Journal of Tropical Crop Science Vol. 11 No. 2, June 2024
www.j-tropical-crops.com

Received 15/01/2024; Revised 31/05/2024; Accepted 11/06/2024
https://doi.org/10.29244/jtcs.11.02.147-154

Modification of The Spectrophotometric Analysis Protocol by Sims D. and Gamon to Analyze Leaf Pigment Content using Green Spinach (*Amaranthus hybridus*) as a Model Plant

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Abstract

The analysis of pigment content in plants, particularly chlorophyll a, chlorophyll b, anthocyanin, and carotenoids, is crucial to assess their physiological performance. The spectrophotometry method by Sims and Gamon offers practicality and reliable results. This research established several variables to achieve more precise results. Green spinach leaf samples were used as a model to analyze their pigment content, determining the minimum sample area or weight, the maximum limit that does not violate Beer's law, and the temperature during analysis. The limit of quantitation (LoQ) is the smallest quantity of analytes in a sample that still meets the criteria for accuracy and precision in testing. The research results show that using a leaf punch with a diameter of 9 mm approaches the LoQ value. To avoid measurements nearing the LoQ limit, it is advisable to use leaf punch diameters of 10- or 12-mm. Leaf punches of 22 mm are still safe to use, as there has been no deviation from Beer's law. Using unchilled acetone at 27°C did not show significant differences compared to using cold acetone at 7°C for the content of chlorophyll a, total chlorophyll, and carotenoids. However, for chlorophyll b and anthocyanin, despite significant differences, the chlorophyll content measured at room temperature was more practical and relatively higher compared to cold acetone, thus disproving concerns about pigment damage.

Keywords: anthocyanin, carotenoid, chlorophyll, spectrophotometry

Introduction

The pigment content of plant leaves provides valuable information about their physiological performance. Chlorophyll absorbs light energy and transfers it to the photosynthetic apparatus. When there is an excess of energy beyond what is needed for photosynthesis, anthocyanins (red and purple pigments) and carotenoids (yellow pigments) protect the leaves from excess light (Sims and Gamon, 2002).

Given the importance of these three pigments—chlorophyll, anthocyanin, and carotenoid—in plant physiology, analyzing pigment content is common in plant studies. Sims and Gamon (2002) have developed methods for pigment analysis, both destructive and non-destructive. The destructive method was adapted from Lichtenthaler's (1987) method. Many studies have utilized Sims and Gamon's destructive method, including investigations into the effects of potassium bicarbonate leaf spraying to mitigate drought stress in sweet basil (*Ocimum basilicum* L.) (Burbulis et al., 2022), biochemical responses and sugar beet yield under drought stress and vermicompost tea application (Ghaffari et al., 2022), physiological and antioxidant responses to drought and heat stress in four eggplant cultivars (Hannachi et al., 2022), the response of rheophyte *Dyckia brevifolia* (Bromeliaceae) seedlings to submersion and recovery (Costa et al., 2022), and reducing ammonium toxicity in *Salvia splendens* 'Vista Red' through silicon supplementation (Song et al., 2022).

To facilitate the use of this method, further research is needed to establish implementation guidelines that improve its accuracy. Determining the minimum and maximum sample sizes is important so that the lowest analyte concentrations can be detected without interference from instrument noise, and high concentrations do not deviate from Beer's law (Riyanto, 2014). In the Sims and Gamon method, the sample is crushed with cold acetone, while Lichtenthaler's (1987) method did not specify this. Some researchers have used Lichtenthaler's method with unchilled acetone (Ali et al., 2021; Dudek et al., 2014; Sun et al., 2021). To establish this method and determine whether working with chilled acetone is

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https://doi.org/10.29244/jtcs.11.02.147-154

crucial, this study was conducted.

Our study utilized green spinach leaves (*Amaranthus hybridus*) as a model due to their rich plant pigments (Chen et al., 2012) and a relatively large leaf size. Ample foliage was necessary to ensure an adequate number of leaf discs for experiments derived from a single leaf. Green spinach leaves were also employed by Johnston et al. (2013) in a study on recycling pigment solvents. Green spinach (*Amaranthus hybridus*) is a widely favored vegetable due to its high nutritional value and widespread availability. Green spinach can be cultivated conventionally or through hydroponic techniques (Aprilia et al., 2022). The ease of acquiring these leaves and their vibrant green color across the extensive leaf surface further enhance their suitability for research, ensuring representative data collection.

