Development of a standardized methodology for quantifying total chlorophyll and carotenoids from foliage of hardwood and conifer tree species

Rakesh Minocha, Gabriela Martinez, Benjamin Lyons, and Stephanie Long

Abstract: Despite the availability of several protocols for the extraction of chlorophylls and carotenoids from foliage of forest trees, information regarding their respective extraction efficiencies is scarce. We compared the efficiencies of acetone, ethanol, dimethyl sulfoxide (DMSO), and N, N-dimethylformamide (DMF) over a range of incubation times for the extraction of chlorophylls and carotenoids using small amounts of unmacerated tissue. Of the 11 species studied, comparable amounts of chlorophyll were extracted by all four solvents from three species and by ethanol and DMF from nine species. In four species, acetone, ethanol, and DMF extracted comparable chlorophyll amounts, while in another two species comparable amounts were extracted by ethanol, DMSO, and DMF. In one species, ethanol extracted significantly greater amounts of chlorophyll compared with all other solvents. The brown coloration of DMSO extracts for some species compromised the calculations of chlorophylls and carotenoids, making DMSO a poor choice. Overall, extraction efficiencies of ethanol and DMF were comparable for analyzing chlorophyll concentrations. However, because DMF is more toxic than ethanol, we recommend ethanol as the better option of these two for chlorophyll extractions. On the other hand, DMF is the most efficient solvent among the four tested for the extraction of carotenoids from these species. The results presented will facilitate the design of multispecies local- and regional-scale ecological studies to evaluate forest health. Additionally, they will enable reliable comparisons of results from multiple laboratories and (or) studies that used different solvents and help validate chlorophyll estimates obtained by remote sensing.

Résumé : Bien qu'il existe plusieurs protocoles pour extraire la chlorophylle et les caroténoïdes du feuillage des arbres forestiers, les informations concernant leur efficacité relative sont rares. Nous avons comparé l'efficacité de l'acétone, de l'éthanol, du diméthylsulfoxyde (DMSO) et du N, N-diméthylformamide (DMF) en utilisant différentes périodes d'incubation pour l'extraction de la chlorophylle et des caroténoïdes à partir de tissus non macérés. Parmi les 11 espèces étudiées, des quantités comparables de chlorophylle ont été extraites avec les quatre solvants chez trois espèces et avec l'éthanol et le DMF chez neuf espèces. Chez quatre espèces, l'acétone, l'éthanol et le DMF ont extrait des quantités comparables de chlorophylle tandis que chez deux autres espèces, c'était l'éthanol, le DMSO et le DMF. Chez une espèce, l'éthanol a extrait des quantités significativement plus élevées de chlorophylle que les autres solvants. Chez certaines espèces le DMSO n'est pas la meilleure option parce que la coloration brune des extraits compromet l'exactitude du calcul des quantités de chlorophylle et de caroténoïdes. Entre deux options comparables pour l'extraction de la chlorophylle chez la plupart des espèces, nous recommandons l'éthanol plutôt que le DMF à cause de la toxicité de ce dernier. Cependant, le DMF est le seul solvant qui peut efficacement extraire les caroténoïdes chez ces espèces. Les données présentées dans cette étude vont faciliter la conception des études écologiques qui comportent plusieurs espèces pour évaluer l'état de santé de la forêt. De plus, elles vont permettre de faire des comparaisons fiables entre les résultats provenant de plusieurs laboratoires ou études qui utilisent différents solvants et aider à valider les estimations de la chlorophylle obtenues par télédétection.

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Introduction

Chlorophylls and carotenoids play a critical role in the process of photosynthesis. Traditionally, changes in their levels in foliage have been used to evaluate photosynthetic activity, and changes in ratios of chlorophyll *a* to chlorophyll *b* have been used as an indicator of abiotic stress in plants (Larcher 1995). The measurement of leaf pigmentation is an important parameter for ecophysiologists because it provides an indirect measure of leaf nitrogen (since chlorophyll contains nitrogen in its structure) and, in turn, nutrient status (Richardson et al. 2002 and references therein).

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More recently, evaluating the relative contribution of both leaf-level chemistry (including chlorophyll) and forest canopy structure over a range of climate and forest types has become necessary for the calculation of total carbon uptake. In addition, chlorophyll and nitrogen measurements are being used as key drivers of photosynthetic capacity in most current gross primary productivity models (Smith et al. 2002; Ollinger and Smith 2005). With the increasing significance of the role of chlorophyll in forest health and the growing concern for the effects of climate change on forest structure, it is prudent to develop a standardized methodology that is capable of complete extraction of whole tissues (without maceration) using the least toxic and most stable solvent that can work equally well across species and environmental conditions. This standardized method would be useful in validating multispecies data gathered simultaneously as part of large-scale ecological studies using various noninvasive methods such as hand-held chlorophyll meters or remote sensing.

The extraction of plant leaf pigments is routinely done using a wide assortment of extraction techniques that vary in the type of solvent used and the duration of extraction. There are several organic solvents that are commonly used to extract chlorophylls and carotenoids from plant tissues, including acetone, methanol, ethanol, dimethyl sulfoxide (DMSO), N, N-dimethylformamide (DMF), and chloroform, each with its own optimal extraction time period. Many studies have compared the photopigment extraction efficiencies of these solvents for organisms ranging from phytoplankton to higher plants (Hiscox and Israelstam 1979; Bowles et al. 1985; Barnes et al. 1992; Tait and Hik 2003; Dunn et al. 2004). Yet, with a few exceptions, most of these studies involved either phytoplankton or nonwoody plants. Also in several studies tissue macerations were required for extractions with acetone.

In the present study we compared efficiencies of four different solvents for extraction of chlorophylls and carotenoids from the foliage of several forest tree species. The study was undertaken to (1) establish optimum extraction times for maximum photopigment extraction by each solvent, and (2) evaluate and compare these solvents for each species to develop a standardized extraction regime across species. This information could potentially be used in designing large-scale ecological studies with multiple tree species where photosynthetic pigments could be extracted using one common method for assessing forest health. A standard method would facilitate reliable comparisons of results from multiple laboratories and (or) studies.

Materials and methods

Plant material

Foliar tissue was collected from the mid to upper canopy in visually healthy mature trees of five conifer species (family Pinaceae): balsam fir (*Abies balsamea* Mill.), Norway spruce (*Picea abies* Karst.), red pine (*Pinus resinosa* Ait.), red spruce (*Picea rubens* Sarg.), and eastern hemlock (*Tsuga canadensis* (L.) Carrière); and six hardwood species: American beech (*Fagus grandifolia* Ehrh.) and black oak (*Quercus velutina* Lam.), family Fagaceae; black cherry (*Prunus serotina* Ehrh.), family Rosaceae; sugar maple (*Acer saccharum* Marsh.), family Aceraceae; yellow birch (*Betula alleghaniensis* Britt.), family Betulaceae; and tulip poplar (*Liriodendron tulipifera* L.), family Magnoliaceae. All trees were sampled in July or August between 2002 and 2004 from our various research sites across northern New England and West Virginia: balsam fir at Macwahoc plantation, Maine; Norway spruce and American beech at Durham, New Hampshire; red pine and black oak at Harvard Forest, Petersham, Massachusetts; red spruce and eastern hemlock at Howland, Maine; sugar maple and yellow birch at Hubbard Brook Experimental Forest, North Woodstock, New Hampshire; black cherry and tulip poplar at Monongahela National Forest, West Virginia. Two to four leaves of hardwoods or branchlets of conifers were pooled from each tree. Needles were chopped into 3–4 mm segments using scissors, and for leaves a paper punch was used to collect 6.35 mm diameter disks, avoiding the major veins. A subsample of well-mixed, chopped or punched tissue from each tree was placed in microfuge tubes in the field, transported to the laboratory on ice in the dark, and stored at –20 °C until analysis. Before use in these experiments, tissues from 5–12 trees of the same species were pooled and mixed thoroughly. Only current-year (CY) needle samples were collected for all conifers except Norway spruce where both current-year and second (past)-year (PY) needles were collected.

