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Rapid Quantification of Lipids in Microalgae *Scenedesmus* sp. Using Fluorescence

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HIGHLIGHTS

- Quantification of lipids in microalgae *Scenedesmus* sp. using 2D fluorescence.
- Chemometric Model Applied to lipid content in *Scenedesmus* sp.

Abstract: Lipids extracted from microalgae for biofuel production have drawn the attention of researchers. Various methods for quantification of neutral lipids, such as solvent extraction or gravimetric, require a large volume of sample and are time-consuming. In this work, Nile Red was employed to evaluate the lipid content in microalgae *Scenedesmus* sp. through 2D fluorescence spectroscopy using chemometric modeling with a selection of wavelength pairs reducing the variable numbers. Cultivation of microalgae for 10 days was performed. Samples were taken starting after the 5th day of cultivation. Microalgae samples were dried using an oven with tangential airflow and the conventional Bligh and Dyer method for lipid extraction was employed. Before the measurement of fluorescence, the samples were diluted in ethanol at 40°C for 10 minutes, and then Nile Red solution (10 µg/mL ethanol) was added. The results of lipid quantification using the proposed technique achieved mean absolute percentage error (MAPE) lower than 5% and correlation coefficients around 0.99 in the test phase. Linear models using four excitation-emission pairs of fluorescence were fitted and were selected based on Pure Spectral Chemometric Modelling (PSCM). The method for lipid quantification using predictive linear models and 2D fluorescence proved to be robust and fast (requiring less than 30 min) compared to the conventional gravimetric method (which takes at least 5 hours) to obtain comparable results.

Keywords: lipids; microalgae; *Scenedesmus* sp.; Nile Red; ant colony optimization.

INTRODUCTION

Microalgae are studied as a possible raw material for biofuels production due to their rapid growth and the ability to accumulate neutral lipids that can be converted into biodiesel. In their composition, microalgae contain carbohydrates, proteins, lipids, and fatty acids [1]. Previous studies have produced biodiesel from algal lipids that had similar properties to those of fossil diesel or other common biodiesels [2].

Finding the proper time for extracting lipids from a culture is an important point for process optimization and it is necessary to monitor the evolution of the concentration of the lipids during the cultivation. There are several methods to determine the lipid content in microalgae. Some of these methods are thin-layer chromatography (TLC), Liquid chromatography (HPLC-MS), gas chromatography (GC-MS), and gravimetric [3]. The most common method is gravimetric extraction, which consists of using solvents such as hexane or chloroform and a known amount of dry biomass. The biggest disadvantage of this method is the necessity of using relatively large quantities of samples and reagents. Furthermore, it requires time for the drying and extraction processes [4]. The efficiency of lipid content quantification from microalgae depends on the species and the method utilized. Thus, it is necessary to evaluate a faster alternative to measure lipids that requires small amounts of sample and employs less hazardous solvents.

An alternative could be to use a faster and less aggressive method such as fluorescence spectroscopy. Two-dimensional fluorescence spectroscopy opens the opportunity to measure biological systems (microorganisms and chemical metabolites). Recently, fluorescence spectroscopy has become one of the preferred methods for monitoring bioprocesses that can easily be implemented for lipid quantification.

The fluorescence spectroscopy may require the use of a fluorescent marker to quantify the lipid content in situ. The marking technique employing spectrofluorescence consists of placing a dye solution in the sample taken from the microalga culture, where the mixture stays in contact for the adequate time for the dye to cross the cell wall of the microalga, creating a dye/lipid interaction that can be analyzed by fluorescence spectroscopy. Commercially available dyes having these characteristics are Nile Red and BODIPY 505/515 [3-6].

The Nile Red (9-diethylamino- 5H-benzo[α]-phenoxazin-5-one) has been widely applied as a marker for lipids [7,8]. Nile Red can be used as a selective probe for neutral lipids by properly selecting the excitation and emission wavelengths or using data-driven chemometric modeling techniques [9–12]. Although the Nile Red method has been frequently used to determine the lipid content of various species, the excitation and emission wavelengths vary considerably for different microalgae (see Table 1).

Table 1. Excitation/Emission wavelengths used in different microalgae lipid determination using Nile Red

Microalgae	Excitation [nm]	Emission [nm]
Desmodesmus quadricauda	530	575
Botryococcus braunii	480	580
Dunaliella	480	590
Scenedesmus sp.	530	568

Adapted from Ren H.-Y. [12]

Table 1 shows that previous studies worked with only one pair of excitation/emission wavelengths to predict the lipid content in a culture. However, the relationship between lipid content and fluorescence data reached a coefficient of determination of 0.85, which does not provide a reliable and accurate adjustment. In the case of the *Scenedesmus* sp. it must be verified if an Ex/Em pair is enough to predict lipid concentration, otherwise, it is necessary to use a multi-wavelength spectrofluorometer and chemometric models.

