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. Jinasa 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 hm². 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 °C 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 (Pk), 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 °C 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·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 α = 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 80% | Chl b 80% | Chl \((a+b)\) 80% | DMSO 80% | Chl a 95% | Chl b 95% | Chl \((a+b)\) 95% | DMSO 95% |
|----------|-------------|-----------|-----------|-----------------|---------|-----------|-----------|-----------------|---------|
| 2        |             |           |           |                 |         |           |           |                 |         |
| 4        |             |           |           |                 |         |           |           |                 |         |
| 6        |             |           |           |                 |         |           |           |                 |         |
| 8        |             |           |           |                 |         |           |           |                 |         |
| 10       |             |           |           |                 |         |           |           |                 |         |
| 12       |             |           |           |                 |         |           |           |                 |         |
| 14       |             |           |           |                 |         |           |           |                 |         |
| 16       |             |           |           |                 |         |           |           |                 |         |
| 18       |             |           |           |                 |         |           |           |                 |         |
| 20       |             |           |           |                 |         |           |           |                 |         |
| 22       |             |           |           |                 |         |           |           |                 |         |
| 24       |             |           |           |                 |         |           |           |                 |         |
| 26       |             |           |           |                 |         |           |           |                 |         |
| 28       |             |           |           |                 |         |           |           |                 |         |
| 30       |             |           |           |                 |         |           |           |                 |         |
| 32       |             |           |           |                 |         |           |           |                 |         |

Table 1.
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 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  | 4  | 8  | 18 | 26 | 32 |
|---------|------|----|----|----|----|----|
| 80%     | Chl a| y=45.338±0.84 | y=41.48±0.79 | y=39.97±0.76 | y=43.25±0.77 | y=43.96±0.77 |
|         | R²=0.75 | R²=0.76 | R²=0.63 | R²=0.69 | R²=0.78 |
|         | Chl b| y=17.29±0.83 | y=15.82±0.76 | y=32.74±0.92 | y=27.92±0.86 | y=28.17±0.84 |
|         | R²=0.69 | R²=0.65 | R²=0.63 | R²=0.69 | R²=0.69 |
|         | Chl (a+b)| y=63.34±0.84 | y=57.04±0.78 | y=68.39±0.80 | y=69.29±0.79 | y=71.48±0.79 |
|         | R²=0.73 | R²=0.73 | R²=0.64 | R²=0.71 | R²=0.76 |
| 95%     | Chl a| y=224.119±1.59 | y=69.19±0.88 | y=28.06±0.65 | y=22.56±0.59 | y=20.94±0.58 |
|         | R²=0.74 | R²=0.79 | R²=0.70 | R²=0.67 | R²=0.66 |
|         | Chl b| y=206.76±1.37 | y=93.87±1.15 | y=43.76±0.94 | y=35.20±0.88 | y=41.95±0.92 |
|         | R²=0.72 | R²=0.75 | R²=0.69 | R²=0.68 | R²=0.70 |
|         | Chl (a+b)| y=411.99±1.25 | y=136.93±0.96 | y=58.37±0.73 | y=47.31±0.68 | y=48.04±0.68 |
|         | R²=0.74 | R²=0.78 | R²=0.71 | R²=0.68 | R²=0.69 |
| DMSO    | Chl a| y=28.84±0.61 | y=21.27±0.55 | y=14.14±0.46 | y=11.92±0.43 | y=11.03±0.42 |
|         | R²=0.72 | R²=0.67 | R²=0.57 | R²=0.54 | R²=0.53 |
|         | Chl b| y=19.31±0.77 | y=12.56±0.65 | y=18.09±0.69 | y=15.36±0.65 | y=14.32±0.64 |
|         | R²=0.58 | R²=0.55 | R²=0.56 | R²=0.56 | R²=0.55 |
|         | Chl (a+b)| y=46.62±0.67 | y=33.32±0.58 | y=29.55±0.54 | y=25.65±0.51 | y=24.08±0.50 |
|         | R²=0.68 | R²=0.64 | R²=0.62 | R²=0.63 | R²=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 tulipfera, 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 °C, 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 °C for the leaves of C. unshiu Marc. cv. Okitsu increased with the exception that Chl a extracted by 80 °C at 48 h decreased slightly [3]. The Chl content for A. sessilis was very stable with the prolonged extraction extension using hot acetone [5]. The Chl extraction was performed with 95% ethanol at 70 °C 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 Fs 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 °C 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 °C, the temperature had little effect on the Chl extraction by 80% acetone [24]. 80% acetone at 60 °C and 65 °C 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 °C. 95%-98% ethanol at 65 °C [9], 70 °C [8], and 80 °C [16] was used to extract Chl from the leaves. The DMSO extracted the Chl was primarily at 65 °C [11, 15, 28] and also at 70 °C [8, 26]. The Chl extraction by DMSO at 40 °C was not complete for the thick, highly cutinized leaves of C. citrates [25] and fern species [1], and 65 °C was required for complete extraction. The Chl (a+b) extraction of the Citrus unshiu cv. Okitsu leaves by DMSO at 60 °C was similar with the highest value at 80 °C [3]. Minocha et al. (2009) also certified that heating solvents at 65 °C for acetone,
ethanol, and DMSO did not alter the Chl stability for the 11 tree species [9]. Therefore, the selection of 65 °C 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
Solvants 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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