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Spectrographic measurement of plant pigments from 300 to 800 nm

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ABSTRACT

The spectral data of many plant pigments available in the literature are limited in their range and resolution. The objective of this project was to obtain improved spectral data that are representative of *in vivo* absorption characteristics for a selection of pigments relevant to leaf reflectance modeling. Absorption spectra of anthocyanins, betacyanins, betaxanthins, chlorophyll a and b, and carotenoids were obtained between 300 and 800 nm at 1-nm intervals. These pigments were extracted from plant material and separated using column and thin layer chromatography. The two methods showed consistent results for all pigments except the chlorophylls which could not be fully separated using simple column chromatography. The anthocyanin and betacyanin spectra were very similar but anthocyanin displayed greater absorption in the UV range.

1. Introduction

Spectral measurements for a variety of plant pigments (including chlorophyll a, chlorophyll b, and beta-carotene) have been collected and their spectra are available through online data bases such as PhotochemCad (Dixon, Taniguchi, & Lindsey, 2005; Du, Fuh, Li, Corkan, & Lindsey, 1998). However, many of these data do not extend into the ultraviolet (UV) range or have been obtained as highly purified *in vitro* measurements, which are often very different from spectra displayed within the plant. This is particularly the case for chlorophylls, whose absorption peak locations and dimensions change quite dramatically depending on extraction solvent or location in the photosynthetic apparatus (Lichtenthaler, 1987). Absorption peaks for anthocyanins, betacyanins, and betaxanthins have been identified but the full absorption spectra have not been published in the range and resolution desired for future study in plant identification or spectral modeling.

The objective of this study was to obtain pigment extracts and report their molar extinction coefficients between 300 and 800 nm at 1-nm intervals that are representative of *in vivo* absorption characteristics for anthocyanins, betacyanins, betaxanthins, chlorophylls, and carotenoids. This range and resolution are desired for use in conjunction with leaf reflectance models and plant identification applications. The extension of these measurements into UV wavelengths is of particular interest as *in vivo* measurements in this range have not yet been published for these pigments. Although UV wavelengths tend not to be important for traditional remote sensing due to atmospheric scattering, there are applications in ground-based and low-altitude measurement and imaging.

Chlorophyll a and b are found in higher plants in varying ratios. The chlorophyll a to chlorophyll b ratio lies between 2.6 and 5 (Maier, 2000) but leaf reflectance models such as PROSPECT assume a constant ratio to simplify calculations (Féret et al., 2008; Jacquemoud & Baret, 1990). In order to account for this variation in pigment ratio, separate collection of chlorophyll a and b would be preferred. Because the chlorophylls and carotenoids are not water soluble, they are extracted using organic solvents such as acetone (Omata & Murata, 1980). Separations of these pigments are commonly performed using silica gel or cellulose columns with a variety of mobile phases (Omata & Murata, 1980).

Chlorophyll absorption measured *in vivo* is known to be influenced strongly by the presence of proteins (Cinque, Croce, & Bassi, 2000; Zucchelli et al., 2002). Thus the spectra available for purified extracts are not suited to leaf optical property modeling. Some researchers have modeled *in vivo* chlorophyll spectra as a summation of Gaussians fit to spectral measurements of photosynthetic reaction centers and light harvesting complexes (Maier, 2000) or fit representative spectra based on leaf reflectance data (Féret et al., 2008; Jacquemoud & Baret, 1990).

Anthocyanins and betacyanins are mutually exclusive families of red pigments. This is of particular interest in plant identification because if one of these pigments is observed in a plant being analyzed, all species

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containing the other pigment can be eliminated (Noble, 2006). The degree to which this is possible for *in situ* spectral measurements of plant tissues is currently unknown. Modeling the leaf reflectance by using a tool such as the PROSPECT model will be useful in identifying detection limits and methods prior to assessment with actual plant reflectance data. The absorption spectra of anthocyanins and betacyanins display slightly different characteristics, especially in the ultraviolet range where anthocyanins absorb more strongly (Harborne, 1958; Zhang et al., 2008). To distinguish between these two pigments *in vivo*, spectral data with high resolution that extends into the UV range to account for the high anthocyanin absorption would be preferred. Cyanidin-3-glucoside is commonly used as the reference in the calculation of anthocyanin concentration as this is a prominent anthocyanin found in many plants (Brockington, Walker, Glover, Soltis, & Soltis, 2011). The yellow pigment betaxanthins are formed from an intermediate product of betacyanin synthesis and are found in almost all plants that contain betacyanins (Brockington, Walker, Glover, Soltis & Soltis, 2011).

The reference molar extinction coefficient data for all pigments of interest in this study are listed in Table 1. The peak wavelength for each pigment is given as well as the molar extinction coefficient at that wavelength. To determine the total molar extinction spectrum, the absorption spectra must be determined, the peaks identified, the concentration calculated, and the spectra normalized to account for the actual sample concentration.