Biological applications have already been carried out and it has been possible to determine the relationship between the fluorescence data and the process variables to be measured [13]. Among the chemometric modeling techniques that are generally applied in data processing, it can be mentioned the techniques of partial least squares (PLS), principal component regression (PCR), principle component analysis (PCA), and neural networks [14–18].

The aim of this study is to propose and evaluate the feasibility of a methodology for lipids quantification from microalgae *Scenedesmus* sp. based on fluorescence spectroscopy measurements, which is fast and uses samples of negligible volume samples, i.e. only 1 mL.

MATERIAL AND METHODS

Microalgae Strain and Culture Conditions

The microalgae *Scenedesmus* sp. was cultivated in Guillard modified culture medium [19]. The culture medium contains two different solutions described in Table 2.

Table 2. Guillard Modified culture medium

Solution	Component	[mg/L]
Macronutrient	$CaCl_2.2H_2O$	367.6
	$MgSO_4.7H_2O$	369.7
	$NaHCO_3$	126
	K_2HPO_4	87.1
	$NaNO_3$	850.1
	$Na_2SiO_3.9H_2O$	284.2
Micronutrient	Na_2EDTA	43.6
	$FeCl_3.6H_2O$	31.5
	$CuSO_4.5H_2O$	0.1
	$ZnSO_4.7H_2O$	0.22
	$CoCl_2.6H_2O$	0.1
	$MnCl_2.4H_2O$	1.8
	$Na_2MoO_4.2H_2O$	0.06

Biomass measurement

The biomass concentration in [g/L] was determined through a curve adjustment between values of optical density and dry weight, measured daily for a total period of 10 days. Optical density was measured at wavelength 570 nm (OD_{570nm}) [20] using a spectrophotometer (UV-1600, Pro-analyze). The dry biomass concentration was measured by weighing samples filtered using 0.7 µm pre-weighed membranes, which were dried in an oven at 100°C for 24 h. The linear adjustment between absorbance values and dry weight is:

$$\text{Biomass [g/L]} = 0.4669 * OD_{570nm} - 0.0243, \quad (1)$$

this equation must be used to convert absorbance values to biomass.

The initial cell concentration of *Scenedesmus* sp. was 0.1 g/L and the algae growth was monitored by measuring the absorbance every day.

Sample Pretreatment for lipid measurement

Samples were conditioned for the lipids extraction of microalgae. First, the samples were obtained by centrifugation (6000rpm, 30 min, 10 ºC). Then, drying was carried out in an oven with a tangential airflow at 40°C. To evaluate the adequate drying time, previous tests were performed using different weights of wet biomass. Figure 1 shows that the samples dried completely in less than 6 hours.