Material and Methods

The materials used in this research include green spinach leaves, acetone solution: Tris buffer pH 7.8 in a volume-to-volume ratio of 80:20, hereafter referred to as 'acetris', distilled water, tissue, and ice cubes. The equipment utilized comprises a Shimadzu UV-VIS spectrophotometer type UV1280, cuvettes, centrifuges, leaf punches with diameters of 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, and 22 mm, volumetric flask 5 ml, a mortar and pestle, centrifuge tubes, a micropipette, funnel, test tubes, marbles, and three thermometers.

The determination of the limit of detection (LoD) and limit of quantitation (LoQ) of the acetris buffer was carried out by measuring the acetris blank absorbance thirteen times at wavelengths of 470, 537, 647, and 663 nm (Sims and Gamon, 2002). The absorbance measurements were conducted in a single measurement mode across the four wavelengths using the UV-Probe software. In determining LoD and LoQ, the acetris blank was read 13 times to obtain the blank absorbance value (Abs) and the standard deviation (SD) value. Magnusson and Örnemark (2014) state that, to obtain an adequate standard deviation, 6 to 15 replications are usually required. The LoD value is the average blank absorbance value plus 3 times the SD, while the LoQ value is the average blank absorbance value plus 10 times the SD (Riyanto, 2014).

To compare cold acetris and unchilled acetris, leaf discs with a diameter of 9 mm as per the Sims and Gamon (2002) method were weighed. Each disc was weighed and ground with acetris until completely pulverized. The grinding process in the cold acetris solution was carried out at an acetris temperature of 1°C, a grinding tray temperature of 2.5°C, and a mortar temperature for the cold acetris treatment of around 7 °C. Grinding with unchilled acetris was conducted at an acetris temperature of 27 °C. After grinding, the sample was transferred into a 5 ml volumetric flask, and any remaining extract in the mortar was rinsed with acetris. The mortar was rinsed until all the chlorophyll had transferred into the volumetric flask. Subsequently, it was topped up to 5 ml with acetris. The filtered solution was collected in 15 ml centrifuge tubes. Centrifugation was conducted at 6000 RPM/4427 G using an Ohaus Type Frontier™ 5000 Series Multi-Type FC5705 230V centrifuge for 7 minutes. The absorbance of the supernatant was measured using a Shimadzu UV-Vis Type 1280 spectrophotometer at wavelengths 470, 537, 647, and 663 nm following the Sims and Gamon (2002) method.

To determine whether there is a deviation from Beer's law, an experiment was conducted using a series of pigment content tests based on leaf area. The leaf punches used had diameters of 3 mm, 4 mm, 5 mm, 6 mm, 7 mm, 8 mm, 9 mm, 10 mm, 12 mm, 14 mm, 16 mm, and 22 mm. After obtaining several leaf discs with areas corresponding to the leaf punch set, pigment content testing was conducted, like the temperature influence experiment method. The recommended size for the leaf disc's extract absorbance value should be above or equal to the LoQ absorbance value. If the extract solution's absorbance reading from larger leaf discs is no longer linear, then those discs cannot be used as they deviate from Beer's law.

Calculation of pigment content using the Sims and Gamon equation (2002) is outlined below.

Pigment Equation (PE):

$$\text{Chlorophyll a } (\mu mol.ml^{-1}) = 0.01373\ A663 - 0.000897\ A537 - 0.003046\ A647$$

$$\text{Chlorophyll b } (\mu mol.ml^{-1}) = 0.02405\ A647 - 0.004305\ A537 - 0.005507\ A663$$

$$\text{Anthocyanin } (\mu mol.ml^{-1}) = 0.08173\ A537 - 0.00697\ A647 - 0.002228\ A663$$

$$\text{Carotenoids } (\mu mol.ml^{-1}) = \frac{(A470 - (17.1 \times (\text{chlorophyll a} + \text{chlorophyll b}) - 9.479 \times \text{carotenoids}))}{119.26}$$

Conversion to $mg.g^{-1}$ is as follows:

$$\text{Pigment } (mg.g^{-1}) = \frac{PE\ \mu mol.ml^{-1} \times 5\ ml \times MW\ g.mol^{-1} \times 0.001\ mg.mol.\mu mol^{-1}.g^{-1}}{\text{sample weight (g)}}$$

According to Sims and Gamon (2002), the units utilized across all equations represent micromoles per milliliter ($mmol.ml^{-1}$). The specified molecular weights are employed to convert gram units in Lichtenthaler's

Received 15/01/2024; Revised 31/05/2024; Accepted 11/06/2024
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work (1987) into mole units: Chl a = 893.5 g.mol^{-1}, Chl b = 907.5 g.mol^{-1}, and total carotenoid = 550 g.mol^{-1}. Leaf pigment contents, expressed in millimoles per square meter (mmol.m^{-2}) (leaf area), result from the calculated solution concentrations, total volume of extraction solution, and total leaf area extracted. MW is molecular weight from Chl a, Chl b, or total carotenoid, and 0.001 μmol^{-1}.g^{-1} is conversion from micromol to milligram per gram.