2. Materials and methods

Extractions for each plant pigment were performed and initial separations and measurements were carried out using thin-layer chromatography (TLC) plates. The results from these plates were used to determine the preferred separation techniques. These techniques were then applied in the column chromatography tests to obtain individual pigments. These extracts were analyzed to verify their spectral identities.

To obtain raw extracts for the pigments, plant material was ground using a pestle and mortar in an appropriate extracting solution. The betacyanin and betaxanthin pigments were extracted by grinding red and yellow beet (*Beta vulgaris* L. sp.), respectively, in water and filtering the mixture. The anthocyanin pigments were obtained in the same way from red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*). Chlorophyll and carotenoids were extracted from *Spinacia oleracea* L. (spinach). *Daucus carota* subsp. *sativus* (carrot) was used as a second source for carotenoids to avoid chlorophyll contamination. Acetone was used in these extractions as the chlorophylls and carotenoids are not water-soluble. Table 2 summarizes the mobile phases which produced the best results for the TLC plates and were used to separate the pigments using column chromatography. The references provided in Table 2 represent where the mobile phase constituents were derived from; however, the ratio between the components was varied during testing in order to produce the greatest separation between the bands on the TLC plate.

2.1. Spectral measurements

Reflectance-factor measurements were taken from all thin-layer chromatography plates using an Ocean Optics Maya 2000PRO spectrometer (Ocean Optics, Dunedin, FL, USA) and a tungsten-deuterium light source. Optical fibers and focusing optics were arranged with a 0°/45° geometry. These spectra were obtained between 300 and 800 nm at approximately 0.5 nm intervals. Measurements were referenced against the TLC plate with the mobile phase developed to the same level as the pigment. These measurements were used to identify the general absorption spectrum of each pigment which was helpful in identifying the absorption peak and provided data to compare against the liquid absorbance measurements. An integrating sphere was not used for these measurements due to the small sample size and relatively large sampling area of integrating sphere equipment available to us. By comparison, the illumination of our reflectance factor probe could be tightly focused onto the pigment bands on the TLC plates. The TLC plate was assumed to be relatively Lambertian and the reflectance-factor measurement therefore taken to be representative of total reflectance. All spectral measurements of solutions were taken using a dual-beam Cary 5G UV–visible-NIR spectrophotometer (Agilent Technologies, Mississauga, Can.) and were sampled at 1-nm intervals between 300 nm and 800 nm. A cuvette of the appropriate mobile phase was used as a zero reference for each spectrum. Quartz cuvettes with a 1-cm path length were used for all sample and reference solutions. Measurements were taken as soon as possible after extraction to avoid degradation of the pigments.

2.2. Calculations

Before the molar extinction coefficient spectrum could be determined for the TLC plate measurements the reflectance factor was converted to absorption using Eq. (1). This is the relationship used for opaque solids in lieu of the more common transmission used for transmitting samples.

$$A(\lambda) = \log\left(\frac{1}{R(\lambda)}\right), \tag{1}$$

where A is the absorption as a function of wavelength and R, the reflectance factor, is equal to the spectral radiance of the pigment spot (in digital units) divided by the spectral radiance of the developed TLC plate (in digital units).

Pigment concentration was calculated using reference wavelengths and absorption values from literature sources (Table 1). This was then used to determine the extinction coefficient for every wavelength in the spectrum. Eq. (2) was used for these calculations.

$$\varepsilon(\lambda) = \frac{A(\lambda)}{c\ell}, \tag{2}$$

where A is the absorption as a function of wavelength (λ), ε [$\mathrm{Lmol^{-1}\,cm^{-1}}$] is the molar extinction coefficient as a function of wavelength, c is the concentration in moles per liter and ℓ is the sample path length in centimeters.

3. Results and discussion

TLC- and liquid-based molar extinction spectra of each pigment were graphically compared. For the TLC plate data only, a 15-point

Table 1
Molar extinction coefficients for plant pigments.

Pigment	Molar extinction coefficient ($\mathrm{Lmol^{-1}\,cm^{-1}}$)	Wavelength (nm)	Reference
Anthocyanin (cyanidin-3-glucoside)	34,300	530	Siegelman & Hendricks, 1958
Betacyanin	65,000	538	Schwartz & von Elbe, 1980
Betaxanthin	48,000	480	Gandía-Herrero, Escribano, & García-Carmona, 2005
Chlorophyll a	71,430	665	Namsaraev, 2009
Chlorophyll b	38,550	652	Namsaraev, 2009
Beta-carotene	139,500	451	Du, Fuh, Li, Corkan & Lindsey, 1998; Dixon, Taniguchi & Lindsey, 2005