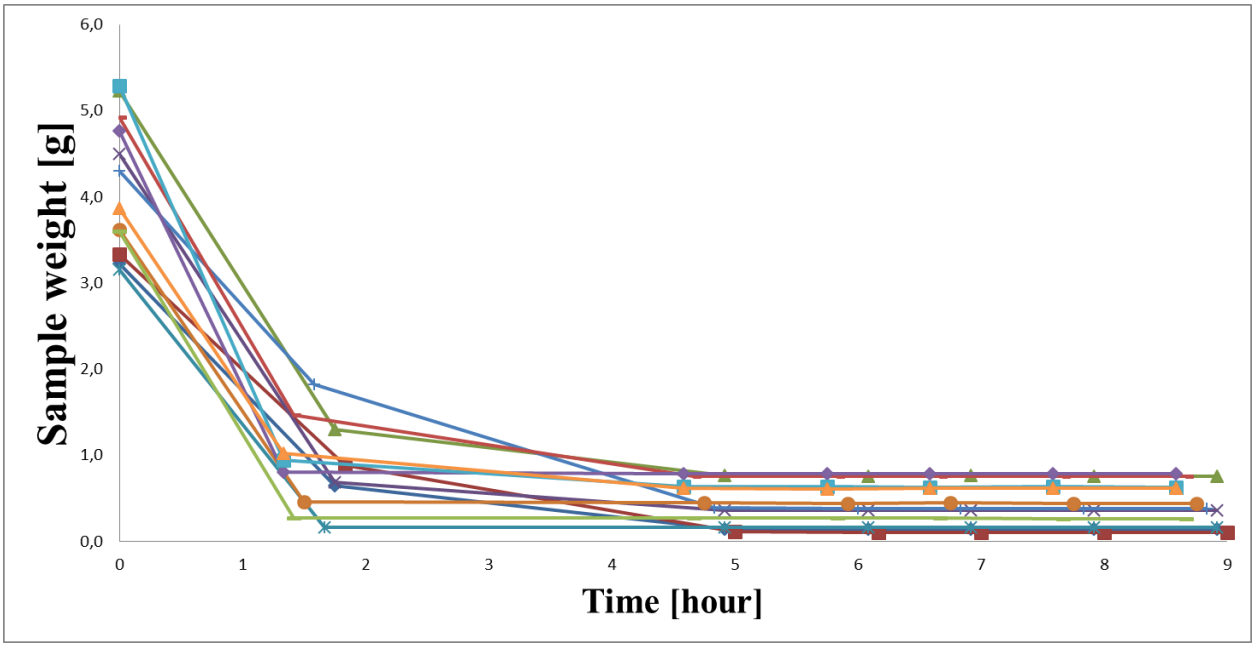

Figure 1. Microalgae drying curve in an oven at 40°C during 9h.

Photobioreactors used for lipid determination

Seven air-lift photobioreactors were installed (more information about the structure is provided in the work of Gris and coauthors [21]. As Xin [22] suggested, the photobioreactors should be maintained at 20°C, the ideal temperature to obtain higher lipid content. Secondly, the airflow was injected into each photobioreactor at a flow rate of 0.75 L/min as well as luminous intensity of 10000 lux (~135 µmol/m^2·s) and photoperiod of 12h light and 12h dark.

Lipid quantification

The lipid content of microalgae was quantified in triplicate from day 5 to day 10 using two methods: gravimetric and fluorescence. The volume required to perform the gravimetric method for lipid quantification between days 5 to 10 corresponds to 48% of the total volume of the experiment. The method of fluorescence spectroscopy required less than 1%. The volume required for the biomass quantification for days 0 to 10 corresponds to 26% of the total culture volume. The lipid quantification was not performed from day 0 to 4 because the gravimetric method required larger sample volumes for those days (> 3L per sample) and the remaining culture (25%) did not have enough volume to provide for all samples.

Lipid determination: Gravimetric method

In order to quantify the microalgal lipid percentage, the gravimetric method was applied. Previous experiments demonstrated that Bligh and Dyer's method provides better recovery of lipids. The method was adapted to be used in microalgae cultivation. For 500 mg of dry biomass, a volume of 30 mL of chloroform was added, followed by 10 mL of methanol. The resulting mixture was transferred to a 50 mL plastic tube and sonicated for 30 minutes (Ultrasonic Bath Unique 1400A MaxiClean 40 kHz, Brazil) at 25 °C. After this, 12 mL of a 1% NaCl solution was added and the tube content was transferred to a separatory funnel. After vigorous stirring, the solution separates into two phases. The lipid phase was filtered. Secondly, the lipid content was transferred to a known mass volumetric flask and placed on a rotary evaporator at 30°C until complete removal of the solvent, and then transferred to a desiccator until it reaches room temperature. Following this process, the flask with the resulting sample was weighed and the number of lipids was obtained by mass difference. All samples were measured in triplicate.

Lipids Determination: Fluorescence

The content of lipids in microalgae was determined using 2D fluorescence after Nile Red staining. Before collecting the two-dimensional fluorescence spectrum, approximately 1mL of microalgae culture was mixed and exposed to 40°C for ten minutes, and then Nile Red solution (10 µg/ml ethanol) was added.

Samples were measured in a quartz cuvette with four polished windows and 1 cm of the optical path. The two-dimensional fluorescence spectra were generated at a range of emission wavelengths between 300 to 800 nm and excitation wavelengths from 300 to 850 nm, with 10 nm increments. The fluorescence spectra were acquired with a spectrofluorometer (FluoroMax-4, Horiba). The fluorescence matrixes were later unfolded as row vectors, where the row represented the sample and each column represented the fluorescence intensity of one Ex/Em pair. All measurements were made in triplicate.

