction amount with the 80% acetone was 1.1-1.4 fold that of DMSO.

Table 1. Correlation coefficients between the *Chl* content extracted by 80% acetone, 95% ethanol or DMSO and the leaf traits including leaf mass area (LMA), leaf water content (LWC), leaf thickness (LT), and primary leaf vein diameter (LVDa) for 12 tree species. ** $P<0.01$, * $P<0.05$, – not significant.

Time (h)	Leaf traits	*Chl a*			*Chl b*			*Chl* (*a*+*b*)		
		80%	95%	DMSO	80%	95%	DMSO	80%	95%	DMSO

4	LMA	-0.78**	-0.86**	-0.83**	-0.74**	-0.81**	-0.70*	-0.77**	-0.85**	-0.80**
	LWC	–	0.64*	0.59*	–	–	–	–	0.62*	–
	LT	-0.85**	-0.91**	-0.91**	-0.81**	-0.85**	-0.78**	-0.84**	-0.89**	-0.88**
	LVDa	-0.82**	-0.91**	-0.85**	-0.77**	-0.85**	-0.66*	-0.80**	-0.89**	-0.80**
8	LMA	-0.78**	-0.86**	-0.82**	-0.70*	-0.82**	-0.67*	-0.76**	-0.85**	-0.78**
	LWC	–	0.66*	–	–	0.60*	–	–	0.64*	–
	LT	-0.82**	-0.91**	-0.90**	-0.75**	-0.85**	-0.76**	-0.81**	-0.90**	-0.87**
	LVDa	-0.86**	-0.93**	-0.84**	-0.70*	-0.88**	-0.62*	-0.82**	-0.92**	-0.78**
18	LMA	-0.73**	-0.80**	-0.74**	-0.69*	-0.77**	-0.65*	-0.73**	-0.80**	-0.76**
	LWC	0.59*	0.61*	–	–	0.59*	–	0.58*	0.61*	–
	LT	-0.72**	-0.87**	-0.83**	-0.69*	-0.81**	-0.73**	-0.73**	-0.85**	-0.86**
	LVDa	-0.82**	-0.91**	-0.79**	-0.71**	-0.85**	-0.61*	-0.80**	-0.90**	-0.77**
26	LMA	-0.75**	-0.78**	-0.71**	-0.73**	-0.75**	-0.62*	-0.75**	-0.78**	-0.75**
	LWC	0.61*	0.59*	–	0.59*	0.59*	–	0.61*	0.60*	–
	LT	-0.75**	-0.85**	-0.81**	-0.73**	-0.80**	-0.69*	-0.75**	-0.84**	-0.85**
	LVDa	-0.84**	-0.91**	-0.75**	-0.75**	-0.84**	–	-0.82**	-0.89**	-0.75**
32	LMA	-0.77**	-0.78**	-0.70**	-0.74**	-0.78**	-0.60*	-0.77**	-0.79**	-0.75**
	LWC	0.67*	0.60*	–	0.61*	0.61*	–	0.66*	0.61*	–
	LT	-0.76**	-0.85**	-0.80**	-0.73**	-0.81**	-0.66*	-0.76**	-0.85**	-0.84**
	LVDa	-0.84**	-0.89**	-0.74**	-0.78**	-0.86**	–	-0.83**	-0.89**	-0.74**

The *Chl a*, *Chl b*, and *Chl* (*a*+*b*) extracted by 80% acetone, 95% ethanol and DMSO over a range of incubation times for the 12 tree species were significantly negatively correlated with the LMA, LT, and LVDa and mostly non-significantly correlated with the LWC (Table 1). Since the LMA, LT, and LVDa were significantly positively correlated with each other, we explored the relationships between the *Chl a*, *Chl b*, and *Chl* (*a*+*b*) with LMA through regression analyses. The power equations described the relationship between *Chl* content and LMA marginally better than the rest of the models (Table 2). There were non-significantly different slopes or intercepts between the different incubation times for the same solvent ($P>0.05$) and between the different solvents at the certain incubation time ($P>0.05$) with the exception that the intercepts for *Chl a* extracted by 95% ethanol were significantly higher than those by 80% acetone and DMSO at incubation times of 4 h and 8 h ($P<0.05$).

