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Eliminating interference by anthocyanins when determining the porphyrin ratio of red plant leaves

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ABSTRACT

Anthocyanins (Ants) are water-soluble secondary metabolites that are responsible for red colour of plant leaves. To determine photosynthetic pigments, 80% acetone was used to extract Ants from Ant-containing leaves of test plants. However, using the 80% acetone extraction method can lead to interference between chlorophylls (Chls) and Ants. Porphyrins, such as protoporphyrin IX (PPIX), Mg-protoporphyrin IX (MgPP), and protochlorophyllide (Pchlide), are Chl biosynthetic intermediates and demonstrate photospectrometric characteristics similar to those of Chl. Although the ether/water extraction method was able to remove Ants interference when detecting porphyrins, the porphyrins extraction efficiency was lower than that of the 80% acetone extraction method. Low Ants levels interfered with individual porphyrin ratios, and the extent of the effect was correlated with Ants concentrations. We developed the three equations could eliminate interference by Ants when determining the porphyrin molecular percentage (%) and were comprehensively applied to all tested species of Ants-containing leaves.

Abbreviations Used

Ants	Anthocyanins
ARI	Ant reflectance index
Cars	carotenoids
Chls	chlorophylls
HPLC	high-performance liquid chromatographic
MgPP	Mg-protoporphyrin IX
NDVI	normalized difference vegetation index
PRI	photochemical reflectance index
Pchlide	protochlorophyllide
PPIX	protoporphyrin IX
ROS	reactive oxygen species

1. Introduction

Porphyrins, such as protoporphyrin IX (PPIX), Mg-protoporphyrin IX (MgPP), and protochlorophyllide (Pchlide), are intermediates of the chlorophyll (Chl) metabolic pathway [1]. They can interact with reactive oxygen species (ROS) which are harmful to cells and cause the peroxidation of membrane proteins [2]. Free porphyrins are extremely harmful compounds, as they can produce cytotoxic free radicals in the presence of light [3]. Therefore, porphyrin synthesis and degradation are adjusted to cellular requirements, and reflect different needs under varying environmental conditions [4].

Anthocyanins (Ants) are water-soluble pigments, characteristic of higher plants, protect plants from excess light and UV irradiation, and serve as ROS scavengers [5]. They are secondary metabolites located in plant vacuoles, are responsible for the red, purple, and blue colors of plant leaves, enhance the antifreeze capacity, and prevent photoinhibition from taking place in chloroplasts [5]. Biosynthesis of Ants is stimulated by developmental signals and environmental stresses, e.g., drought, pathogen infections, and insect attacks. Red leaves contain Ants in vacuoles, cell walls of epidermal cells, and palisade parenchyma, where they are most effective as light screens and modify the photosynthetic profile by restricting the absorption of light by chloroplasts [6]. Photosynthesis is lower in red leaves than in green leaves [7]. Ants, porphyrins, Chls, and carotenoids (Cars) participate in light

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absorption in particular bands, and can be assessed with absorption and reflectance spectroscopy. The absorption spectra of Ant-containing plant tissues indicated strong overlapping of Ant and Chl absorption, and interfered with Chl detection [8].

Measurement of spectral reflectance provides a fast, nondestructive method for the *quantitative analysis* of pigments. A large number of spectral indices have been developed for estimating leaf pigment contents. For instance, the photochemical reflectance index (PRI) is related to Car/Chl ratios in green leaves [9]. Moreover, the normalized difference vegetation index (NDVI) is a measure of the difference between near-infrared light and red light reflected from plants, and is used to identify the health status of plants [10]. In Ant-containing leaves, the Ant reflectance index (ARI) is considered Chl's contribution to reflectance in this spectral region and the retrieved Ant content from reflectance over a wide range of pigment contents and compositions [11]. A previous study also demonstrated that the red region did not significantly affect estimation of Chl from spectral reflectance [12]. However, a new equation was created to eliminate the distorting effect of Ants on Chl contents, regardless of the Ant concentration [13]. It is difficult to detect trace pigments, such as PPIX, MgPP, and Pchlide, using spectral reflectance. A high-performance liquid chromatographic (HPLC) analysis is a traditional method for pigment analysis, but it is time-consuming and expensive, and is not suitable for analysis of large numbers of samples. Consequently, photospectrometry is recommended due to its fast and economic analysis [13].

In the present research, we showed that the absorption spectrum of Ants overlapped with peaks of porphyrins when using 80% of acetone, and the effects of Ant interference was reduced using a modified equation. The new method was able to correct ratios of each porphyrin, and was applied to test Ant-containing leaves.

2. Materials and Methods

2.1. Growth Conditions

Ant-containing leaves, including *Acalypha wilkesiana*, *Plectranthus scutellarioides*, red-leaf cultivar of *Ipomoea batatas*, *Perilla frutescents, Excoecaria cochinchinensis, Stromanthe sanguinea* 'Tricolor', *Alternanthera ficoidea, Alternanthera dentate* (Fig. 1A-H), were used in this study. Leaves of *Bidens pilosa* were used as a control because they are free of Ant content (Fig. 1I). Plants were grown in a greenhouse at 28 °C with illumination by natural sunlight.