The search for the specific excitation-emission (Ex/Em) pairs that had a greater correlation with the lipid content in the samples was done using the PSCM algorithm (Pure Spectra Chemometric Modelling), developed by Ranzan and coauthors [23]. The algorithm is based on Ant Colony Optimization, a methodology that tries to mimic the indirect communication between real ants in a colony using chemical tracers, called pheromones. Briefly, the aim of the method is to use pheromones to optimize the variable selection of spectral features that will be used to fit a linear model capable to predict an output of interest, similarly as ants use pheromone trails to converge to the shortest route between a food source and the nest. Figure 2 presents a schematic representation of the ACO implementation used in this study.

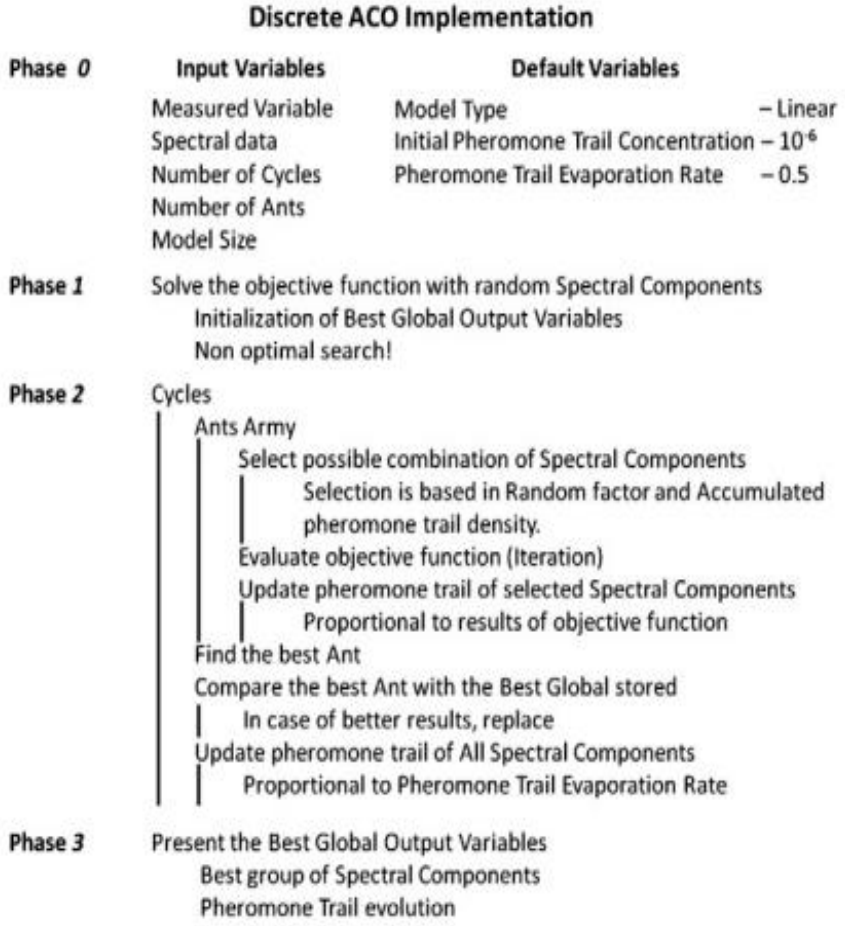

Figure 2. Schematic ACO algorithm. Extracted from Ranzan and coauthors [23].

As can be seen in Figure 2, the algorithm is divided into 4 phases. Phase 0 supplies the input data and defines the optimization parameters: in this study, the spectral data is the matrix of unfolded two-dimensional fluorescence spectra, and the measured variable is the corresponding percentage of the lipid content of each sample (analytical result obtained by the traditional Bligh and Dyer method), the number of cycles was defined as 300 and the number of ants as 400. As for model size (number of Ex/Em pairs each ant will select as input for the linear models), the algorithm was run multiple times, keeping the same parameters, but varying the model size between 1 to 4, to evaluate the predictive performance of models with different complexities. By default, in the initial pheromone trail, every Ex/Em pair has the same pheromone concentration. All fitted models will be linear regressions and the pheromone evaporation rate (amount of pheromone evaporated at the end of each cycle) is 50%.

In phase 1, totally random Ex/Em pairs are selected and a linear model is fit to predict the output variable. The selected inputs and the model's predictive metrics are stored to initialize the global solution.