Sample preparation and extraction

Three leaf disks (2–15 mg depending on species) for hardwoods and 15 mg of chopped needles for conifers were taken from thawed pooled tissue collections of each species and placed in 2 mL microfuge tubes (Eppendorf Safe Lock, Eppendorf North America, Westbury, New York). One and a half millilitres of each of the four solvents (95% ethanol (EtOH), 80% acetone (Mallinckrodt Baker Inc., Phillipsburg, New Jersey) buffered with 2.5 mmol/L sodium phosphate at pH 7.8, DMSO (Sigma-Aldrich, St. Louis, Missouri), DMF (Sigma-Aldrich, St. Louis, Missouri) were added to three replicate tubes for each combination of species, solvent, time period. Samples were incubated in the dark, in a water bath at 65 °C for 2, 4, 6, 8, 16, 24, and 30 h for hardwoods and 4, 8, 16, and 24 h for conifers, for all solvents except DMF. Samples extracted with DMF were incubated at room temperature (25 °C) in the dark for 24, 48, 72, and 96 h for both hardwoods and conifers because DMF cannot be heated safely at 65 °C owing to its low flash point.

Heated samples were removed from the water bath, allowed to come to room temperature, vortexed at slow speed for 1 min, and centrifuged for 5 min at 13 500 *g*. Using 0.7 mL aliquots in quartz microcuvettes (Quartz Suprasil, Hellma Cells Inc., Plainview, New York), we recorded absorbances in the range of 350 to 710 nm with a Hitachi U-2010 spectrophotometer (Hitachi Ltd., Tokyo, Japan; spectral bandwidth 2 nm, wavelength accuracy of +0.3 nm, wavelength setting reproducibility of ±0.1 nm; with Hitachi UV Solutions software version 2.0 program). For each species, the extraction procedure was repeated two or three times using three replicates per treatment, with a few exceptions noted for carotenoids in the figure legends. For calculations of total chlorophyll, chlorophyll *a*, chlorophyll *b*, and carotenoids for the four solvents, appropriate equations

(Table 1) from published papers were used: Lichtenthaler (1987) for 95% ethanol and buffered acetone, Wellburn (1994) for 100% DMSO, and Porra et al. (1989) for 100% DMF.

Statistical analyses

For each species, data for each solvent were first analyzed as a series of one-way analysis of variance (ANOVA) to determine whether statistically significant differences existed among times of extraction for the solvent. Then, the data for each solvent for the incubation times that were statistically similar for extracting maximum amounts were pooled. These pooled data were then used for solvent comparisons using ANOVA.

When F values were significant, differences in treatments were tested with Tukey's multiple comparisons tests using Systat for Windows version 10.2 (SYSTAT Inc., Evanston, Illinois), and a p value ≤ 0.05 was used unless otherwise specified.

Results

Extractions in acetone, DMSO, and ethanol were carried out at 65 °C using incubation times between 2 and 30 h. However, incubations with DMF were carried out at room temperature and generally for much longer time periods (24–96 h) compared with the other three solvents. Figures 1 and 2 represent data from incubation times tested irrespective of their statistical differences. Time ranges reported in tables refer to the window of time in which each solvent extracted statistically similar maximum amounts of the targeted pigment. The stability of sugar maple and red spruce extracts in all four solvents was verified after 2 weeks of storage at –20 °C. The tested extracts were stable in all four solvents except for sugar maple extracts in DMSO. In this case chlorophyll *b* content decreased by approximately 40%.

Chlorophylls

Hardwoods

Although the time ranges for the maximum extraction of total chlorophyll with acetone, DMSO, and ethanol varied among species, all six shared a common extraction period of 4–16 h (Table 2). The only exception was American beech, for which the only suitable common time among the three solvents for maximal yield was 16 h incubation (Table 2). For DMF, similar results were obtained for incubation periods between 24 and 96 h for all hardwood species except for American beech, which required a minimum of 72–96 h for maximum chlorophyll extraction (Table 2).

Chlorophyll was extracted by all four solvents from the six hardwood species examined (Table 2). However, the efficiency of extraction varied among the four solvents and also among species (Figs. 1*a*–1*f*). All four solvents extracted statistically similar amounts of total chlorophyll from yellow birch foliage. DMSO, DMF, and ethanol extracted statistically similar amounts from both black cherry and tulip poplar. Statistically, no one solvent consistently extracted significantly higher amounts of total chlorophyll from all six species (Table 2).

In American beech, ethanol extracted significantly higher

Table 1. Equations used with each of the four solvents to calculate chlorophyll and total carotenoid concentrations.

Solvent	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll	Total carotenoids
95% ethanol (Lichtenthaler 1987)	(13.36 ×A664) – (5.19 × A649)	(27.43 × A649) – (8.12 × A664)	Chl *a* + Chl *b*	Cx+c = (1000A470 – 2.13Ca – 97.64Cb)/209
80% buffered acetone (Lichtenthaler 1987)	(12.25 × A664) – (2.79 × A647)	(21.50 × A647) – (5.10 × A664)	Chl *a* + Chl *b*	Cx+c = (1000A470 – 1.82Ca – 85.02Cb)/198
100% DMSO Wellburn 1994	(12.47 × A665) – (3.62 × A649)	(25.06 × A649) – (6.50 × A665)	Chl *a* + Chl *b*	Cx+c = (1000A480 – 1.29Ca – 53.78Cb)/220
100% DMF (Porra et al. 1989); carotenoids (Wellburn 1994)	(12 × A664) – (3.11 × A647)	(20.78 × A647) – (4.88 × A664)	Chl *a* + Chl *b*	Cx+c = (1000A480 – 1.12Ca – 34.07Cb)/245

Note: Cx+c, concentration of xanthophylls and carotenes; Ca, chlorophyll *a*; Cb, chlorophyll *b*.

Fig. 1. The effects of incubation time on the extraction of total chlorophyll from foliage of hardwood and conifer tree species using four different solvents. The data are means + SE (n = 6) pooled by each combination of time period, solvent, species from two representative experiments each with n = 3. Summaries of statistical comparisons among time points for each solvent as well as comparison of solvents within each species are described in Tables 2 and 4.

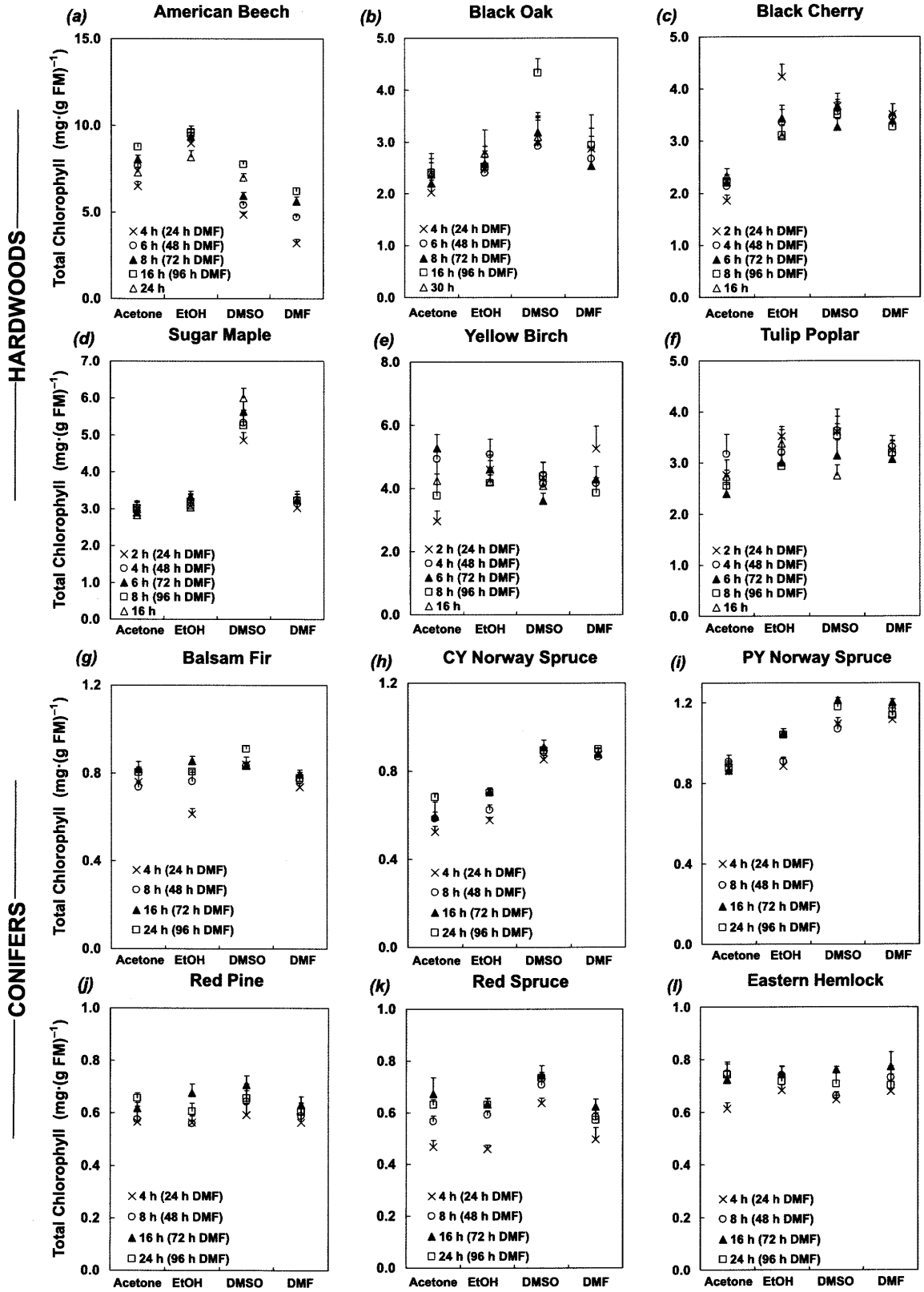

Fig. 2. The effects of incubation time on the extraction of total carotenoids from foliage of hardwood and conifer tree species using four different solvents. The data are means + SE (n = 6) pooled by each combination of time period, solvent, species from two representative experiments each with n = 3 with the exceptions of sugar maple, black oak, red spruce, and eastern hemlock, for which data from only one experiment are given. Summaries of statistical comparisons among time points for each solvent as well as comparison of solvents within each species are described in Tables 2 and 4.

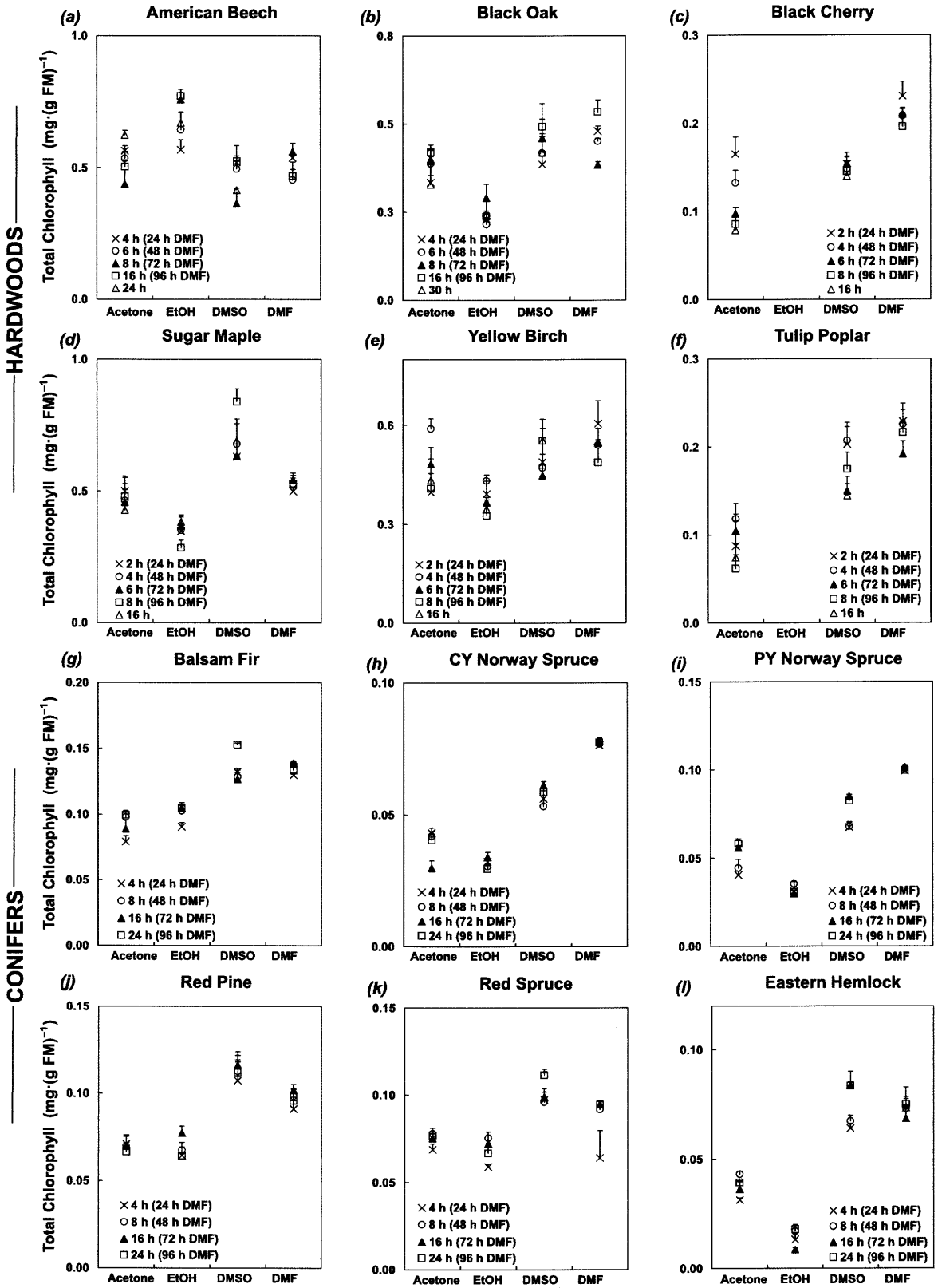

Table 2. Incubation time ranges for chlorophyll and carotenoid concentrations (mean ± SE) with statistically similar maximum yields per solvent for each hardwood species.

	American beech			Black oak			Black cherry			Sugar maple			Yellow birch			Tulip poplar		
		Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)	
	Time (h)	Mean	SE	Time (h)	Mean	SE	Time (h)	Mean	SE	Time (h)	Mean	SE	Time (h)	Mean	SE	Time (h)	Mean	SE
Total chlorophyll																		
Acetone	8–16	8.41a	0.18	2–30	2.21a	0.11	2–16	2.15a	0.06	2–16	2.94a	0.08	4–16	4.56a	0.27	2–16	2.72a	0.12
Ethanol	4–24	9.21b	0.15	2–30	2.51a	0.12	4–16	3.25b	0.11	2–16	3.16a	0.09	2–16	4.54a	0.17	2–16	3.21b	0.11
DMSO	16	7.77a	0.18	4–30	3.32b*	0.21	2–16	3.54b	0.08	16	6.00b*	0.26	2–16	4.11a	0.16	2–16	3.34b	0.14
DMF	72–96	5.92c	0.17	24–96	2.76ab	0.21	24–96	3.41b	0.08	24–96	3.18a	0.08	24–96	4.39a	0.24	24–96	3.21b	0.10
Chlorophyll *a*																		
Acetone	8–16	5.61a	0.11	2–30	1.55a	0.08	2–16	1.49a	0.04	2–16	2.00a	0.05	4–16	3.11a	0.19	2–16	1.84a	0.08
Ethanol	4–24	6.00b	0.10	2–30	1.70a	0.08	4–16	2.12b	0.08	2–16	2.06a	0.05	2–16	3.02a	0.11	2–16	2.06ab	0.07
DMSO	16	5.27a	0.14	4–30	2.18b	0.14	2–16	2.39c	0.05	16	2.84b	0.14	2–16	2.87a	0.11	2–16	2.31b	0.10
DMF	72–96	4.59c	0.13	24–96	1.96ab	0.15	24–96	2.34bc	0.06	24–96	2.09a	0.06	24–96	3.06a	0.16	24–96	2.24b	0.07
Chlorophyll *b*																		
Acetone	8–16	2.80ab	0.07	2–30	0.66a	0.04	2–16	0.66a	0.02	2–16	0.94a	0.03	4–16	1.45ab	0.09	2–16	0.89a	0.04
Ethanol	4–24	3.21b	0.06	2–30	0.82a	0.04	4–16	1.12b	0.04	2–16	1.10b	0.03	2–16	1.52b	0.06	2–16	1.16b	0.04
DMSO	16	2.50a	0.09	4–30	1.14b	0.07	2–16	1.15b	0.03	16	3.16c	0.13	2–16	1.24a	0.05	2–16	1.03ab	0.05
DMF	72–96	1.33c	0.05	24–96	0.80a	0.06	24–96	1.07b	0.03	24–96	1.09b	0.03	24–96	1.33ab	0.08	24–96	0.96a	0.03
Total carotenoids																		
Acetone	4–8	0.51a	0.02	2–30	0.36a	0.02	4–16	0.10a	0.01	2–16	0.47a	0.02	2–16	0.46a	0.03	2–16	0.09a	0.01
Ethanol	4–24	0.68b	0.02	2–30	0.25b	0.01	na	na	na	2–16	0.35b	0.02	2–16	0.37b	0.01	na	na	na
DMSO	4–24	0.47a	0.02	2–30	0.42ac*	0.02	2–16	0.15b	0.00	2–16	0.69c*	0.04	2–16	0.50a	0.02	2–16	0.18b	0.01
DMF	72–96	0.51a	0.02	24,48,96	0.49c	0.02	24–96	0.21c	0.01	24–96	0.52a	0.01	24–96	0.54a	0.02	24–96	0.22c	0.01

Note: For solvent comparisons, data were pooled for time ranges given in the table. Letters denote statistical differences between solvents at $p < 0.05$. $n = 6$ for each combination of time period, solvent, species for chlorophyll and carotenoids with the exception of sugar maple and black oak ($n = 3$). * indicates that the extract was brown; na indicates no pigment extracted with solvent.

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amounts of total chlorophyll, and DMF extracted the lowest amount (Table 2). Although in black oak and sugar maple DMSO extracted higher amounts of total chlorophyll than the other solvents, the extract was brown compared with the green color obtained with the other solvents (Figs. 3*c*, 3*d*). This suggests that DMSO may have extracted other compounds (e.g., tannins and other phenolics) besides chlorophyll that affected the calculations for chlorophylls because of higher absorbance values at 649 or 665 nm (wavelength scans, Figs. 3*c*, 3*d*). Even though the other three solvents yielded statistically comparable amounts, acetone and DMF extracts also had a slightly brown coloration.

All four solvents extracted chlorophyll *a* and chlorophyll *b* from all six species within the same time ranges discussed for total chlorophyll (Table 2). Similar trends were observed among total chlorophyll, chlorophyll *a*, and chlorophyll *b*. Statistically, the results for chlorophyll *a* and chlorophyll *b* were similar to the results for total chlorophyll, with a few differences (Table 2).

We also converted our total chlorophyll data for hardwoods from a fresh-mass basis to an area basis (Table 3) so that these data are available for comparisons to researchers who have and continue to obtain area-based chlorophyll data.

Conifers

As with hardwoods, all conifer species shared a common incubation time of 16 h for the maximum extraction of chlorophylls in acetone, DMSO, and ethanol (Table 4). However, in three species (red spruce, balsam fir, and red pine), the common window of time for maximum extraction of chlorophylls was 8–24 h using these three solvents. Statistically similar amounts of total chlorophylls were extracted in 24–96 h with DMF from all conifers (Table 4).

In the five conifer species, total chlorophyll was extracted by all four solvents (Figs. 1*g*–1*l*). As was observed in the hardwoods, no single solvent proved to be significantly more efficient at extracting total chlorophyll from all conifer species. In fact, all four solvents extracted similar amounts from red pine and eastern hemlock foliage (Table 4).

Acetone, DMF, and ethanol extracted similar amounts of total chlorophyll from both red spruce and balsam fir, while DMSO extracted slightly higher amounts in these species. However, the extract with DMSO was brown compared with the green colored extracts obtained with the other solvents (Figs. 4*a*, 4*d*). This brown coloration indicates that, as with sugar maple and black oak, DMSO extracted other compounds besides chlorophylls, which, in turn, affected its quantitation (wavelength scans, Figs. 4*a*, 4*d*). DMSO and DMF extracted similar amounts from current-year (CY) and past-year (PY) Norway spruce needles, and these amounts were significantly higher than those obtained with acetone or ethanol (Table 4).

All four solvents also extracted chlorophyll *a* and chlorophyll *b* (Table 4) from all five species within the same time frames discussed for total chlorophyll. Total chlorophyll, chlorophyll *a*, and chlorophyll *b* all demonstrated similar trends, but statistical significances varied slightly for chlorophyll *a* and chlorophyll *b* compared with total chlorophyll in some species (Table 4).

Carotenoids

Hardwoods

As was observed with chlorophylls, common time windows for maximum extraction of carotenoids from hardwood foliage varied among solvents and species; however, acetone and DMSO did share a common incubation time of 8 h (Table 2). Additionally, a 4–16 h window was common to most species when using ethanol with the exception of black cherry and tulip poplar, for which ethanol did not extract any carotenoids (Table 2). For maximum carotenoids extraction with DMF, a 24–96 h time range worked well for all species except for American beech (72–96 h) and black oak (24, 48, or 96 h) (Table 2).

The extraction of carotenoids from the six hardwoods was more variable than the chlorophyll extraction among the different solvents and species (Figs. 2*a*–2*f*). DMSO and DMF tended to extract higher carotenoids amounts than acetone and ethanol in most species, although differences among solvents were not always statistically significant. DMSO extracted the highest amounts of carotenoids from sugar maple, and DMF extracted the second highest. As with chlorophylls, the brown color of DMSO extracts also affected the calculations for carotenoids because of higher absorbance values at 470 nm (wavelength scans, Figs. 3*c*, 3*d*). Based on our data, no single solvent was statistically better than the others at extracting carotenoids from all the hardwoods.

DMF extracted the most carotenoids from tulip poplar and black cherry, while DMSO extracted more than acetone, and ethanol did not extract any (Table 2). Acetone, DMSO, and DMF all extracted similar amounts of total carotenoids from yellow birch, with ethanol extracting the least (Table 2). Of the four solvents, ethanol was the least efficient and the most variable for the extraction of carotenoids in most hardwoods tested. The only exception to this result was observed in American beech, for which ethanol extracted significantly higher amounts of carotenoids than the other three solvents (Table 2).

Conifers

Acetone, DMSO, and ethanol had a common maximum extraction time of 16 h in almost all conifers except for balsam fir, for which 24 h was the minimum time needed for maximum extraction with DMSO. For extraction of carotenoids using DMF, 24–96 h was common for all five conifers (Table 4).

Carotenoids were extracted from all conifer species by all four solvents (Figs. 2*g*–2*l*). In red spruce, eastern hemlock, red pine, and balsam fir DMSO extracted significantly higher amounts of carotenoids than the other three solvents (Table 4). In these four species the absorbance at 470 nm increased because of the brown coloration of the DMSO extracts, which, in turn, affected the quantitation of carotenoids (wavelength scans, Figs. 4*a*–4*d*). In balsam fir and eastern hemlock the amounts of carotenoids extracted varied significantly among all solvents, with DMSO extracting the most (brown extract), followed by DMF, and either ethanol or acetone extracting the least amount. Acetone and ethanol extracted similar amounts from both red spruce and red pine (Table 4). In red spruce, DMF also extracted

Fig. 3. Comparison of color and wavelength scans of extracts and color of residual tissue of leaves of six hardwood species extracted by four different solvents. The solvents (L–R) used were 95% ethanol, 80% acetone, DMSO, and DMF. Incubation time was 16 h for acetone, 95% ethanol, and DMSO and 72 h for DMF. Scale bar = 5 mm. * indicates brown coloration of DMSO extract.

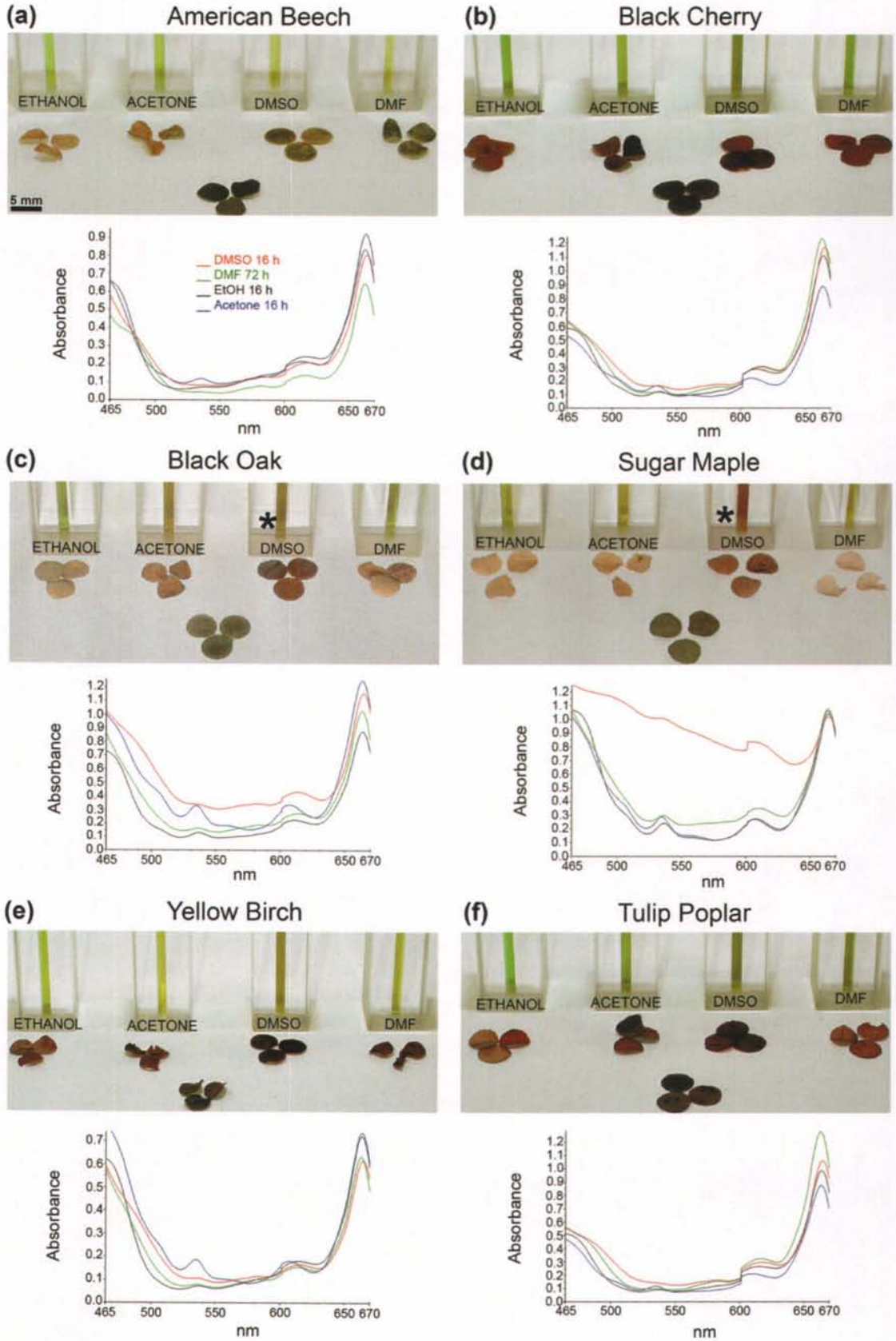

Table 3. Total chlorophyll (mean ± SE) data (mg·mm^{-2}) for hardwoods.

Species	Acetone	EtOH	DMSO	DMF
American beech	191.56±5.77	250.15±2.44	154.87±7.43	131.97±7.45
Black oak	284.74±15.25	319.77±19.19	414.64±26.33	337.37±27.56
Black cherry	188.10±5.26	277.67±4.66	297.60±3.27	295.02±4.05
Sugar maple	255.74±3.31	279.38±3.30	562.15±20.35	286.97±5.88
Yellow birch	370.64±21.23	353.52±9.11	351.46±9.67	376.98±23.75
Tulip poplar	237.89±4.52	288.14±4.66	291.61±5.59	286.69±4.47

Note: For solvent comparisons data were pooled from time ranges given in Table 2 for total chlorophyll. n = 6 for each combination of time period, solvent, species. Summaries of statistical comparisons among these solvents within each species are described only on a milligrams per gram fresh mass basis in Tables 1 and 2.

amounts similar to those obtained with acetone. Additionally, DMSO extracted significantly higher amounts of carotenoids from CY and PY Norway spruce than acetone and ethanol; DMF extracted the highest amounts (Table 4).

Discussion

Based on the detailed data for individual solvents (Tables 2 and 4), we have been able to summarize for each species the solvents that yield statistically comparable maximum amounts of different pigments within the window of time common to all four solvents (Table 5). Of the 11 species tested, comparable chlorophyll amounts were extracted by all four solvents in three species and by two solvents (ethanol and DMF) in nine species. In four species, acetone, ethanol, and DMF extracted comparable chlorophyll amounts, while in another two species, comparable amounts were obtained with ethanol, DMSO, and DMF.

A few studies have reported the extraction of comparable amounts of chlorophylls between DMF and acetone. However, in these studies the tissue was macerated for extracting chlorophylls in acetone (Barnes et al. 1992; Tait and Hik 2003). In our study no foliar tissues were macerated, and acetone still extracted amounts comparable to those obtained with ethanol and DMF in seven species, although in three other species DMF did extract more pigment than acetone. In these three species (tulip poplar, black cherry, and Norway spruce) maceration may be required for maximum extraction. Therefore, in our study DMF and ethanol were more efficient than acetone with nonmacerated tissues. Acetone has a high vapor pressure that causes it to evaporate easily during and after extraction, thus yielding more variable data sets especially when the volume used is low (Stiegler et al. 2005).

Since DMSO is often used in various protocols to solubilize tannins and other types of phenolics (Liu et al. 2008), it is not surprising that in our study DMSO extracts of foliage from sugar maple, black oak, red spruce, and balsam fir had a brown coloration, which affected calculations for chlorophylls. Thus, in this study DMSO was not a viable choice for the extraction of pigments from these four species. Sugar maple and black oak extracts in acetone and DMF were also slightly brown, but the amounts of total chlorophyll extracted were statistically similar between these two solvents and to ethanol, indicating little interference from the brown pigments.

The choices of solvents for the extraction of carotenoids were limited. The data presented in Table 5 indicate that on the basis of statistical significances DMSO should be the obvious choice in the case of most conifers. However, upon closer examination, DMF is actually the preferred solvent because the DMSO extracts were brown in four conifer and two hardwood species (Table 5). DMF outperformed other solvents for carotenoid extractions from most hardwood species (Table 5).

With the exception of American beech, DMF extracted the maximum amount of chlorophylls and carotenoids within a 24 h period from 10 of 11 species tested. Ethanol also extracted maximum amounts of chlorophyll from 10 species (nine species in common with DMF) within 2–30 h, depending on the species, with a 16 h incubation period working well for all 10 species. The incubation times for both DMF and ethanol are convenient, and in general, no interfering compounds were detected in the wavelength scans using these solvents. Both solvents did not require tissue maceration and centrifugation. A comparison of wavelength scans for solvents for which heating was required (acetone, ethanol, and DMSO) and DMF, for which heating was not required, reveals that heating at 65 °C did not negatively affect the stability of extracted chlorophylls (Figs. 3, 4). Overall, extraction efficiencies of ethanol and DMF were comparable for analyzing chlorophyll concentrations. However, DMF is more toxic than ethanol (Ponec et al. 1990; Gescher 1993) — it may be fatal if ingested, inhaled, or absorbed through skin, and it is a mutagen and a teratogen. Thus, keeping personnel safety in mind, we recommend ethanol as the better option of these two solvents for chlorophyll extractions. However, if carotenoids also need to be extracted, DMF is the only choice for the species described here, since ethanol was not very effective at extracting carotenoids.

We highly recommend that the information reported here be validated before applying it to a species not tested in this report. Although acetone and DMSO are not the preferred choices in this study because they extracted significantly lower amounts, they can still extract quite reliable and reproducible amounts of chlorophylls (as observed in repeat experiments). Therefore, even these solvents may be used for comparative studies as long as the extract is not brown. The stability of sugar maple and red spruce extracts in all four solvents were checked after 2 weeks of storage at –20 °C, and all of the tested extracts were stable in all four solvents except for sugar maple extracts in DMSO. In this case chlorophyll b content decreased by approximately 40%.

Table 4. Incubation time ranges for chlorophyll and carotenoid concentrations (mean ± SE) with statistically similar maximum yields per solvent for each conifer species.

	Balsam fir			Norway spruce (current year)			Norway spruce (past year)			Red pine			Red spruce			Eastern hemlock		
		Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)			Concentration (mg·(g FM)$^{-1}$)	
	Time (h)	Mean	SE	Time (h)	Mean	SE	Time (h)	Mean	SE	Time (h)	Mean	SE	Time (h)	Mean	SE	Time (h)	Mean	SE
Total chlorophyll																		
Acetone	4–24	0.78a	0.018	8–24	0.62a	0.026	4–24	0.88a	0.016	4–24	0.60a	0.020	8–24	0.62a	0.025	4–24	0.71a	0.021
Ethanol	8–24	0.81ab	0.017	16–24	0.71b	0.012	16–24	1.05b	0.018	8–24	0.61a	0.021	8–24	0.62a	0.012	4–24	0.72a	0.014
DMSO	4–24	0.85b*	0.012	4–24	0.89c	0.011	16–24	1.20c	0.013	4–24	0.65a	0.023	8–24	0.73b*	0.018	16–24	0.74a	0.023
DMF	24–96	0.77a	0.011	24–96	0.88c	0.007	24–96	1.16c	0.013	24–96	0.60a	0.015	24–96	0.57a	0.019	24–96	0.72a	0.023
Chlorophyll *a*																		
Acetone	4–24	0.57a	0.011	8–24	0.48a	0.019	4–24	0.62a	0.012	4–24	0.45a	0.014	8–24	0.48ac	0.012	4–24	0.56a	0.015
Ethanol	8–24	0.60ab	0.011	16–24	0.52a	0.007	16–24	0.70b	0.011	8–24	0.45a	0.015	8–24	0.49a	0.008	4–24	0.54a	0.011
DMSO	4–24	0.60b	0.007	4–24	0.62b	0.008	16–24	0.81c	0.009	4–24	0.50a	0.017	8–24	0.53b	0.009	16–24	0.54a	0.017
DMF	24–96	0.59ab	0.005	24–96	0.64b	0.005	24–96	0.83c	0.009	24–96	0.45a	0.011	24–96	0.44c	0.012	24–96	0.56a	0.017
Chlorophyll *b*																		
Acetone	4–24	0.21a	0.011	8–24	0.14a	0.008	4–24	0.27a	0.007	4–24	0.16a	0.007	8–24	0.14a	0.014	4–24	0.15a	0.006
Ethanol	8–24	0.21a	0.007	16–24	0.19b	0.006	16–24	0.34b	0.008	8–24	0.16a	0.006	8–24	0.13a	0.008	4–24	0.18bc	0.004
DMSO	4–24	0.23b	0.007	4–24	0.27c	0.003	16–24	0.39c	0.004	4–24	0.15a	0.009	8–24	0.20b	0.009	16–24	0.20c	0.007
DMF	24–96	0.17c	0.007	24–96	0.24d	0.002	24–96	0.32b	0.004	24–96	0.14a	0.004	24–96	0.12a	0.008	24–96	0.16ab	0.006
Total carotenoids																		
Acetone	4–24	0.091a	0.003	8–24	0.04a	0.002	16–24	0.06a	0.002	4–24	0.07a	0.003	4–24	0.07ac	0.002	4–24	0.04a	0.002
Ethanol	8–24	0.104b	0.002	4–24	0.03b	0.001	4–24	0.03b	0.002	4–24	0.07a	0.002	4–24	0.07a	0.003	4–24	0.01b	0.001
DMSO	24	0.153c*	0.001	4–24	0.06c	0.001	16–24	0.08c	0.001	4–24	0.11b*	0.004	4–24	0.10b*	0.003	16–24	0.08c*	0.003
DMF	24–96	0.135d	0.001	24–96	0.08d	0.001	24–96	0.10d	0.001	24–96	0.10c	0.002	24–96	0.09c	0.005	24–96	0.07d	0.003

Note: For solvent comparisons, data were pooled for the common time ranges shown in the table. Letters denote statistical differences between solvents at $p < 0.05$. $n = 6$ for each combination of time period, solvent, species for chlorophyll and carotenoids with the exception of red spruce and eastern hemlock ($n = 3$). * indicates that the extract was brown.

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Fig. 4. Comparison of color and wavelength scans of extracts and color of residual tissue of needles of five conifer species extracted by four different solvents. The solvents (L–R) used were 95% ethanol, 80% acetone, DMSO, and DMF. Incubation time was 16 h for acetone, 95% ethanol, and DMSO and 72 h for DMF. Scale bar = 5 mm. * indicates brown coloration in DMSO extract.

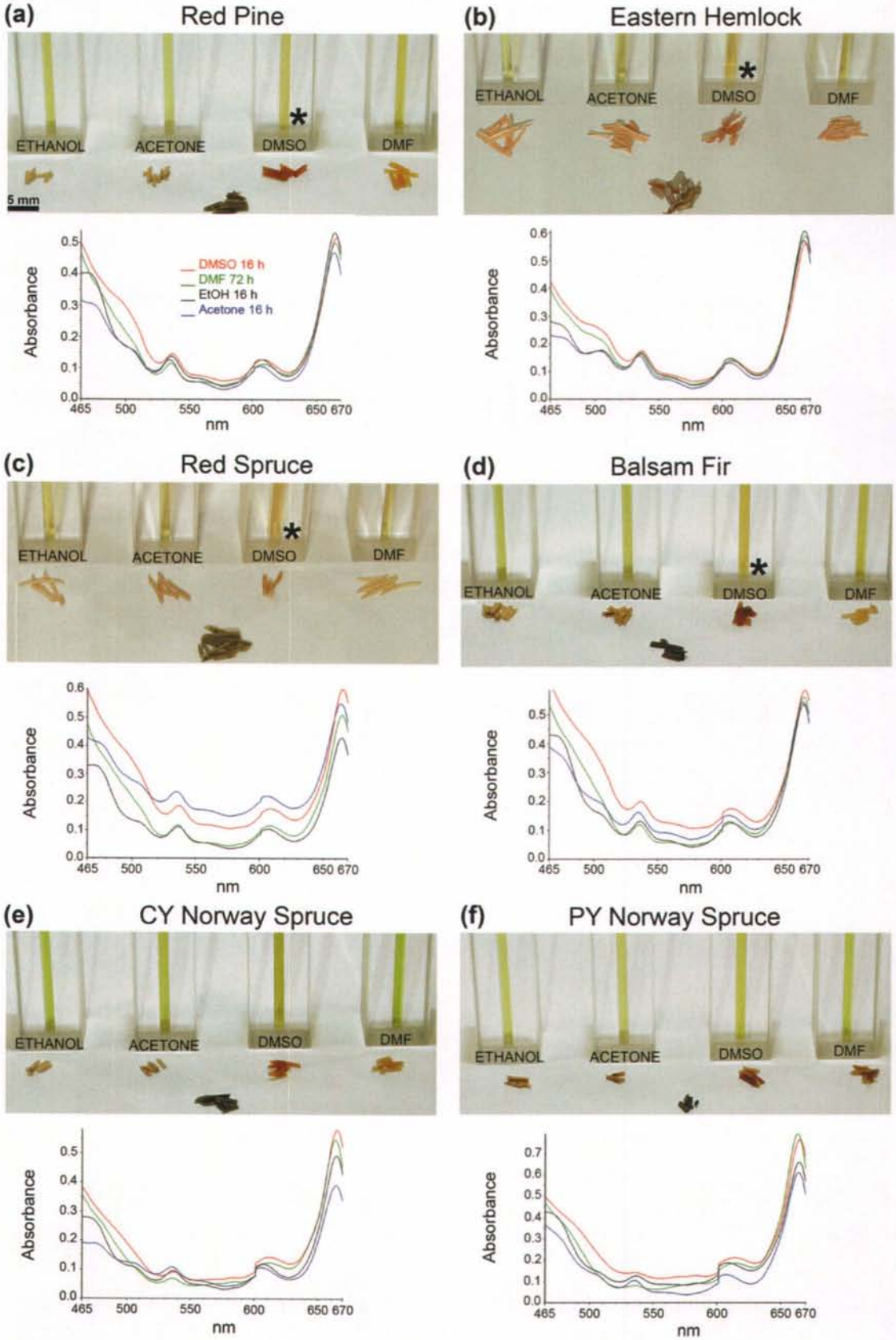

Table 5. Summary of recommended solvent(s) that yield maximum but statistically similar results for extraction of total chlorophyll and (or) carotenoids within the window of times given below.

	Total chlorophyll		Total carotenoids		Chlorophyll+carotenoids	
Species	Time*	Solvent(s)	Time	Solvent(s)	Time	Solvent(s)
Hardwoods						
Yellow birch	4–16	A, E, D, DMF	2–16	A, D, DMF	2–16	A, D, DMF
Sugar maple†	2–16	A, E, DMF	2–16	A, DMF	2–16	A, DMF
Black oak†	4–30	A, E, DMF	4–30	D, DMF	4–30	DMF
Tulip poplar	2–16	E, D, DMF	24–96	DMF	24–96	DMF
Black cherry	4–16	E, D, DMF	24–96	DMF	24–96	DMF
American beech	4–24	E	4	E	4	E
Conifers						
Red pine‡	8–24	A, E, D, DMF	4–24	DMF	4–24	DMF
Eastern hemlock‡	16–24	A, E, D, DMF	16–24	DMF	16–24	DMF
Balsam fir†	8–24	A, E, DMF	24	DMF	24	DMF
Red spruce†	8–24	A, E, DMF	4	A, DMF	8	A, DMF
Current-year Norway spruce	4–24	D, DMF	24–96	DMF	24–96	DMF
Past-year Norway spruce	4–24	D, DMF	24–96	DMF	24–96	DMF

Note: A, acetone; E, ethanol; D, DMSO. For extraction amounts and other details refer to Tables 2 and 4.

*This time window is for solvents other than DMF. Times for DMF are not given in this table, since all tested incubation times for DMF (24–96 h) worked equally well. The only two exceptions were American beech (72–96 h) and black oak (24–48 and 96 h).

†DMSO was ruled out as a choice for chlorophyll and carotenoids extraction because of the brown coloration of the extract that significantly affected the absorbance readings in sugar maple, black oak, balsam fir, and red spruce. Although for balsam fir, the effects of DMSO on chlorophyll were not as pronounced as in the other three species, they were high enough to cause a significant difference between DMSO and the other comparable solvents.

‡In eastern hemlock and red pine, DMSO extract also had slightly brownish color that affected the extraction of carotenoids but did not significantly affect chlorophyll extractions compared with the other three solvents. Therefore, we do not recommend DMSO for carotenoids extraction even for these species.

To verify that our extraction values fell within the normal range for particular species we needed to compare our results with published literature. However, chlorophyll values for a given species are known to vary within a wide range because leaf structure, plant age, physiological status, light, temperature, and growth season (Faria et al. 1998; Lamontagne et al. 2000) all affect the chlorophyll levels in foliage. A wide range of chlorophyll values were reported for mature sugar maple growing at different sites (Ellsworth and Liu 1994) and for red pine trees under different levels of physiological stress (Bauer et al. 2004). Another factor that complicates these types of comparisons is the use of different spectrophotometers (variable resolution vs. 2 nm fixed resolution diode array) across laboratories, which also affect chlorophyll measurements, as discussed in Wellburn (1994). Finally, chlorophyll content has been expressed in various ways in the published literature, including in milligrams per gram fresh mass, milligrams per gram dry mass, and milligrams per square metre. To roughly compare our data ($mg \cdot (g\ FM)^{-1}$) with published data expressed as milligrams per gram dry mass, it was necessary to multiply our data by a factor of two to accommodate the average moisture content (40%–60%, data not shown) in the species we tested for the conversion of fresh mass to dry mass.

Despite all of these limitations, the extraction values obtained in our study did compare well with those cited in the published literature for the solvents used with red spruce, sugar maple, and Norway spruce (Amundson et al. 1992; Ellsworth and Liu 1994; Liu et al. 1997; Soukupova et al. 2000). We also converted our total chlorophyll data for hardwoods from a fresh-mass to an area basis (Table 3) so that these data are available for comparisons to researchers who have and continue to obtain area-based chlorophyll data.

