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Spectrofluorimetric determination of chlorophyll a in arugula extracts

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ABSTRACT

As a principal pigment in plants, chlorophyll a (Chl a) is widely used to evaluate quality changes and senescence process during storage of leafy vegetables. The determination of Chl a in plant extracts by spectrophotometric methods using various empirical equations is often unreliable. Considering the sensitivity of fluorescence detection, we report here a simple, inexpensive spectrofluorimetric method that can detect and quantify Chl a in plant extracts. The fluorescence standard used for the quantitative determination of Chl a was isolated in our laboratory from the extract of *Anthrospira platensis* (Spirulina). The method proved to be reliable, fast, and low cost in a study of the influence of the most commonly used domestic storage conditions on Chl a degradation in fresh-cut arugula.

KEYWORDS

chlorophyll a, arugula extracts, spectrofluorimetry, storage conditions, degradation

1. INTRODUCTION

Chlorophylls, besides playing a crucial role in photosynthesis, are used in industry as stable, non-toxic, and physiologically safe colourants for dairy products, edible oils, soups, beverages,

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cosmetics, and medicines. Various therapeutic properties of chlorophyll have been reported, such as anti-inflammatory effect, acceleration of wound healing process, immunomodulatory properties, etc (Chernomorsky and Segelman, 1988). Due to its antioxidant, anti-inflammatory, and detoxifying properties, chlorophyll and its derivatives are used in medicines and food supplements (Ferruzzi and Blakeslee, 2007). To date, 12 types of chlorophylls are known, of which chlorophyll a (Chl a) and chlorophyll b (Chl b) are the most abundant (Pareek et al., 2017). Chl b always occurs in lower amounts than Chl a, and their ratio varies from 1:2 to 1:5 (Strain and Svec, 1966) depending on variety of factors including genetic (species, cultivar) and environmental factors. Chlorophylls are considered relatively unstable compounds, especially in the presence of light and oxygen. It has been shown that acids, temperature, various pollutants, storage methods, or various enzymes can lead to the degradation of chlorophylls (Ngamwonglumlert et al., 2017). Destruction of the chemical structure of chlorophyll results in the loss of the green colour of leaves through the formation of metal-free derivatives such as the grey-coloured phaeophytin, the yellow-brown pheophorbide, and other colourless products (Hendry et al., 1987). Inherent instability of chlorophyll reduces the commercial value and shortens the shelf life of leafy green vegetables during the storage period. The Chl a content in extracts of various plant materials is routinely determined spectrophotometrically by measuring absorption maxima in blue and red spectral ranges followed by application of empirical equations (Lichtenthaler and Buschmann, 2001). The accuracy of UV–Vis spectroscopic measurements depends on sample, solvent system, and spectrophotometer used. Bearing in mind the higher sensitivity and selectivity of fluorimetric assays (Maxwell and Johnson, 2000) compared with conventional UV–Vis methods, the aim of this study was to establish and test a simple low-cost spectrofluorimetric method, capable of detecting and quantifying Chl a in plant extracts. Experiments were performed on Arugula (*Eruca sativa*), an edible annual plant in the *Brassicaceae* family that is popular as salad vegetable. In recent years, many scientific research papers have been published describing the potential health benefits of the phytochemicals found in arugula (Bell and Wagstaff, 2019). Due to its low caloric content, high content of vitamin C, phenolic compounds, glucosinolates, and other biologically active compounds (Guijarro-Real et al., 2019), it is an important ingredient of some diets. The objective of the present work is spectrofluorimetric determination of Chl a content in extracts of arugula stored under the most commonly used home storage conditions.

2. MATERIALS AND METHODS

2.1. General

The analytical grade solvents were obtained from Gram-Mol (Zagreb, Croatia), J.T. Baker (Philipsburg, United States), and Carlo Erba (Val de Reuil, France) and used without additional purification. Silica gel plates for thin-layer chromatography (TLC) containing a fluorescent indicator were purchased from Merck (Darmstadt, Germany).

2.2. Plant materials

Spirulina powder used to isolate the standard was purchased from Encian, Lučko, Croatia. Fresh arugula samples (2 g) were purchased at a local market in Zagreb, Croatia, bagged in commercial

plastic bags, and stored at atmospheric pressure as follows: (*i*) in the light at room temperature (~20 °C), (*ii*) in the dark at room temperature (~20 °C), (*iii*) in the refrigerator at ~ 4 °C, and (*iv*) in the freezer at ~ −20 °C. The duration of storage under the different conditions was based on the kinetics of Chl a degradation.

2.3. Extraction and isolation of Chl a standard from spirulina

1 g of spirulina powder was suspended in 20 mL of acetone and 300 mg of $CaCO_3$ was added. After vortex homogenisation for 1 min in a Falcon test tube wrapped with aluminium foil, the acetone extract was filtered and concentrated on a rotary vacuum evaporator at 25 °C. Preparative TLC isolation of the standard was preceded by chromatography of the extract on small silica gel plates to determine the mobile phase in which Chl a is separated from the remaining pigments, and a mixture of petroleum ether and acetone in a 75:25% ratio proved optimal. Separation and isolation of Chl a by preparative TLC was performed using the same eluent ($R_f = 0.65$). To minimise photodegradation, TLC was performed in the dark. Chl a was desorbed from the silica gel by multiple washes with acetone, and the resulting solution was evaporated to dryness.

2.4. Extraction of pigments and isolation of chlorophylls from stored arugula

In a similar way as described for the standard, chlorophylls were extracted from stored samples of arugula. 5 g of plant material was homogenised with 20 mL of acetone and 300 mg of $CaCO_3$ using a mortar and pestle for 5 min. After filtration, the acetone extract was evaporated to dryness under reduced pressure at 25 °C. The residue was dissolved in 30 mL of petroleum ether, washed with distilled water, dried over anhydrous sodium sulphate, filtered, and evaporated to dryness. Isolation of chlorophylls from the remaining pigments in the extracts was performed by preparative TLC in the same manner as described for the isolation of the Chl a standard. Depending on the storage conditions, the extractions were performed at different time intervals, as shown in Table 1. All extraction experiments were performed in duplicate, and the average values are reported for reproducibility.

2.5. Determination of Chl a concentration using fluorescence spectroscopy

The concentration of the methanolic solution of a Chl a standard was determined with a PerkinElmer Lambda 25 UV/ViS spectrophotometer by measuring the absorbance at 660 nm and applying the Beer-Lambert law ($\lambda = 32{,}600\ dm^3\ mol^{-1}\ cm^{-1}$, $l = 1$ cm). Standard solutions of Chl a were prepared by serial dilution with the same solvent to give the concentrations

Table 1. Experimental design for study of Chl a degradation in arugula stored under various conditions

Sampling days								
Storage	2	4	8	11	16	23	30	43
Light, ~20 °C	+	+	+					
Dark, ~20 °C	+	+	+					
Refrigerator	+	+	+	+	+	+	+	+
Freezer	+	+	+	+	+	+	+	+

ranging from $1 \cdot 10^{-7}$ mol dm^{-3} to $2 \cdot 10^{-6}$ mol dm^{-3}. A standard fluorescence calibration curve was generated using an excitation wavelength of 405 nm (Wesolowski, 2014) and an emission wavelength of 673 nm, ex. slit of 10 nm and em. slit of 5 nm, with a PerkinElmer LS 55 fluorimeter (Fig. 1).

The concentration of Chl a in the arugula extracts was determined using the following equation:

$$c(\text{Chl a}) = \frac{\text{rel. int. fluo}(\lambda_{\max} = 673 \text{ nm}) - 14.59}{1.38 \cdot 10^8 \text{ dm}^3 \text{ mol}^{-1}}$$

All measurements were run in triplicate (Table 2).

3. RESULTS AND DISCUSSION

The first part of this study was devoted to the modification of the fluorimetric method (Wesolowski, 2014) for the determination of Chl a concentration in acetone extracts of arugula. The construction of the calibration curve was preceded by the isolation of the Chl a standard from Spirulina. Spirulina was chosen as the standard source because it produces only the

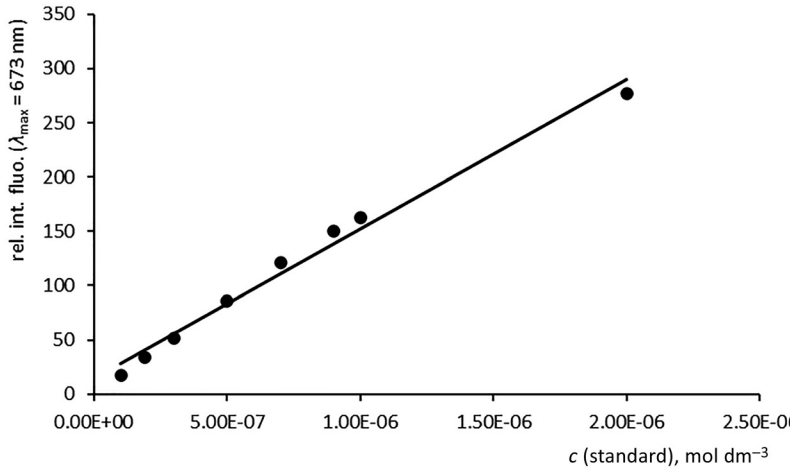

Fig. 1. Chl a calibration curve

Table 2. Concentration of Chl a in arugula extracts stored under various conditions. Initial concentration of Chl a is $(7.1 \pm 0.6) \cdot 10^{-7}$ mol dm^{-3}

10^7 c (Chl a), mol dm^{-3}								
Storage days	2	4	8	11	16	23	30	43
Light, ~20 °C	4.4 ± 0.4	2.4 ± 0.2	n.d.	–	–	–	–	–
Dark, ~20 °C	4.1 ± 0.3	2.1 ± 0.1	0.9 ± 0.1	–	–	–	–	–
Refrigerator	6.3 ± 0.5	5.1 ± 0.4	3.9 ± 0.3	2.6 ± 0.2	1.8 ± 0.1	2.6 ± 0.2	1.8 ± 0.2	0.5 ± 0.1
Freezer	6.7 ± 0.6	4.7 ± 0.4	4.6 ± 0.4	4.8 ± 0.4	3.2 ± 0.2	3.4 ± 0.3	4.1 ± 0.3	3.3 ± 0.2

evolutionarily more distant Chl a (Bauer et al., 2019), thus avoiding overlap of Chl a and Chl b lines in TLC and absorption bands in UV spectra. Acetone was used as a solvent for the extraction of pigments from spirulina and arugula samples because of its high volatility, which allows it to evaporate easily at reduced pressure and room temperature, preventing the degradation of chlorophyll at elevated temperature. To prevent the possible acid-catalysed conversion of chlorophyll to pheophytin, calcium carbonate was added during the extraction of all samples. Since chromatographic separation can be affected by even small amounts of water, after evaporation of the acetone extract of arugula, water and petroleum ether were added to the residual mixture to separate the organic phase from the water derived from the fresh leaves. Prior to the determination of Chl a content, the carotenoids known to "quench" the fluorescence of chlorophyll (Shinkarev and Govindjee, 1993) were removed from the arugula extracts by preparative TLC. It is important to emphasize that arugula contains both types of chlorophylls, but fluorimetric determination of Chl a concentration need not be preceded by its complete separation by TLC because only Chl a fluoresces due to excitation at 405 nm. The second part of the study is devoted to the determination of Chl a concentration in extracts of fresh arugula stored under different conditions (Table 2) using a fluorimetric method. The Chl a content was determined four times within eight days for the samples stored at room temperature, as the amount of remaining Chl a fell below 15% of the initial concentration after eight days.

As shown in Fig. 2 left, the degradation of Chl a in arugula stored in the light at room temperature is relatively rapid, as less than 5% of the Chl a is retained after eight days. A similar trend was observed in the arugula sample stored at room temperature in the dark (Fig. 2 right), with the difference that the amount of Chl a still present after eight days of storage is significantly higher in the case of the sample stored in the dark.

The concentration of Chl a in arugula samples stored in the refrigerator at ~ 4 °C and in the freezer at ~ −20 °C was determined nine times during 43 days (Fig. 3 left and right). The degradation of Chl a in arugula stored at ~ 4 °C is relatively slow, and after eleven days the Chl a content decreased to a value below 50% of the initial value. From the eleventh to the thirtieth day of storage of the sample in the refrigerator, the concentration of the remaining Chl a is approximately constant with slight fluctuations (25–40% of the initial value). After the thirtieth day, the concentration of Chl a begins to decrease sharply and at the end of the measurement (after 43 days of storage) is less than 10% of the initial value. The degradation of Chl a in arugula stored at ~ −20 °C is slow, and after four days the Chl a content has decreased to below 70% of the

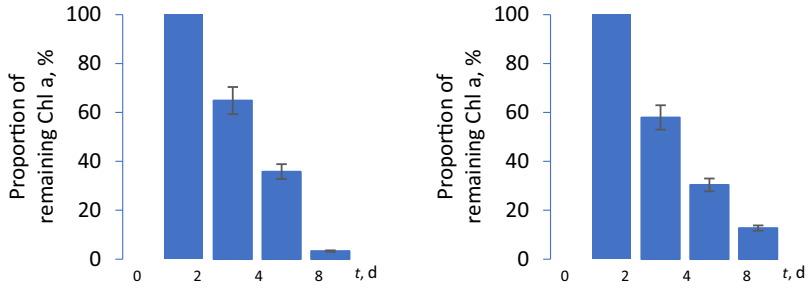

Fig. 2. Proportion of remaining Chl a in extract of arugula stored at ~ 20 °C for 8 days, exposed to light (left) and kept in dark (right)

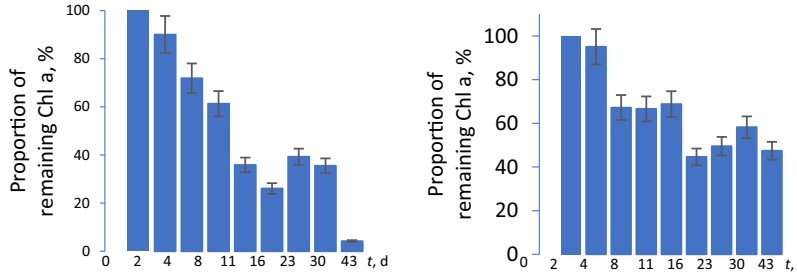

Fig. 3. Proportion of remaining Chl a in extract of arugula stored at ~ 4 °C (left) and ~ −20 °C (right) for 43 days

initial value. From the fourth to the eleventh day of storage, the concentration of the remaining Chl a is approximately constant (65–70% of the initial value) with slight fluctuations. After the eleventh day of storage, the amount of Chl a decreased slightly again and then remains approximately constant (45–60% of the initial value) with slight fluctuations until the end of the measurement. Comparing diagrams in Fig. 3, we find that the degradation of Chl a occurs at about the same rate during the first eight days of storage, and that thereafter the degradation of Chl a is much faster in the sample stored in the refrigerator than in that stored in the freezer.

The results of this study are consistent with previous reports of rapid degradation of chlorophyll in green leafy vegetables stored at 10 °C or above (Vina and Chaves, 2003; Manolopoulou and Varzakas, 2016), and of slower decline in chlorophyll content in both refrigerated and frozen storage (Bergquist et al., 2006; Lisiewska and Gebczynski, 2007; Prabhu and Barrett, 2009). However, it should be emphasised that in similar previous studies, the concentration of total chlorophylls was determined spectrophotometrically, whereas in this study, the concentration of Chl a was determined by the fluorimetric method. Although Chl a fluorescence measurement methods are versatile, noninvasive, and sensitive, and are commonly used to determine the concentration of phytoplankton in aquatic environments (Popik and Gamayunov, 2015) and in plant science (Govindjee, 2004), there are few examples in the literature in which the degradation of Chl a in leafy vegetables was monitored fluorimetrically. By measuring the fluorescence of Chl a, Ferrante and Maggiore (2007) evaluated the quality of *Valeriana lettuce* stored at 4 or 10 °C for 15 days, and the quality changes and senescence process during storage of lettuce and spinach were determined by Baldassarre et al. (2011). Schofield et al. (2005) investigated the storage potential of iceberg lettuce using Chl a fluorescence. In addition to the above examples, Chl a fluorescence has been used to monitor the quality of stored broccoli (Toivonen and DeEll, 2001) and apples (Moshou et al., 2005). In all of the above studies, Chl a fluorescence was determined using a portable, handy fluorometers, and as far as we know, this is the first example of a precise determination of Chl a content in plant extracts that can be routinely performed in all laboratories equipped with fluorimeter.

4. CONCLUSIONS

The aim of this study was to develop a simple, fast, and inexpensive spectrofluorimetric method for the detection and quantification of Chl a in plant extracts. The method was successfully

tested in monitoring Chl a degradation in arugula stored under different conditions. Therefore, we can conclude that the tested method is an excellent alternative to spectrophotometric determination of chlorophyll concentration in plant extracts.

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