Phase 2 is the core of the optimization: each ant will, in parallel, select n (model size) Ex/Em pairs, based on a random trigger and in the accumulated pheromone trail - although every variable has a chance to be selected, the more pheromone a variable has, the greater its chance to be selected. Then, each ant will use its selected pairs to fit a linear model to predict the output of interest. The residual error between the measured output and the predicted output is calculated (in this case, the root mean square error - RMSE), and the ant will deposit an increment of pheromone in the selected pairs that are inverse to the residual error: the greater the error, the smaller the pheromone increment. After every ant has fitted its models and deposited its pheromones, the ant whose model has the smallest residual error is found and compared to the global solution: if it is better, this ant's model becomes the new global solution. The pheromone trail is then multiplied by the pheromone trail evaporation rate, to penalize variables that were not selected or that participated in models with high errors. This routine is repeated *i* (number of cycles) times. As the cycle passes, variables that have a greater correlation with the desired output receive more pheromones and are selected more times.

In phase 3 the global solution is presented, with its selected inputs and metrics, and the final pheromone trail.

To truly evaluate the predictive power of the methodology, the complete sample dataset was split into training and test subsets. The training subset is the only one supplied to the ACO algorithm. Once the global solution is obtained, the model is used to predict the percentage of lipid content in samples never seen before by the model - the test subset. If the test metrics are consistent with the training metrics, the ability of the model to deal with new data is confirmed, and the models can be considered more robust and reliable. The train/test split was based on the y-rank methodology: the samples were sorted in ascending order of the output of interest (in this case, the percentage of lipid content), and then the samples were allocated to the train and test subsets following the pattern 2 (train) to 1 (test) [24,25]. The split resulted in a training subset with 66% of the total samples (8 samples) and a test subset with 34% (4 samples).

To compare and evaluate models, three metrics are used throughout this work: (i) mean absolute percentage error (MAPE) defined as

$$MAPE = \frac{100\%}{n}\sum_{i}^{n} \left|\frac{(y_i - \hat{y}_i)}{y_i}\right| \quad (2)$$

where *n* is the number of samples, y_i the real value of the output and $\hat{y}_i$ the predicted value of the output; (ii) root mean square error (RMSE) defined as

$$RMSE = \sqrt{\frac{1}{n}\sum_{i} (y_i - \hat{y}_i)^2} \quad (3)$$

and (iii) the coefficient of determination (R^2) defined in Equation (4),

$$R^2 = 1 - \frac{\sum_i (y_i - \hat{y}_i)^2}{\sum_i (y_i - \bar{y})^2} \quad (4)$$

where $\bar{y}$ is the mean value of y_i.

In addition, it was evaluated two different approaches for the selection of pairs: (i) The use of the whole spectra (WS) and (ii) the use of only the spectra's area of greater fluorescence (AGF) - Emission between 550 and 750 nm - as can be seen in Figure 3.

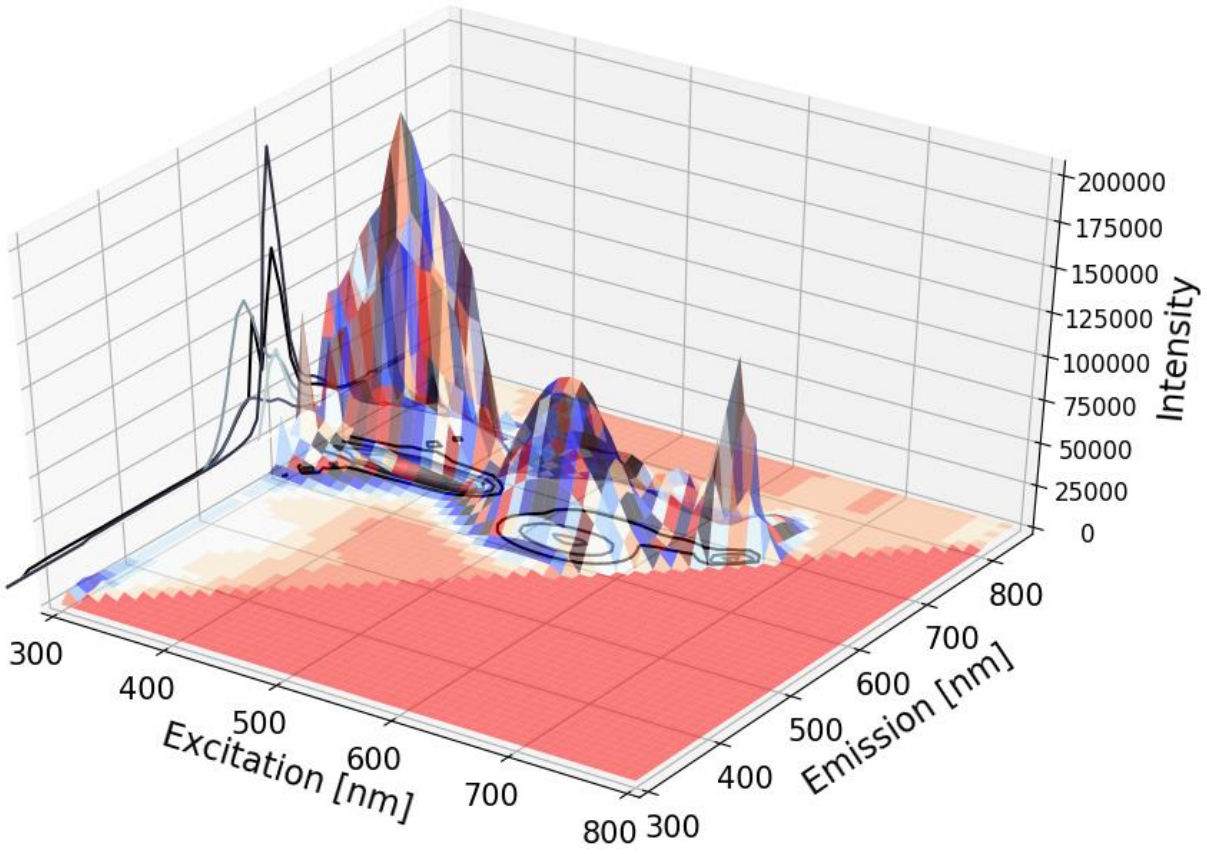

Figure 3. Mean fluorescence spectra of all microalgae samples.

RESULTS

Microalgae Growth Curve

Measurement of the biomass concentration in the exponential growth phase was performed, as shown in Figure 4.

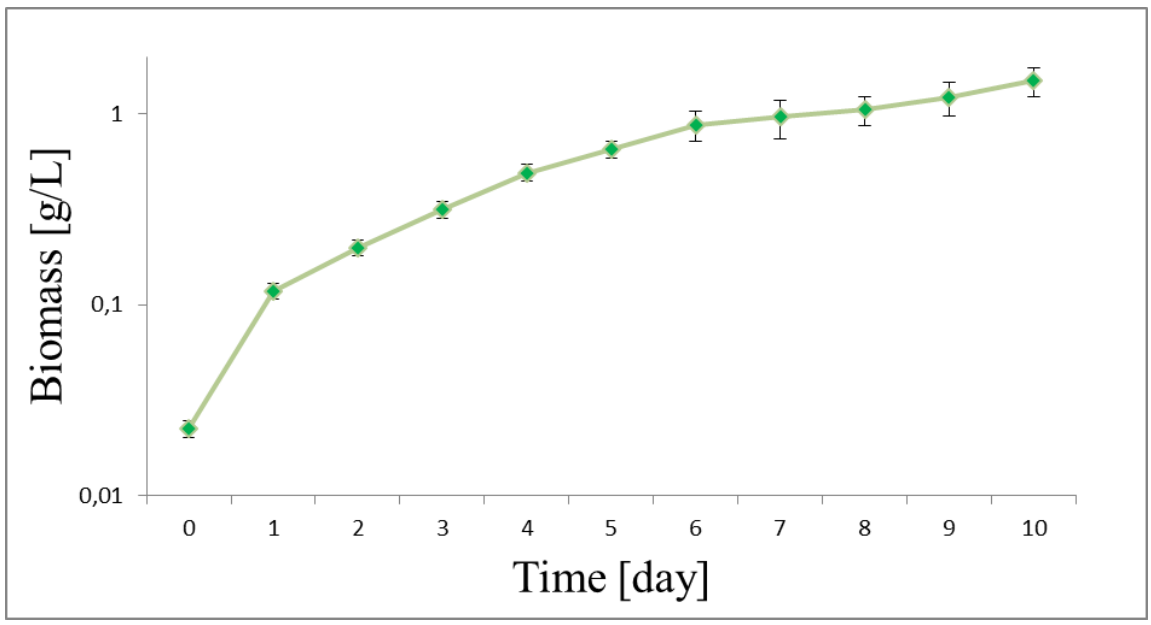

Figure 4. Growth logarithmic curve microalgae *Scenedesmus* sp. in air-lift photobioreactors under conditions of 20°C and 10.000 lux.

Lipid Content: Gravimetric Method

Figure 5 presents the lipid extraction results obtained by the traditional method Bligh & Dyer. It is observed that the contents reached maximum concentration on day 7, and the percentage was similar during the followed days.

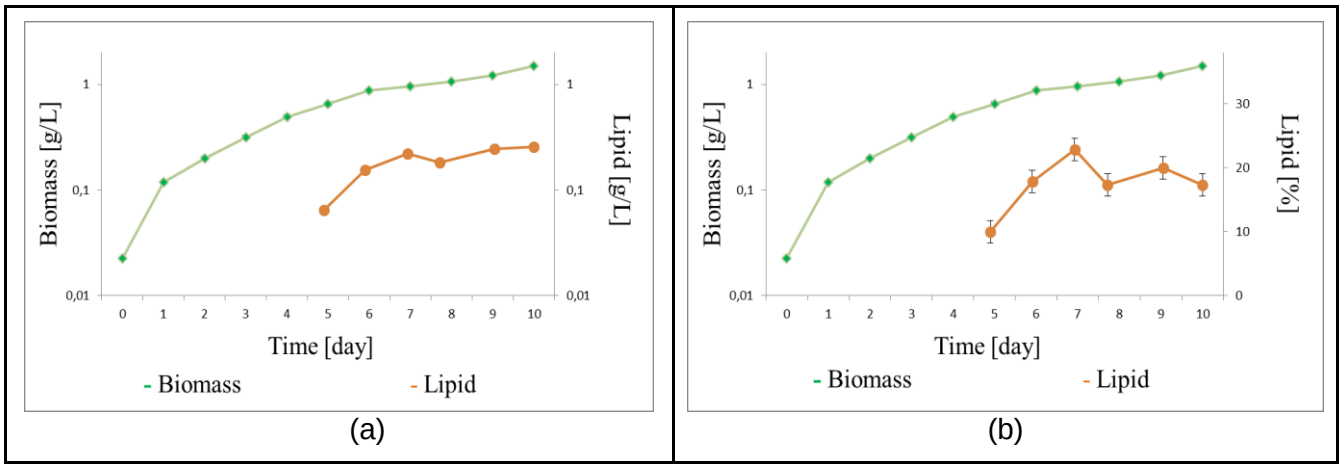

Figure 5. Biomass and lipid content of microalga Scenedesmus sp. (a) Comparison between biomass concentration and lipid concentration; (b) Comparison between biomass concentration and lipid percentage.

Figure 5a shows the biomass concentration [g/L] and the behavior of the concentration of the lipids throughout the cultivation. It was observed that the lipid content increased with the biomass concentration.

Between days 5 to 7, the concentration of the lipids doubled per day (i.e.,100%) but between days 7 to 10 only increased by 1.3 times. Similarly, Figure 5b shows the behavior of the lipids percentage [%] for a sample of 500 mg per day. This allowed us to see that the percentage reached a maximum on day 7, and, in the following days, it was reduced by 5%. Therefore, it may be useful to extract the lipids on day 7 (or add more culture medium in a new photobioreactor for a faster increase in lipids) or continue the culture to obtain larger amounts of biomass.

The results of the analytical method of Bligh and Dyer (seen in Figures 5a and 5b) were used as measured variables for the characterization of lipid concentration using two-dimensional fluorescence spectra and PSCM. Models were trained with 1 to 4 excitation/emission pairs of fluorescence, using the 8 samples selected for the training subset and then tested with the 4 samples of the test subset. Table 3 shows the metrics for the global solution models of each size, and the Ex/Em pairs relative to those models. The results are divided by the search throughout the whole spectra and the search focused on the area of greater fluorescence.

Table 3. Metrics for the best predictive model of each size and the selected Ex/Em pairs

	Whole Spectra						Area of Greater Fluorescence					
	Train			Test			Train			Test		
MS	R^2	MAPE	RMSE	R^2	MAPE	RMSE	MS	R^2	MAPE	RMSE	R^2	MAPE	RMSE
1	0.399	27.54	4.51	0.933	15.15	2.78	1	0.336	29.48	4.74	0.767	21.40	3.47
2	0.940	4.94	1.42	0.448	14.25	2.80	2	0.775	15.04	2.76	0.953	7.42	1.07
3	0.990	2.24	0.59	0.963	5.97	0.85	3	0.995	1.60	0.42	0.762	10.68	1.91
4	0.997	1.32	0.34	0.999	0.59	0.11	4	0.998	1.37	0.25	0.987	4.43	0.78

MS	Selected Excitation/Emission Pairs	MS	Selected Excitation/Emission Pairs
1	460/740	1	540/700
2	310/330 370/480	2	550/620 540/630
3	370/480 580/750 760/770	3	340/650 550/700 430/710
4	370/480 610/700 580/750 760/770	4	460/650 310/660 470/660 530/700

Figure 6a and Figure 6b illustrate the location of the selected pairs, plotted upon a contour map of the mean fluorescence spectra of all microalgae samples. Figure 6a shows all the selected pairs for the whole spectra models and Figure 6b the selection for the AGF.