In the field, chlorophylls are often measured using noninvasive optical methods (handheld devices) that are based on absorbance and (or) reflectance of light by the intact leaf (Gamon and Surfus 1999; Markwell 2002). Data from these methods yield a chlorophyll index instead of absolute chlorophyll content (Richardson et al. 2002). More recently, the advent of new aircraft-based and space-based hyperspectral sensors makes it possible to record reflected radiation over hundreds of continuous and narrow spectral bands, often covering a spectral range from 400 to 2500 nm. These sensors make it possible to quantitatively assess leaf pigment at the canopy scale. With this superior spectral coverage, the recorded data may be used to detect subtle forms of ecological variation, including leaf pigment and chemical constituents (Smith et al. 2002, 2003; Ollinger and Smith 2005). Evaluating the effectiveness of these remote sensing approaches as well as other noninvasive field methods requires that the data obtained by these methods be carefully validated by traditional laboratory methods of chlorophyll determination. For a regional-scale study, only one specific chlorophyll extraction method should be used to validate and compare the field data for all conifers and hardwoods. Our study provides sufficient information for choosing an appropriate extraction solvent and corresponding incubation time range of extracting chlorophylls and (or) carotenoids from foliage of multiple species of trees.

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References

Amundson, R.G., Hadley, J.L., Fincher, J.F., and Fellows, S. 1992. Comparisons of seasonal changes in photosynthetic capacity, pigments, and carbohydrates of healthy sapling and mature red spruce and of declining and healthy red spruce. Can. J. For. Res. **22**: 1605–1616. doi:10.1139/x92-213.

Barnes, J.D., Balaguer, L., Manrique, E., Elvira, S., and Davison, A.W. 1992. A reappraisal of the use of DMSO for the extraction and determination of chlorophylls *a* and *b* in lichens and higher plants. Environ. Exp. Bot. **32**: 85–100. doi:10.1016/0098-8472(92)90034-Y.

Bauer, G.A., Bazzaz, F.A., Minocha, R., Long, S., Magill, A., Aber, J.D., and Berntson, G.M. 2004. Effects of chronic N additions on tissue chemistry, photosynthetic capacity, and carbon sequestration potential of a red pine (*Pinus resinosa* Ait.) stand in the NE United States. For. Ecol. Manage. **196**: 173–186. doi:10.1016/j.foreco.2004.03.032.

Bowles, N.D., Paerl, H.W., and Tucker, J. 1985. Effective solvents and extraction periods employed in phytoplankton carotenoid and chlorophyll determinations. Can. J. Fish. Aquat. Sci. **42**: 1127–1131.

Dunn, J.L., Turnbull, J.D., and Robinson, S.A. 2004. Comparison of solvent regimes for the extraction of photosynthetic pigments from leaves of higher plants. Funct. Plant Biol. **31**: 195–202. doi:10.1071/FP03162.

Ellsworth, D., and Liu, X. 1994. Photosynthesis and canopy nutrition of four sugar maple forests on acid soils in northern Vermont. Can. J. For. Res. **24**: 2118–2127. doi:10.1139/x94-272.

Faria, T., Silverio, D., Breia, E., Cabral, R., Abadia, A., Abadia, J., Pereira, J., and Chaves, M. 1998. Differences in the response of carbon assimilation to summer stress (water deficits, high light and temperature) in four Mediterranean tree species. Physiol. Plant. **102**: 419–428. doi:10.1034/j.1399-3054.1998.1020310.x.

Gamon, J., and Surfus, J. 1999. Assessing leaf pigment content and activity with a reflectometer. New Phytol. **143**: 105–117. doi:10.1046/j.1469-8137.1999.00424.x.

Gescher, A. 1993. Metabolism of N,N-Dimethylformamide: Key to the understanding of its toxicity. Chem. Res. Toxicol. **6**: 245–251. doi:10.1021/tx00033a001. PMID:8318646.

Hiscox, J.D., and Israelstam, G.F. 1979. A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. **57**: 1332–1334. doi:10.1139/b79-163.

Lamontagne, M., Bigras, F.J., and Margolis, H.A. 2000. Chlorophyll fluorescence and CO2 assimilation of black spruce seedlings following frost in different temperature and light conditions. Tree Physiol. **20**: 249–255. PMID:12651461.

Larcher, W. 1995. Physiological plant ecology. Springer Verlag, Berlin, Heidelberg. pp. 424–426.

Lichtenthaler, H.K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. **148**: 350–382. doi:10.1016/0076-6879(87)48036-1.

Liu, X., Ellsworth, D., and Tyree, M. 1997. Leaf nutrition and photosynthetic performance of sugar maple (*Acer saccharum*) in stands with contrasting health conditions. Tree Physiol. **17**: 169–178. PMID:14759871.

Liu, J., Ando, R., Shimizu, K., Hashida, K., Makino, R., Ohara, S., and Kondo, R. 2008. Steroid 5α-reductase inhibitory activity of condensed tannins from woody plants. J. Wood Sci. **54**: 68–75. doi:10.1007/s10086-007-0905-9.

Markwell, J. 2002. Nondestructive chlorophyll assessment. New Phytol. **153**(1): 7–8. doi:10.1046/j.0028-646X.2001.00309.x.

Ollinger, S.V., and Smith, M.-L. 2005. Net primary production and canopy nitrogen in a temperate forest landscape: an analysis using imaging spectroscopy, modeling and field data. Ecosystems (N. Y., Print), **8**: 760–778. doi:10.1007/s10021-005-0079-5.

Ponec, M., Haverkort, M., Soei, Y.L., Kempenaar, J., and Bodde, H. 1990. Use of human keratinocyte and fibroblast cultures for toxicity studies of topically applied compounds. J. Pharm. Sci. **79**(4): 312–316. doi:10.1002/jps.2600790408. PMID:2352141.

Porra, R.J., Thompson, W.A., and Kriedemann, P.E. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochim. Biophys. Acta, **975**(3): 384–394. doi:10.1016/S0005-2728(89)80347-0.

Richardson, A.D., Duigan, S.P., and Berlyn, G.P. 2002. An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytol. **153**(1): 185–194. doi:10.1046/j.0028-646X.2001.00289.x.

Smith, M., Ollinger, S., Martin, M., Aber, J., Hallett, R., and Goodale, C. 2002. Direct estimation of aboveground forest productivity through hyperspectral remote sensing of canopy nitrogen. Ecol. Appl. **12**: 1286–1302. doi:10.1890/1051-0761(2002)012[1286:DEOAFP]2.0.CO;2.

Smith, M., Martin, M., Plourde, L., and Ollinger, S. 2003. Analysis of hyperspectral data for estimation of temperate forest canopy nitrogen concentration: comparison between an airborne (AVIRIS) and a spaceborne (Hyperion) sensor. IEEE Trans. Geosci. Rem. Sens. **41**: 1332–1337. doi:10.1109/TGRS.2003.813128.

Soukupova, J., Cvikrova, M., Albrechtova, J., Rock, B.N., and Eder, J. 2000. Histochemical and biochemical approaches to the study of phenolic compounds and peroxidases in needles of Norway spruce (*Picea abies*). New Phytol. **146**: 403–414. doi:10.1046/j.1469-8137.2000.00666.x.

Stiegler, J.C., Bell, G.E., and Maness, N.O. 2005. Comparison of acetone and N, N-dimethylformamide for pigment extraction in turfgrass. Commun. Soil. Sci. Plant Anal. **35**: 1801–1813. doi:10.1081/CSS-200026796.

Tait, M.A., and Hik, D.S. 2003. Is dimethylsulfoxide a reliable solvent for extracting chlorophyll under field conditions? Photosynth. Res. **78**(1): 87–91. doi:10.1023/A:1026045624155. PMID:16245067.

Wellburn, A.R. 1994. The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J. Plant Physiol. **144**(3): 307–313.