Anthocyanin is stated in μmol.g^{-1}, the calculation:
Anthocyanin (μmol.g-1) = (PE μmol.ml-1 x 5 ml)/ sample weight (g)

Results and Discussion

Experiment to Determine Limit of Detection and Limit of Quantitation Using Acetone Tris Buffer Blank

LoD (Limit of Detection) is the smallest amount of an analyte in a sample that can be detected, while LoQ (Limit of Quantitation) is the smallest amount of an analyte in a sample that still meets the criteria for accuracy and precision in testing.
LoD = x + 3 SD
LoQ = x + 10 SD
where x is the average concentration of the blank and SD is the standard deviation of the blank (Riyanto, 2014).

The determination of LoD and LoQ involved reading the acetris blank absorbance thirteen times, resulting in the blank's absorbance (Abs) value. Based on the experiment, the LoQ values for wavelengths (WL) were found to be as follows: for WL 470, Abs 0.000; for WL 537, Abs 0.011; for WL 647, Abs 0.025; and WL 663, Abs 0.028, as shown in Table 1. If the LoQ obtained in Table 1 is related to the pigment content series experiment based on area, as shown in Table 2, the smallest punch size usable from Table 2 would be a 9 mm diameter or an area of 0.64 cm^2. The 9 mm leaf punch has a LoQ value greater than the minimum required value in Table 1 for all wavelengths (λ). The 9 mm diameter aligns with the diameter used in the Sims and Gamon method (2002). It's essential to consider optimization for specific plant leaf samples to ensure that the measurement results are not lower than the LoQ value for each wavelength used.

When using sample weight measurements for spectrophotometric analysis, the minimum sample weight should be around 2 mg. Various researchers employ different sample-to-solvent ratios to enable accurate spectrophotometric measurement. For instance, Ali et al. (2021) used 10 mg of sample per ml of 80% acetone for chlorophyll analysis in chili and tomato plants. Dudek et al. (2014) used ethanol at concentrations ranging from 10 to 20 mg.ml^{-1} for chlorophyll analysis in herbaceous plant classification.

Table 1. Acetris blank readings (Blk) for determining LoD and LoQ values.

Blank values	λ 470	λ 537	λ 647	λ 663
Blk 1	0.000	0.002	0.006	0.012
Blk 2	0.000	0.002	0.006	0.012
Blk 3	0.000	0.000	0.002	0.008
Blk 4	0.000	0.003	0.008	0.014
Blk 5	0.000	0.003	0.008	0.013
Blk 6	0.000	0.003	0.008	0.013
Blk 7	0.000	0.003	0.008	0.013
Blk 8	0.000	0.003	0.008	0.013
Blk 9	0.000	0.002	0.008	0.013
Blk 10	0.000	0.003	0.008	0.013
Blk 11	0.000	0.002	0.008	0.013
Blk 12	0.000	0.003	0.008	0.014
Blk 13	0.000	0.002	0.008	0.014
Average (x)	0.000	0.002	0.007	0.013
SD	0.000	0.001	0.002	0.002
LoD x+(3*SD)	0.000	0.005	0.012	0.017
LoQ x+(10*SD)	0.000	0.011	0.025	0.028

Note: Blk = blank, LoD = limit of detection, LoQ = limit of quantitation, SD = standard deviation

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Table 2. Leaf punch series absorbance values

Leaf punch diameter (mm)	WL470.0	WL537.0	WL647.0	WL663.0
3	0.023*	nd	nd	0.016
4	0.041	0.000	0.005	0.032*
5	0.063	0.000	0.016	0.061
6	0.080	0.000	0.019	0.075
7	0.140	0.006	0.048*	0.145
8	0.156	0.006	0.049	0.154
9*	0.199	0.016*	0.079	0.226
10	0.228	0.016	0.084	0.247
12	0.313	0.022	0.122	0.353
14	0.420	0.034	0.174	0.481
16	0.641	0.055	0.285	0.772
22	1.177	0.107	0.513	1.348

Note: nd = not detected; * = LoQ acetris blank, 9 mm Leaf punch; WL = wavelength. The star symbols are determined based on Table 1, the leaf puncher used must have a LoQ value greater than the minimum value across all wavelengths (λ). The choice falls on a leaf puncher sized 9 mm.

Sumanta et al. (2014) employed 5 mg of sample per ml of various solvents for chlorophyll and carotenoid analysis in ferns.

Compared to these methods, the method discussed here appears quite diluted, meaning the solvent-to-sample ratio is relatively high. Similarly, Palta's textbook method (1990), which uses 40 mg/ml of acetone, indicates a significantly higher sample concentration. This method approaches the LoQ limit, still allowing the use of one or two larger hole punch sizes.

Comparison between Cold Acetone and Unchilled Acetone Solutions in Affecting Pigment Content of Fresh Samples

In the Sims and Gamon method (2002), grinding is performed using cold acetone, but the authors did not specify the purpose, or the exact temperature used. The use of cold acetone raises questions about whether it minimizes acetone evaporation or prevents pigment degradation. This experiment compares processing done with cold acetone at 7 °C and unchilled acetone at 27 °C.

Based on Tables 3, 4, and 5, the F-test results with a 5% confidence interval indicate significant differences in chlorophyll b and anthocyanin pigments per wet weight, per leaf area, and dry weight between experiments conducted with cold and unchilled acetone. However, for chlorophyll a, total chlorophyll, and carotene, there were no significant differences. Despite significant differences in chlorophyll b and anthocyanin content between cold and unchilled acetone processing, the levels of these pigments processed with cold acetone were not higher than those with unchilled acetone. This suggests no damage occurred to these pigments during processing with unchilled acetone.

This finding is supported by Sun et al. (2021), who found that in 80% acetone, the temperature range of 8 to 30 °C has little effect on chlorophyll extraction. Zhengfu et al. (2018) demonstrated the influence of temperature on visible light absorbance values in chemical oxygen demand (COD) measurements, indicating that variations in chlorophyll b and anthocyanin content might be due to temperature differences between measurements conducted with cold acetone samples and the blanks used.

Maintaining the temperature of acetone until spectrophotometer reading is challenging, so readings are taken at room temperature (27 °C). The cold acetone is stored on ice at 1 °C, with the tray containing the mortars kept at 2.5 °C and the mortars themselves at approximately 7 °C. Due to the difficulty in maintaining a consistent cold acetone temperature, using unrefrigerated acetone is a practical alternative.

A Series of Tests for Pigment Content Based on Leaf Area

The mathematical equations developed by Sims and Gamon for calculating chlorophyll a, chlorophyll b, anthocyanin, and carotenoid content have streamlined the analysis process and reduced costs. These equations eliminate the need for rare and expensive chlorophyll standards for each analysis, allowing

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https://doi.org/10.29244/jtcs.11.02.147-154

Table 3. The effect of cold acetris and unchilled acetris on pigment content per fresh weight

Parameter	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids	Anthocyanins
	(mg.g^{-1})				(µmol.g^{-1})
CA	1.0496	0.3198b	1.3694	0.1938	0.2972b
UA	1.0387	0.3896a	1.4282	0.2017	0.3986a
F test 5%	0.7959	0.0000	0.2560	0.6665	0.0482
CV (%)	7.42	5.70	6.59	17.04	24.78

Note: CA = cold acetris; CV = coefficient of variation.

Table 4. The effect of cold acetris and unchilled acetris on pigment content per area

Variable	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids	Anthocyanins
	(mg.g^{-1})				(µmol.g^{-1})
CA	0.0185	0.0056b	0.0241	0.0034	0.0052
UA	0.0178	0.0067a	0.0244	0.0034	0.0069
F test 5%	0.2745	0.0003	0.6593	0.9293	0.0833
CV (%)	6.32	16.31	5.85	17.13	26.63

Note: CA = cold acetris; CV = coefficient of variation.

Table 5. The effect of cold acetris and unchilled acetris on pigment content per dry weight

Variable	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids	Anthocyanins
	(mg.g^{-1})				(µmol.g^{-1})
CA	4.8493	1.4777b	6.3270	0.8954	1.3732b
UA	4.7987	1.7998a	6.5985	0.9321	1.8414a
F test 5%	0.7962	0.0000	0.2562	0.6670	0.0482
CV (%)	7.42	5.70	6.59	17.04	24.78

Note: CA = cold acetris; CV = coefficient of variation.

Table 6. Summary of coefficient of determination (R^2) values for pigment test series based on leaf area

Pigment	R^2 values for pigment test series based on leaf area		
	1	2	3
Chlorophyll a	0.9961*	0.9904*	0.9968*
Chlorophyll b	0.9854	0.9891	0.9975*
Total chlorophyll	0.9943*	0.9903*	0.9971*
Anthocyanins	0.9847	0.9576	0.8835
Carotenoids	0.9919*	0.9957*	0.9978*

Note: The asterisk is given to R^2 determination values that are good, such as 0.99, which is close to the value of 1

pigment content to be expressed in milligrams per unit leaf area.

A leaf punch set with 12 diameter sizes (3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 22 mm) was used in this experiment. The results showed that the pigment content measured was linearly related to the leaf punch size used, indicating no deviation from Beer's law, which states that absorbance vs. concentration forms a straight line with slope b (Day et al., 1999).

The proportional response of chlorophyll content concentration in the sample was determined by using a series of leaf area measurements. The presence of a linear relationship was evaluated using the coefficient of correlation R^2 in linear regression analysis, represented by the equation $y = a + bx$, where: a in the intercept, b is the slope, x represents leaf area, and y is the amount of chlorophyll.

Using unrefrigerated acetone for pigment analysis at room temperature is a practical and reliable option,

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https://doi.org/10.29244/jtcs.11.02.147-154