2.2. Ants Determination

Fresh leaves (0.2 g) were frozen in liquid nitrogen and ground in a mortar, and then 4 ml methanol/1% HCl was added and mixed thoroughly. After centrifuging at 8000 ×*g* for 10 min and 4 °C, the supernatant was determined photometrically (Hitachi U2800, Tokyo, Japan). Ants were calculated using $A_{530}-0.333 \times A_{657}$, [14] where A_{530} and A_{657} are absorbances at 530 and 657 nm, respectively.

2.3. Porphyrin Determination

Fresh leaves (0.2 g) were frozen in liquid nitrogen and ground in a mortar, and then 4 ml of 80% acetone (v/v) was added. After being centrifuged at 8000 ×*g* for 10 min and 4 °C, the supernatant was determined photometrically using the following Eqs. [15]:

$$\text{PPIX (nmol ml}^{-1}) = 196.25 \times A_{575} - 46.6 \times A_{590} - 58.68 \times A_{628}$$

$$\text{MgPP (nmol ml}^{-1}) = 61.81 \times A_{590} - 23.77 \times A_{575} - 3.55 \times A_{628}, \text{ and}$$

$$\text{Pchlide (nmol ml}^{-1}) = 42.59 \times A_{628} - 34.22 \times A_{575} - 7.25 \times A_{590}$$

where A_{575}, A_{590}, and A_{628} are absorbances at 575, 590 and 628 nm, respectively.

2.4. Ant Concentrations Affect Porphyrins

Ants were extracted from a red-leaf cultivar of sweet potato (*I. batatas*). Fresh leaves (5 g) were frozen in liquid nitrogen and ground in a mortar, and then 100 ml of water was added. After centrifuging at 8000 ×*g* for 10 min and 4 °C, the supernatant was freeze-dried and stored at −20 °C until use.

Porphyrins were extracted from fresh *B. pilosa* leaves. Samples (0.5 g) were frozen in liquid nitrogen and ground in a mortar, and then 10 ml of 80% acetone was added. After centrifuging at 8000 ×*g* for 10 min and 4 °C, the supernatant was mixed with different concentrations of Ant freeze-dried samples (Fig. S1). Measurements of concentrations of individual porphyrins were previously described [15].

2.5. Porphyrins Extracted with Ether

Fresh leaves (0.2 g) were frozen in liquid nitrogen and ground in a mortar. First, Ants were extracted with 2 ml of distilled water, and porphyrins extracted with 2 ml ether were added and gently mixed. After centrifuging at 1800 ×*g* for 10 min and 4 °C, the upper supernatant was collected and dried with nitrogen gas. Thereafter, the dried residue was re-suspended in 4 ml of 80% acetone, the optical density value was determined, and the equations used were the same as previously described [15].

3. Results and Discussion

3.1. Comparisons of Extract Efficiencies Between the 80% Acetone Method and Ether/Water Method

Fig. 2 shows that in test Ant-containing leaves, Ant was extracted with 80% acetone, and the absorption spectra of Ant overlapped with absorption peaks of porphyrins, where PPIX at 575 nm is represented by a blue arrow, MgPP at 590 nm by a red arrow, and Pchlide at 628 nm by a green arrow. Ants are water soluble, but insoluble in ether. Therefore, the ether/water extraction method was used to remove Ant interference from porphyrin detection. However, Fig. 3A shows that the ether/water method had much lower extraction efficiency (75% of acetone extraction efficiency in *B. pilosa*) and was more time-consuming compared to the 80% acetone method. Furthermore, both the ether/water and acetone methods exhibited similar results for analyses of individual porphyrin ratios of *B. pilosa* (Fig. 3B), indicating that there were no influences on porphyrin ratios between these two extraction methods. Hence, in the study, the ether/water method was used as an individual porphyrin ratio standard.

3.2. Interference Between Porphyrins and Ants

Since the relationships among spectral indices were influenced by the presence of Ants (Fig. 4), individual porphyrin concentrations and ratios (%) with different concentrations of Ants were tested. Ants were extracted from freeze-dried samples of a red-leaf cultivar of sweet potato leaves with distilled water, followed by mixing with the 80% acetone extract of *B. pilosa* leaves. Fig. 4A shows that Ants interfered with individual porphyrin ratios when using equations of individual porphyrin contents [15] in Ant-containing leaves. PPIX ratios (%) increased with a high Ant concentration, but Pchlide ratios (%) decreased as Ants increased. MgPP ratios (%) slightly changed with different concentrations of Ants. Absorbance values of any solution should be < 2 when using a photospectrometric method, and even if a reading is > 1.5, the solution should be diluted for accuracy (Fig. S1). Therefore, we *corrected* the *errors* of individual porphyrin ratios by the slope of each porphyrin ratio with low Ant levels (< 1.4 units/ml), which interfered with individual porphyrin ratios, and the extent of the effect was correlated with Ant concentrations. The modified equations of individual porphyrin concentrations were as follows: