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Development of a Rapid UV-Visible Spectrophotometry Method to Assess of Total Carotenoid Content in a Green Microalgae, *Scenedesmus armatus*

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Abstract

The benefits of microalgae are due to the promising sources of pigments influencing researchers to focus on optimizing the culture conditions for the high-yield pigment of microalgae culture. However, conventional procedures to determine microalgae pigments required a large sample volume and toxic chemicals. Due to the drawback of conventional procedures for determining the pigment of microalgae, this research aims to develop a new UV-visible spectrophotometry method using the Remote Diffuse Reflectance Accessory (RDRA) equipped with a UV-visible spectrophotometer for assessment of the total carotenoid content in green microalgae, *Scenedesmus armatus*. Using an optimal preparation of *S. armatus* on the Whatman GF/C™ glass microfiber (GF/C™) filter and scanning UV-visible spectra using RDRA, the characteristic peak of carotenoid at 480 nm demonstrates good analytical characteristics. It exhibits a strong linear relationship with cell concentrations ranging from 2-20 x 10^7 cells/mL ($R^2 = 0.9884$). The developed method yields a total carotenoid content of 2.16±0.58 ng/10^4 cells for *S. armatus*. A paired t-test at a 95% confidence level indicates no significant difference ($P \geq 0.05$) between total carotenoid content obtained using the developed method and a conventional method (1.96±0.24 ng/10^4 cells). In summary, the developed method shows promise for estimating total carotenoid content in green microalgae. Furthermore, the developed method offers advantages over the conventional method by reducing sample processing time and eliminating the need for hazardous reagents and a large volume of samples.

Introduction

Nowadays, the word "food security" has gained significant importance due to the impacts of climate change. According to food security, there is a global focus on the search for highly nutritious food sources. Microalgae are considered one of the best candidates for high-nutrition food sources. Microalgae are recognized for their ability to provide abundant amounts of various nutrients, including essential vitamins, minerals, proteins

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and fatty acids. Additionally, microalgae are particularly rich in pigments such as chlorophyll, carotenoids and phycobiliproteins. Pigments are widely used in many industries, such as pharmaceuticals, food and functional food and supplements. Pigments serve multiple purposes in the industry as sources of color, antioxidants and bioactive compounds with potential health benefits. *Scenedesmus armatus,* green microalgae, is interested in aquaculture due to its high nutritional value and suitability as a feed source for aquatic organisms due to its high-fat content of 27.4-29.7% of dry weight (Ryckebosch et al., 2011). In addition, *S. armatus* has been an attractive interest because of its potential to produce highly nutritious food and various medical applications due to the high concentration of bioactive compounds such as pigments and carotenoids (Goodwin & Britton, 1988). Carotenoids found in algae are responsible for their colors and possess bioactive properties. Therefore, carotenoids are widely used in many applications, such as food, medicine, cosmetics and aquatic products, due to antioxidant and immune-boosting benefits (Jalal et al., 2013). According to the increasing demands, numerous researchers are conducting studies aimed at optimizing culture conditions for pigments, such as carotenoid production, to maximize pigment yield (Pavel et al., 2016; Razi Parjikolaei et al., 2013; Rise et al., 1994; Seyfabadi et al., 2011). The quantification or determination of pigment content is an essential step in this field of research.

The analysis of each type of pigment in microalgae culture process, particularly carotenoids, involves many techniques, such as flow cytometry (Chen et al., 2017), high performance liquid chromatography tandem mass spectrometry (Soares et al., 2019) and particularly extraction methods using organic solvents (Hyoung, 2001). In 1972, Strickland & Parsons (1972) reported spectrophotometric assays to estimate pigments such as chlorophyll a, b, c and total carotenoid content using acetone as an organic solvent for extraction. These methods have served as standard procedures for estimating pigments in algae, such as carotenoids and chlorophyll. However, these methods are time-consuming and require a large volume of samples and toxic chemical organic solvents for extraction. Therefore, extraction methods do not work well for pigment analysis in microalgae. Due to the large volume of samples and time-consuming pigment estimation in biological samples, spectrophotometric measurement of solid-state samples using diffuse reflectance accessory (DRA) has been considered an effective tool due to rapid and non-invasive sample preparation (Blitz, 1998). DRA and RDRA are widely used in near-infrared (NIR) and UV-visible spectrometers to obtain spectra of various solid-state samples. By means of specific wavelength chemical bond absorption, NIR reflectance spectroscopy is applied for the analysis of plant and animal tissue (Foley et al., 1998) and food quality control applications (Cen et al., 2007; Huang et al., 2008; Nicolai et al., 2007). Diffuse reflectance UV-visible spectroscopy is used in biological research as an in vivo study (Leštan et al., 1993). Besides spectral analysis, DRA is commonly used for color measurement of plant and fruits sample (Kasajima, 2019). For pigment analysis, diffuse reflectance spectroscopy has been applied for carotenoids contents in maize (Brenna et al., 2004), banana and plantain fruit pulp (Davey et al., 2009) and yellow-fleshed watermelon (Davis et al., 2004). In the field of algae research, NIR reflectance spectroscopy has been applied for an estimate and quantitative analysis of protein, carbohydrate and lipid content in microalgae (Dean et al., 2010; James et al., 2011; Larens & Wolfrum, 2013). However, diffuse reflectance UV-visible spectroscopy has rarely been used for studies in pigment analysis for microalgae research (Duppeti et al., 2017). Therefore, this research aims to develop a new method for pigment content estimation, focusing on total carotenoid content in microalgae, *S. armatus*. to create a practical and reliable assay for quantifying total carotenoids in microalgae research. Herein, we describe a protocol for the optimal fabrication of fresh cells on a solid support for absorbance measurements using an RDRA. Moreover, our developed method has been validated by comparing results with the conventional method reported by Strickland and Persons (Strickland & Parsons, 1972).

Materials and methods

1. Apparatus

All UV-visible spectra were recorded on a Cary 60 UV-visible spectrometer (Agilent Technology, Inc. USA) with a xenon flash lamp and a photomultiplier tube (PMT) as a light source and detector, respectively. In all conventional method experiments, sample solutions were carried out with UV-visible spectra measurements using an ultra-microvolume cuvette with an 1 mm path length lid (Hellma GmbH & Co. KG, Germany). UV-visible spectra of solid support samples in developed method

experiments were measured using a VideoBerrelino Remote Diffuse Reflectance Accessory (RDRA) (Harrick Scientific Products, Inc. USA).

2. Reagents and materials

All chemicals in microalgae cultivation were purchased as analytical grade (Sigma Aldrich, USA). Spectroscopic grade acetone (Merck, Germany) was used for the conventional method experiments. Water used for the experiments was purified with a Milli-Q filtration system (Millipore, USA).

3. *S. armatus* cell preparations

The inoculum of *S. armatus* used in the experiment was previously isolated from open-air tilapia tanks by Kamperayanon (2013). According to Chen's modified method, *S. armatus* was cultivated by immobilization techniques using sodium alginate matrices (Chen, 2007). *S. armatus* was immobilized in stable gel beads with a 3-4 mm diameter and an average weight of 0.2 g per bead. The microalgae in gel beads were cultured in Chu's Medium (Chu,1942; Stein, 1973) at 26±2 °C under 3,000 lux light conditions until reaching the stationary growth stage. After removing the algae beads from the media, 3 g of the gel beads were dissolved using 5 mL of 5% $(NaPO_3)_6$ solution (Chen, 2007). The algae suspension was centrifuged at 4,000 rpm for 5 min and the supernatants were discarded. After washing the pellet twice with Milli-Q water and removing the supernatants, the cell pellet was resuspended in 5 mL of Milli-Q water to be used in the subsequent experiments.

4. Determinations of the optimum conditions for preparing samples using RDRA

4.1 Effect of solid supports

Into 1.5 mL Eppendorf tube, 1.0 mL cell solution with the desired number of cells was centrifuged at 4,000 rpm for 5 min and the supernatants were discarded. Then 1.0 mL of Milli-Q water was added to obtain $2x10^7$ cells/mL. To fabricate a solid-state sample for RDRA, 5 mL of cell solution was introduced to three types of solid supports, including the GF/C™ glass microfiber (GF/C™) filter (Whatman), Whatman filter paper No.1 (Whatman) and nylon membrane filter 0.45 μm (ANOW). Then samples were left to dry in the dark at room temperature for 30 min. UV-visible spectra of solid support samples were scanned in the 250-750 nm range using RDRA.

4.2 Effect of volumes of sample

To fabricate a solid-state sample for RDRA, a $2x10^7$ cells/mL cell solution was introduced to the GF/C™ filter with a range of 1-50 mL volume. After drying in the dark at room temperature for 30 min, UV-visible spectra of solid support samples were scanned in the 250-750 nm range using RDRA.

4.3 Effect of temperature

To fabricate a solid-state sample for RDRA, a 5 μL of $2x10^7$ cells/mL cell solution was introduced to the GF/C™ filter. To evaluate the effect of drying temperature, samples were dried in the dark under three conditions at room temperature, 40°C and 60°C for 30 min. Then, UV-visible spectra of solid support samples were scanned in the 250-750 nm range using RDRA.

5. Effect of *S. armatus* concentrations on carotenoids determination under optimum conditions for RDRA

Into a 1.5 mL Eppendorf tube, 1.0 mL of cell solution with the desired number of cells was centrifuged at 4,000 rpm for 5 min and the supernatant was discarded. Milli-Q water (50 – 500 μL) was added to obtain various concentrations as 2 to 20×10^7 cells/mL. To fabricate a solid-state sample for RDRA, 5 μL of the cell solution was introduced onto the GF/C™ filter. Samples were left to dry in the dark at room temperature for 30 min. UV-visible spectra of the solid support samples were scanned in the 250-750 nm range using RDRA.

6. Total carotenoid content determination using developed method

Into a 1.5 mL Eppendorf tube, $1.2x10^7$ cells of *S. armatus* were centrifuged at 4,000 rpm for 5 min and the supernatant was discarded. Then 300 mL Milli-Q water to resuspend cells into a solution. Under optimal conditions, solid-state samples were fabricated using 5 μL of the cell solution onto a GF/C™ filter. Samples were left to dry in the dark at room temperature for 30 min. UV-visible spectra of the solid support samples were scanned in the 250-750 nm range using RDRA.

7. Total carotenoid content determination using conventional method

The conventional method was modified by the Strickland & Parsons (1972) method. Into a 1.5 mL Eppendorf tube, 4.0×10^7 cells of the same bulk sample of *S. armatus* were centrifuged at 4,000 rpm for 5 min. After discarding supernatants, the pellet of *S. armatus* cell was extracted with 400 μL of 90% acetone in water. The suspension was placed on a vortex mixer for 1 min and then centrifuged was allowed for phase separation. The acetone crude extracted solution was separated and transferred into a 2 mL vial. The acetone crude extracted was evaporated under reduced pressure using a rotary evaporator and freeze-drying to remove solvent for dryness. Subsequently, the acetone crude extracted was

redissolved with 400 µL spectroscopic acetone. A 5 µL acetone-crude extract solution was introduced to an ultra-microvolume cuvette with a 1 mm path length lid and then UV-visible spectra in the range of 250-750 nm were recorded.

8. Estimation of total carotenoid content

The total carotenoid content for the developed method and conventional method were estimated using the eq. (1) given by Strickland & Persons calculation (Strickland & Parsons, 1972) as follows:

$$\text{Total carotenoid (ng/104 cells)} = \frac{4xA_{480}xVx1000}{cell\ density} \quad (1)$$

where A_{480} is an equivalent absorbance at 480 nm, V is a volume of cell solution and the cell density is a unit of 10^4 cells. The equivalent absorbance at 480 nm (A_{480}) was calculated using eq. (2) as follows:

$$A_{480} = A^*_{480} \times 10 \quad (2)$$

where A^*_{480} is an absorbance recorded by the ultra-microvolume cuvette with a 1 mm path length lid and RDRA.

9. Statistical analysis

All measurements were carried out for replication samples (N = 3), and the results are expressed as mean values ± standard deviation. Linear least squares regression analyses and Paired t-test (N=30) were performed using the Data analysis tool of Microsoft® Excel 2019.

Results and discussion

1. Determinations of the optimum conditions for preparing samples using RDRA

1.1 Effect of solid supports

Three types of materials were examined to study the proper solid support for the preparation of solid-state samples for RDRA measurement, including GF/C™ filter, Whatman filter paper No.1 and nylon membrane filter 0.45 µm for recorded UV-visible spectra. UV-visible spectra and absorbance at 480 nm of carotenoid characteristic peak response are presented in Fig.1. According to the controlled fabrication conditions with a 5 µL of 2×10^7 cells/mL *S. armatus* died out in the dark at room temperature for 30 min, all types of solid supports exhibited absorption bands at 440-480 nm and 670 nm, contributed to carotenoid and chlorophyll, respectively (Fig. 1a). Moreover, UV-visible spectra of solid-state samples compared to the spectrum of acetone crude extract solution of *S. armatus* (8×10^7 cells/mL), all the bands of solid-state sample had longer wavelength than the acetone crude extract solution (Fig. 2). Consideration on the absorbance at 480 nm, which corresponds to the carotenoid characteristic peak, the result revealed that the highest response was observed on the GF/C™ filter, followed by the nylon membrane 0.45 µm. The lowest response was observed on the Whatman filter paper No.1 filtration. According to properties presented on the product website studies as previous studies, retention sizes and hydrophobic properties of GF/C™ filter (Hickel, 1984; Logan et al., 1993; Cytiva Life Sciences, n.d.), Whatman filter paper No.1 (Cytiva Life Sciences, n.d.; Nishat et al., 2021) and nylon membrane filter 0.45 mm (Hangzhou Anow Microfiltration Co., Ltd., n.d.); Zhang et al., 2010) could be considered for this result. As shown in the image of the GF/C™ filter sample in Fig. 1c, the small pore size of 1.2 µm pore diameter and the hydrophilic polarity of the GF/C™ filter allowed for the even dispersion of *S. armatus* cells on the surface, fitting well within the sample spot size of RDRA (1.5 mm) resulting in the highest response. Despite the Whatman filter paper No.1 having a hydrophilic polarity, which can create a uniform distribution of cells, the retention size of the Whatman filter paper No.1 is 11 mm which means the cells did not retain on the surface, resulting in the lowest absorbance. The pore size of the nylon membrane (0.5 µm pore diameter) is smaller than the size of microalgae cells. The cells can retain well on the surface. However, due to its hydrophobicity, a wider distribution of cells exceeded the sample spot size of RDRA, resulting in a lower signal.

1.2 Effect of sample volumes

This experiment aimed to investigate the effect of *S. armatus* sample volumes on the fabricated GF/C™ filter. As shown in Fig. 3, the absorbance of UV-visible spectra of *S. armatus* at a concentration of 2×10^7 cells/mL increased with the increase of sample volume due to increasing cell concentrations. However, the linearity of the signal and concentrations should be considered. Fig. 3b demonstrates that the absorbance response at 480 nm and the concentration of cells exhibit low linearity and low precision with increasing standard deviations. The measurement uncertainty associated with high sample volumes results from larger diameters, leading to different cell distributions within each volume. Therefore, it can be concluded from this experiment that the optimum sample volume is 5 µL which is an appropriate volume for a sample window of RDRA.

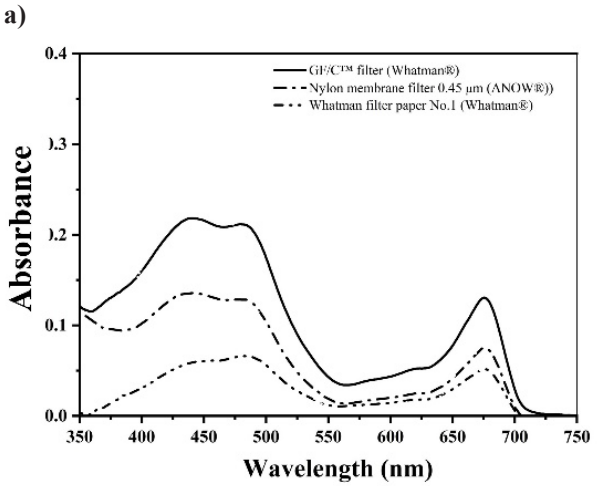

Fig. 1 Effect of solid supports on UV-visible spectra (a) and absorbance at 480 nm (b) of S. armatus 5 µL, 2×107 cells/mL fabricated onto GF/C™ filter (Whatman), Whatman filter paper No.1 (Whatman) and nylon membrane filter 0.45 mm (ANOW); and solid-state samples images of S. armatus fabricated onto solid supports (c).

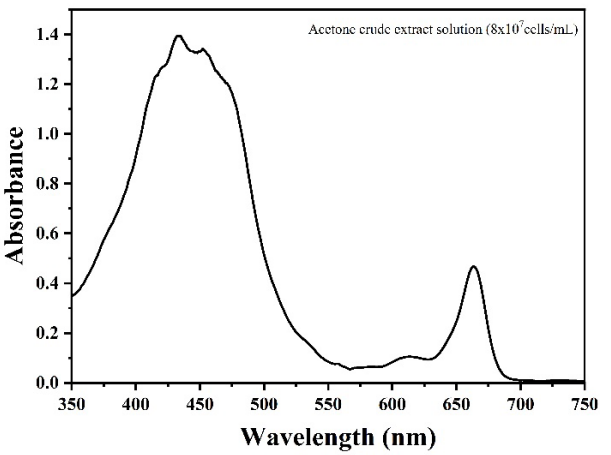

Fig. 2 UV-visible spectrum of acetone-crude extract solution of 8×107 cells/mL *S. armatus*

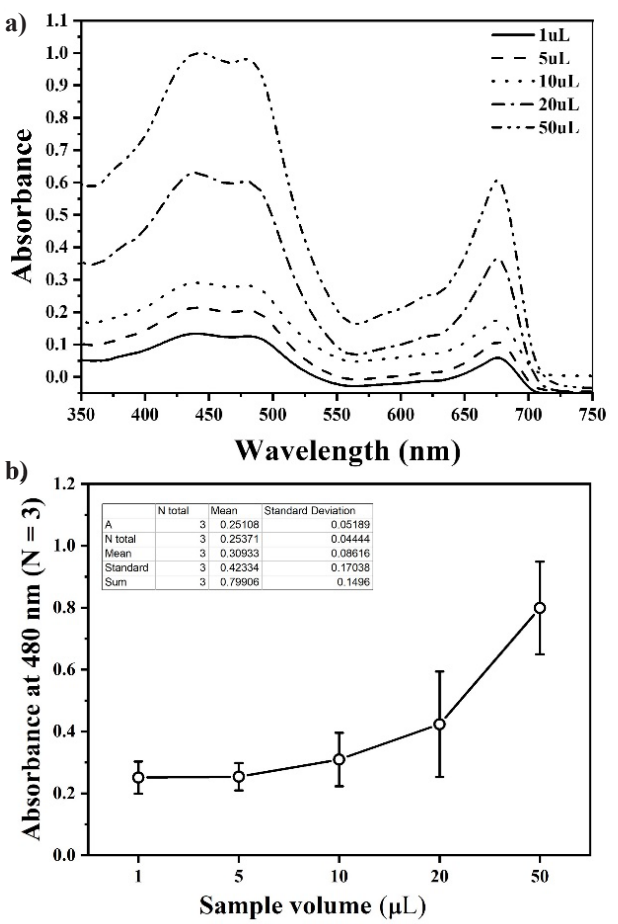

Fig. 3 Effect of sample volumes on UV-visible spectra (a) and absorbance at 480 nm of S. armatus (b) fabricated onto GF/C™ filter (Whatman) with different sample volumes in the range of 1 – 50 mL (cell concentration = 2×107 cells/mL).

1.3 Effect of temperature

In the solid-state sample for RDRA measurement, the sample needs to be dried to minimize the reflection effect of solvent. Therefore, this experiment investigated the effect of temperature on a drying process using an optimal volume of 2 × 10^7 cells/mL introduced on the GF/C™ filter. The samples were dried under a cover at different temperatures, including room temperature, 40°C and 60°C, for 30 min. As shown in Fig. 4, absorbance bands of 40°C and 60°C conditions were lower than room temperature conditions and corresponded to thermal degradation of carotenoids at high temperatures (Sun et al., 2023).

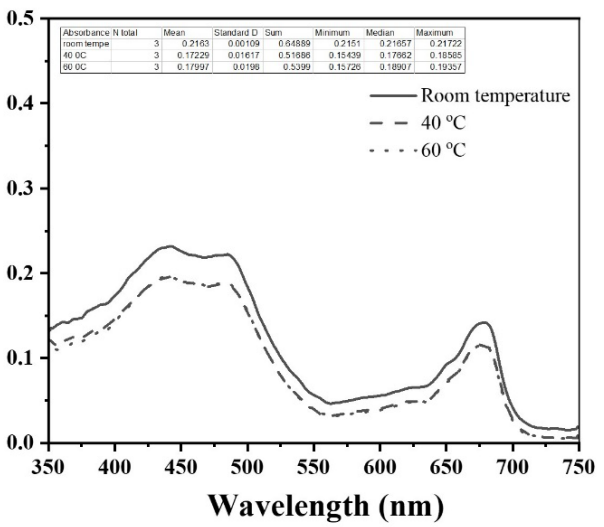

Fig. 4 UV-visible spectra of 5 µL 2×10^7 cells *S. armatus* fabricated onto GF/C™ filter with drying at different temperatures: room temperature, 40°C and 60°C, respectively

2. Possibility to apply the developed method for the analysis of carotenoid content

To apply the method for quantitative analysis, the effect of cell concentrations on the carotenoid response at 480 nm was investigated using the optimum measurement conditions of 5 µL volume sample fabricated on GF/C™ filter and dried in the dark at room temperature for 30 min. The solid-state sample was carried out using optimal conditions as presented in Fig. 5.

Fig. 5 Solid-state sample image of *S. armatus* cells with optimal conditions as the volume of sample 5 µL fabricated onto GF/C™ filter and dried at room temperature for 30 min

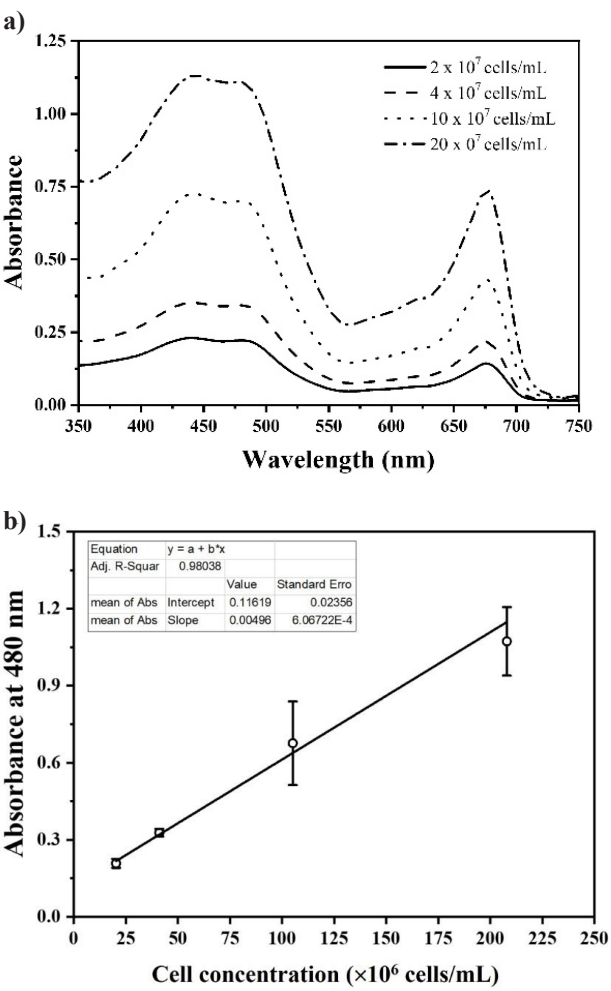

Fig. 6 Effect of cell concentrations on UV-visible spectra (a); and calibration plot of absorbance at 480 nm of S. armatus 2-20×107 cells/mL with optimal conditions as the volume of sample 5 mL fabricated onto GF/C™ filter and dried at room temperature for 30 minutes.

As shown in Fig. 6a, the absorbance band at 480 nm increases with an increase of cell concentrations. The response of the carotenoid characteristic peak at 480 nm in Fig. 6b exhibits a strong linear relationship with cell concentrations ranging from 2-20×10^7 cells/mL (R^2 = 0.9884). These results can be noted that the developed method can be applied to quantitative applications.

3. Comparison of the total carotenoid content of the developed method with the conventional method of Strickland and Parsons

To compare the analytical results of the developed method with the conventional method of Strickland & Parsons (1972), the carotenoid content of both methods were analyzed using the same bulk sample of *S. armatus*. The total carotenoid content of both methods were estimated using the equivalent absorbance at a wavelength of 480 nm (A480) and Eq. (1). The total carotenoid concentration obtained by the developed method under optimum conditions was 2.16±0.58 ng/10^4 cells (n = 30). In comparison, according to the conventional method, the total carotenoid content in *S. armatus* were determined to be 1.96±0.24 ng/10^4 cells (n = 30). A paired t-test at the 95% confidence level was performed to compare the results of total carotenoid analysis by the developed and standard methods. The analysis showed that there was no significant difference ($P \geq 0.05$) between the total carotenoid content obtained by the developed method (2.16±0.58 ng/10^4 cells) and the conventional method (1.96±0.24 ng/10^4 cells). The results of this study demonstrate that the developed method allows for the analysis of equivalent absorbance at 480 nm using a solid-state measured with RDRA to estimate total carotenoid content for microalgae. However, this developed method also provides a low precision as a high standard deviation of the analytical results. The measurement uncertainty may be obtained from the sample in the form of cell suspension. There may be some effect on cells transferred in a variable of each sample on solid support. The high standard deviation of the result also appears in the conventional method. However, both methods offer the same range in the standard deviation (≈25% deviation form means). The high variation of the result of this method may be derived from the loss of sample during the extraction process.

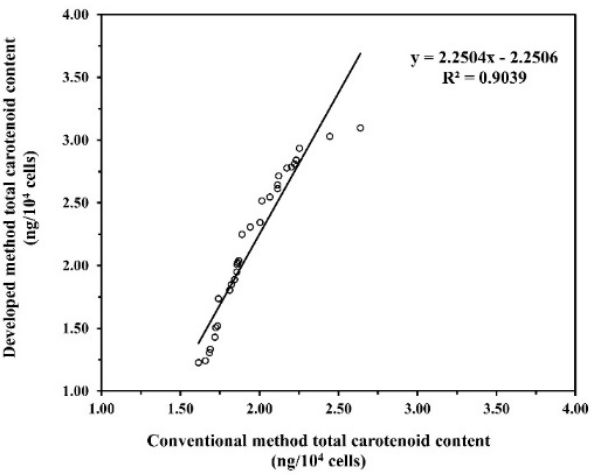

Fig. 7 Total carotenoid content of *S. armatus* (n = 30) using optimal condition of developed method compared to total carotenoid content of the similar bulk sample *S. armatus* (n = 30) using conventional method

Furthermore, the estimated total carotenoid content of *S. armatus* using the developed method was plotted against the total carotenoid content estimated using the conventional method (Fig. 7). A linear relationship was obtained between the estimated two methods with an R^2 of 0.9039. According to the results, the developed method overcomes the drawback of the conventional method for carotenoid estimating for microalgae in terms of large-scale sample requirement, time-consuming and not generating toxic chemicals in the extraction process. It can be concluded that the new development method could replace the conventional method for total carotenoid content estimation for *S. armatus*.

Conclusion

In conclusion, we have successfully developed a new practical method for estimating the total carotenoid content in *S. armatus* using RDRA with a UV-visible spectrometer. The optimal sample preparation on GF/C™ filter as a solid support has demonstrated a characteristic carotenoid peak at 480 nm, indicating promising quantitative application with a well-established linear relationship between the signal and cell concentrations. To assess the suitability of the proposed method for pigment production in microalgae cultivation, we compared the total carotenoid content of *S. armatus* obtained using the developed method with a conventional method using a paired t-test at a 95% confidence level. The results showed that the developed

method exhibits no significant differences in total carotenoid values compared to the conventional method and offers an improvement, including non-hazardous waste generating, saving time and low sample needs. The developed method can serve as a total carotenoid estimating assay in microalgae research due to its simplicity, rapidity and cost-effectiveness. Its advantageous features makes it a practical choice for an efficient and reliable method to routinely estimate carotenoid content in microalgae.

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