Table 2. The best equations between LMA and the *Chl a*, *Chl b*, and *Chl* (*a*+*b*) extracted by 80% acetone, 95% ethanol and DMSO for 12 tree species.

Solvent	*Chl*	Incubation time (h)				
		4	8	18	26	32
80%	*Chl a*	$y=45.338x^{-0.84}$ $R^2=0.75$	$y=41.48x^{-0.79}$ $R^2=0.76$	$y=39.97x^{-0.76}$ $R^2=0.63$	$y=43.25x^{-0.77}$ $R^2=0.69$	$y=43.96x^{-0.77}$ $R^2=0.78$
	Chl b	$y=17.29x^{-0.83}$ $R^2=0.69$	$y=15.82x^{-0.76}$ $R^2=0.65$	$y=32.74x^{-0.92}$ $R^2=0.63$	$y=27.92x^{-0.86}$ $R^2=0.69$	$y=28.17x^{-0.84}$ $R^2=0.69$
	Chl (*a*+*b*)	$y=63.34x^{-0.84}$ $R^2=0.73$	$y=57.04x^{-0.78}$ $R^2=0.73$	$y=68.39x^{-0.80}$ $R^2=0.64$	$y=69.29x^{-0.79}$ $R^2=0.71$	$y=71.48x^{-0.79}$ $R^2=0.76$
95%	*Chl a*	$y=224.119x^{-1.19}$ $R^2=0.74$	$y=69.19x^{-0.88}$ $R^2=0.79$	$y=28.06x^{-0.65}$ $R^2=0.70$	$y=22.56x^{-0.59}$ $R^2=0.67$	$y=20.94x^{-0.58}$ $R^2=0.66$
	Chl b	$y=206.76x^{-1.37}$ $R^2=0.72$	$y=93.87x^{-1.15}$ $R^2=0.75$	$y=43.76x^{-0.94}$ $R^2=0.69$	$y=35.20x^{-0.88}$ $R^2=0.68$	$y=41.95x^{-0.92}$ $R^2=0.70$
	Chl (*a*+*b*)	$y=411.99x^{-1.25}$ $R^2=0.74$	$y=136.93x^{-0.96}$ $R^2=0.78$	$y=58.37x^{-0.73}$ $R^2=0.71$	$y=47.31x^{-0.68}$ $R^2=0.68$	$y=48.04x^{-0.68}$ $R^2=0.69$
DMSO	*Chl a*	$y=28.84x^{-0.63}$ $R^2=0.72$	$y=21.27x^{-0.55}$ $R^2=0.67$	$y=14.14x^{-0.46}$ $R^2=0.57$	$y=11.92x^{-0.43}$ $R^2=0.54$	$y=11.03x^{-0.42}$ $R^2=0.53$
	Chl b	$y=19.31x^{-0.77}$ $R^2=0.58$	$y=12.56x^{-0.65}$ $R^2=0.55$	$y=18.09x^{-0.69}$ $R^2=0.56$	$y=15.36x^{-0.65}$ $R^2=0.56$	$y=14.32x^{-0.64}$ $R^2=0.55$
	Chl (*a*+*b*)	$y=46.62x^{-0.67}$ $R^2=0.68$	$y=33.32x^{-0.58}$ $R^2=0.64$	$y=29.55x^{-0.54}$ $R^2=0.62$	$y=25.65x^{-0.51}$ $R^2=0.63$	$y=24.08x^{-0.50}$ $R^2=0.63$