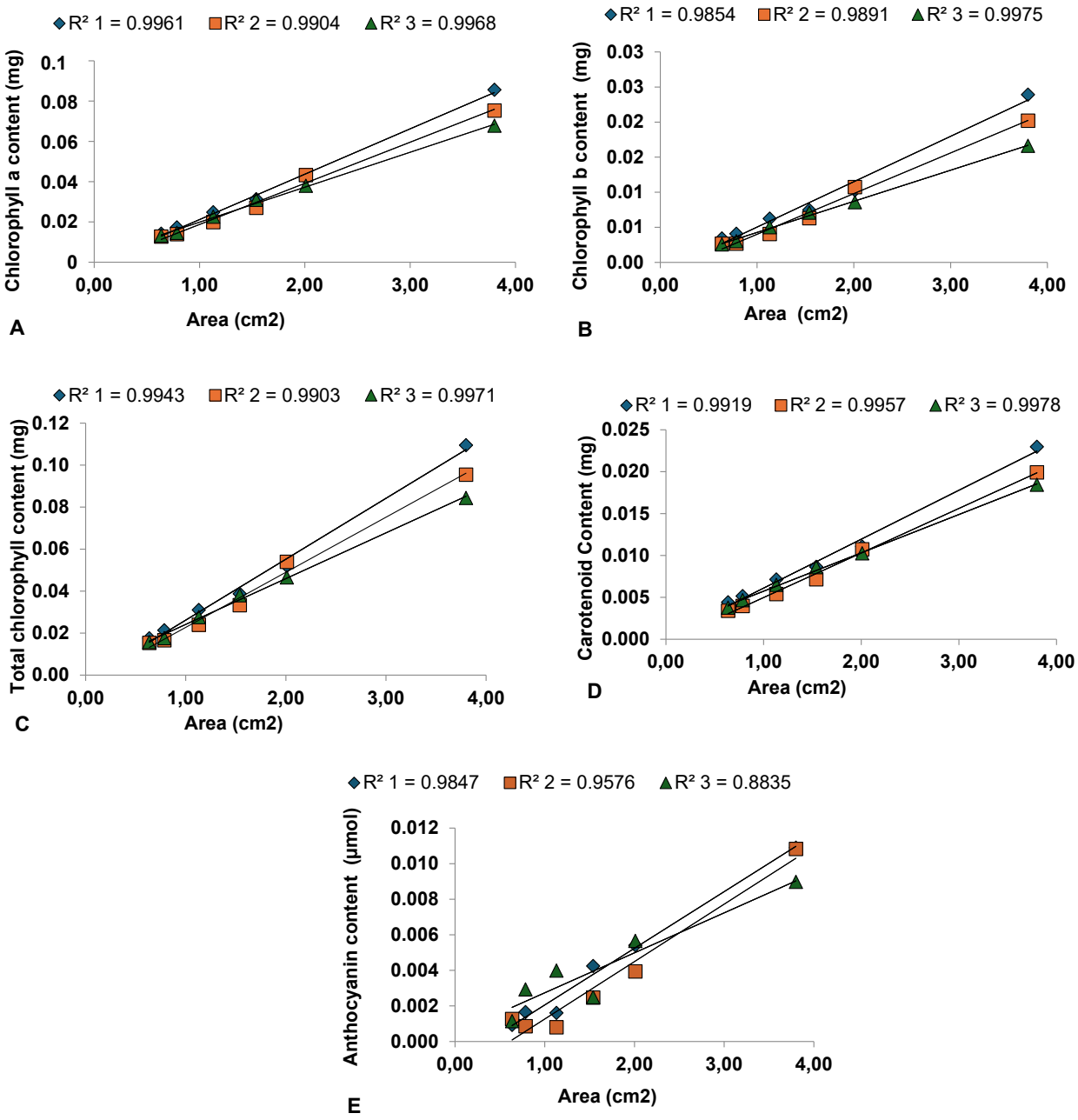

Figure 1. Coefficient of determination (R^2) values for pigment test based on leaf area: chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), carotenoid (D), and anthocyanin (E) measurement series based on leaf area.

as maintaining a consistently cold temperature throughout the analysis process is challenging. The Sims and Gamon equations provide a cost-effective method for determining pigment content without the need for expensive standards, and the linear relationship observed in pigment content measurements across different leaf punch sizes confirms the method's accuracy and adherence to Beer's law.

The summary of slope data for each pigment is presented in Table 6, and the corresponding graph is shown in Figure 1. The variation in slope values within each series could be attributed to using different leaves, as each leaf tends to have different pigment contents due to the influence of agroclimatic factors. Hazrati et al. (2016) stated that water stress and light intensity affect Aloe vera's chlorophyll and anthocyanin contents. Similarly, Kong and Nemali (2021) observed that the presence of far-red and blue light affects the chlorophyll (a) and carotenoid ratios in lettuce plants. Meanwhile, Shah et al. (2017) reported that salinity influences the chlorophyll and carotenoid content in wheat plants.

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An interesting observation from this data is that the coefficient of determination (R^2) for all pigments shows values above 0.9, except for one trial of anthocyanin, which falls below 0.9. An R^2 value of 0.9 is considered good, indicating linearity in the sample, particularly when using the largest punch diameter of 22 mm (3.8 cm^2) or a fresh sample weight of around 75 mg (15 $mg \cdot ml^{-1}$ acetone). The use of a 15 $mg \cdot ml^{-1}$ acetone sample does not deviate from Beer›s law, where R^2 approaches 1, aligning well with the sample weight used by Dudek et al. (2014). An R^2 value of 0.9 is quite satisfactory, considering the pigment distribution within leaves, as shown by Zeng et al. (2021) through Raman spectroscopy, which reveals lower concentrations of pigments around leaf veins and edges.

Sims and Gamon (2002) state that although anthocyanin content can be computed from existing equations, these results are not reliable due to the diverse chemical structures of anthocyanins and their degradation within buffers. However, considering the R^2 values ranging from 0.88 to 0.98 in this experiment, they are still practical for most purposes. For better accuracy specifically for anthocyanin, it is advisable to use a solvent like methanol/HCl/water (90:1:1, v:v) following methods as presented by Sims and Gamon (2002).

Conclusions

Our study demonstrated the results using a leaf punch with a diameter of 9 mm is close to the the smallest amount of analyte in a sample that meets the criteria for accuracy and precision in testing LoQ value. It is recommended to use a leaf punch with a diameter of 10 or 12 mm. A leaf punch of 22 mm is still safe to use, as there is no deviation from Beer's law. Unchilled acetone (27 °C) did not show significant differences compared to using cold acetone (7 °C) for the content of chlorophyll a, total chlorophyll, and carotenoids. However, for chlorophyll b and anthocyanin, despite significant differences, the chlorophyll content measured at room temperature is more practical and was relatively higher compared to cold acetone, thus disproving concerns about pigment damage.

Acknowlegment

The authors thank the Directorate of Human Resources at IPB University for providing research funding through the Competitive Grant Fund for Functional Positions in 2019.

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Received 15/01/2024; Revised 31/05/2024; Accepted 11/06/2024
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