4. Discussion

The Chl extraction efficiency by the solvents differed depending on the plant materials. The *Chl* (*a*+*b*) extracted by DMSO was higher [1, 9, 20], lower [9, 25] or similar [1, 4, 25] compared with 80%

acetone and 95% ethanol. A comparison between ethanol and acetone also indicated differences between the species [9, 19]. For example, Minocha *et al*. (2009) [9] found the *Chl* extracted by DMSO was the highest for five conifer tree species, but the data for six broadleaved species were different. The *Chl* (*a*+*b*) extracted by 95% ethanol for *Fagus grandifolia* and DMSO for *Q. velutina* was the highest. For *Prunus serotina* and *Liriodendron tulipifera*, the *Chl* (*a*+*b*) extracted using 95% ethanol and DMSO was similar and significantly higher than that extracted by 80% acetone. There was no significant difference between the extractions of *B. alleghaniensis* and *Tsuga canadensis* using 80% acetone, 95% ethanol and DMSO. Our results showed DMSO was a better solvent for the *Chl* extraction with the exception of the highest extraction of *Chl* of *Pa*, *Fm*, and *Ta* obtained with 80% acetone, and these results supported the concept that DMSO extracted the conifer species efficiently as indicated by the results of Minocha *et al*. (2009) [9] and Barnes *et al*. (1992) [1].

The extraction time of *Chl* is based on the diffusivity of solvents within the particular intact plant tissue. The solvent extraction times varied from 15 min to 7 h [4, 11, 21, 28], and 26 h [25] for DMSO. At 65 ºC, *Chl* (*a*+*b*) was extracted from the leaves of *Trifolium subterraneum* with DMSO, and over 99% of the *Chl* was extracted at 1 h [20]. However, the *Chl* (*a*+*b*) at 7, 26, and 48 h for *P. virginiana*, *Helianthus annuus*, *Fragaria vesca*, *Andropogon gerardii*, and *Cymbopogon citrates* were similar [25]. With the increase in the extraction time from 4, 6, 8, 26, and 48 h, the *Chl a* and *Chl b* contents extracted by DMSO at 25, 40, 60, and 80 ºC for the leaves of *C. unshiu* Marc. cv. Okitsu increased with the exception that *Chl a* extracted by 80 ºC at 48 h decreased slightly [3]. The *Chl* content for *A. sessilis* was very stable with the protracted extraction extension using hot acetone [5]. The *Chl* extraction was performed with 95% ethanol at 70 ºC for 30 min for the birch, beech, ash, and sycamore. Our study showed that the *Chl* contents extracted by 80% acetone and 95% ethanol for most of the tree species increased with the prolonged extraction time and reached the highest value at least for 18 h. The *Chl* content extracted by DMSO for the thicker conifer leaves of *Pks*, *Pkn*, and *Ps* was the highest from 18 to 32 h. More than 90% of the *Chl* was extracted at 2-6 h for the rest of the nine tree species, although the *Chl a* for *Jm* and the *Chl b* for *Fm* decreased and increased with the extraction extension, and the *Chl* (*a*+*b*) remained stable.

The particular sample tissues are incubated in solvents determined by the leaf thickness, degree of cutinization [1, 10, 21, 22] because mechanical disruption of the cells does not take place. As Hiscox & Israelstam (1979) [4] and Barnes *et al*. (1992) [1] delineated, the *Chls* extraction from the leaf tissues with DMSO requires incubation for various times, depending on the degree of cutinization and thickness of the leaf. Nikolopoulos *et al*. (2008) made the first attempt to determine the influence of the leaf anatomy on the extraction efficiency of DMSO for 19 plant species [10]. They observed that the linear correlation between each specific anatomical parameter and the extraction efficiency of the DMSO was poor (R^2 = 0.35 for SLA, R^2 = 0.44 for leaf density and R^2 = 0.28 for LT). Our study showed that the LMA, LT and LVDa were significantly negatively correlated with the extraction efficiency of the solvents but not the LWC in most cases. The result supported the hypothesis that the extraction time of *Chl* is based on the diffusivity of the solvents within the particular intact plant tissues depending on the leaf thickness and degree of cutinization.

The temperature used in the *Chl* extraction with solvents differed depending on the references. 80% acetone and 95%-98% ethanol were often used at 4 ºC or room temperature to extract the *Chl* for at least 26, 48, or 72 h, resulting in poor pigment stability or incomplete extraction, which could be solved by heating the solvents [14]. In the range of 8 to 30 ºC, the temperature had little effect on the *Chl* extraction by 80% acetone [24]. 80% acetone at 60 ºC and 65 ºC was used to extract *Chl* from the leaves of *A. sessilis* [5] and walnut [30], resulting in slightly lower values of *Chl* (*a*+*b*) than the highest ones obtained at 50 ºC. 95%-98% ethanol at 65 ºC [9], 70 ºC [8], and 80 ºC [16] was used to extract *Chl* from the leaves. The DMSO extracted the *Chl* was primarily at 65 ºC [11, 15, 28] and also at 70 ºC [8, 26]. The *Chl* extraction by DMSO at 40 ºC was not complete for the thick, highly cutinized leaves of *C. citrates* [25] and fern species [1], and 65 ºC was required for complete extraction. The *Chl* (*a*+*b*) extraction of the *Citrus unshiu* cv. Okitsu leaves by DMSO at 60 ºC was similar with the highest value at 80 ºC [3]. Minocha *et al*. (2009) also certified that heating solvents at 65 ºC for acetone,

ethanol, and DMSO did not alter the *Chl* stability for the 11 tree species [9]. Therefore, the selection of 65 ºC as the optimum temperature for the *Chl* extraction is feasible.

Prolonged heating may result in a lower *Chl* value due to the destruction of *Chl*. It was reported that *Chl a* was less thermally stable than *Chl b*. Scott & Robson (1991) found that the *Chls* were undisturbed by additional incubation for 2 h, but an extraction time of 3 h or longer would destroy the extracted *Chl a*, resulting in a decrease in *Chl a* and a slight increase in *Chl b* under the conditions of the extract (65 °C) in DMSO [20]. However, Hiscox & Israelstam (1979) suggested extraction times as long as 6 h for *Chl* from pine needles [4]. Barnes *et al*. (1992) also clearly demonstrated that the period of incubation in warm DMSO resulted in a lack of significant degradation of *Chl a* or *Chl b* [1]. Jinasena *et al*. (2016) also showed that the *Chl* content for *A. sessilis* was very stable with prolonged extraction using hot acetone, and there was no *Chl* degradation for a long period of time while heating [5].

The *Chl* absorption wavelength and the various calculation formulas used would also lead to different results for the same solvent. For example, the readings were 646 and 663 nm [26] and 649 and 665 nm [25] for the *Chl* extracted by DMSO, and the *Chl* content was calculated based on the formula of Wellburn (1994) [29]. Some researchers believed that the DMSO absorption spectrum of *Chl a* and *Chl b* were the same as that in 90% acetone [4, 18, 22] and suggested determining the value of 645 and 663 nm and using the classical Arnon formula to calculate the *Chl* content [15, 20, 28]. It has been noted that there is a significant error in the calculations of the *Chl* extracted by DMSO based on the formula described above [1, 13] because the Arnon formula is 80% instead of 90% acetone. Furthermore, Barnes *et al*. (1992) found that the *Chl* content extracted by DMSO was underestimated by approximately 10% using the Arnon formula [1]. Parry *et al*. (2014) also found that the *Chl* content extracted by DMSO from 22 types of plants calculated by the acetone formula (absorption wavelength 646.6, 663.6 nm) was underestimated by 7.84% compared with the DMSO formula (absorption wavelength 649.1, 665.1 nm) [11]. Therefore, the wavelengths measurement and the corresponding formula should be strictly followed whether using acetone, ethanol, or DMSO [6].

5. Conclusions

Solvents play a major role in the process of extracting *Chl*. The spectrophotometric absorbance properties of the *Chl* molecules facilitate their qualitative and quantitative analysis using different solvents, and the contribution of these solvents to the extraction in various species was compared. Furthermore, suitable solvents related to the leaf traits on *Chl* were selected. Our results clearly indicated that the *Chl* extraction by DMSO, 80% acetone and 95% ethanol are dependent on the leaf morphological characteristics, such as the thickness, LMA and degree of cutinization. This study revealed that DMSO was the most effective solvent to extract the most significant amount of *Chl* for most of the species sampled.

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