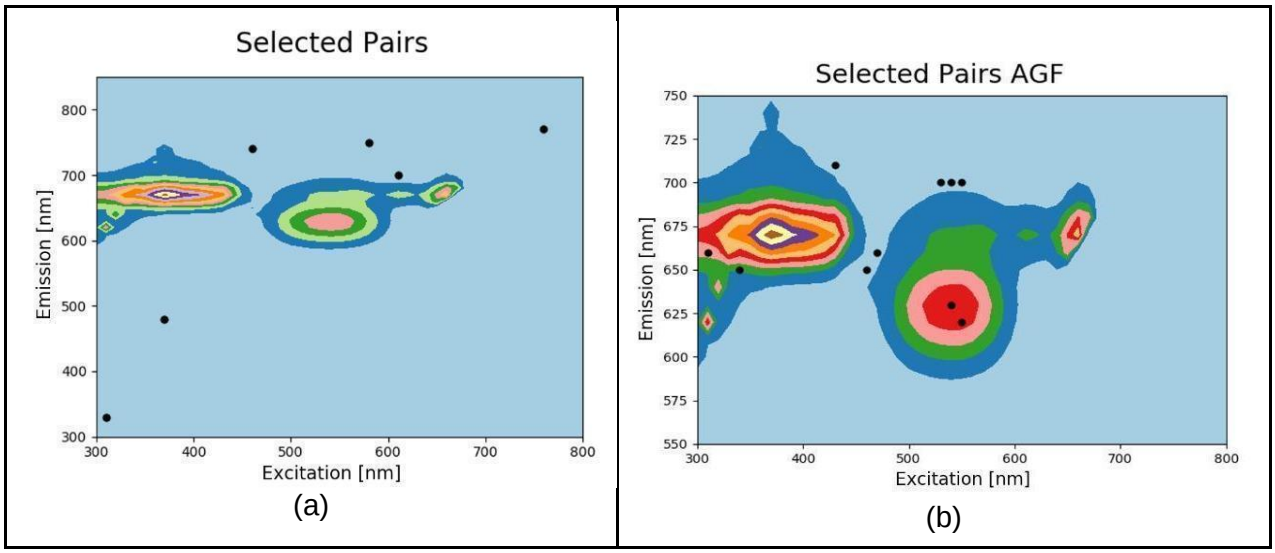

Figure 6. All the selected pairs for (a) the whole spectra models and (b) the AGF models.

DISCUSSION

The percentage of lipids present in the samples of this work matches the lipid content of *Scenedesmus* sp. presented in the bibliography, with values between 8% and 30%, which depend on the cultivation conditions [26–28]. With the use of Nile Red, we achieved results similar to those obtained by Balduyck and coauthors [9] who used Nile Red to determine lipids in Nannochloropsis oculata and T-Isochrysis lutea, comparing results of lipid content using Nile Red for fluorescence measures and gravimetric method. Balduyck and coauthors fitted models using only one Ex/Em pair with R^2 of 0.91, while in our study, the implemented models were able to achieve better model results, i.e. R^2 of 0.99 using four Ex/Em pairs.

Ren and coauthors [12] have noted the importance of breaking the cell membrane of *Scenedesmus* sp. and proposed using ultrasound as a pre-treatment of samples before being analyzed in the spectrofluorometer. They obtained satisfactory results in the excitation range from 500 to 600 nm and emission from 550 to 620 nm. These results are within the range of analysis proposed in this work, demonstrating that it is possible to obtain equivalent results using our procedure without the need to break the cell wall as their proposal.

Sá and coauthors [29] evaluated the effectiveness of data processing and the application of PCA (principle component analysis) and PLS (partial least squares) methods, achieving predictive results with R^2 superior to 0.81, proving that chemometric methods can be useful to quantify lipids. In our study, the application of the PSCM method, associating chemometric models, and the use of the ACO algorithm to optimize the selection of Ex/Em pairs, we were able to predict lipid concentration with R^2 above 0.99, using only 4 Ex/Em pairs. Our results indicate that using the PSCM methodology it is possible to predict the concentration of the lipids satisfactorily, with mean absolute errors smaller than 5%. The use of the whole spectra yielded the best overall models, selecting Ex/Em pairs that have relatively small fluorescence intensity. The AGF models also satisfactorily predicted the percentage of lipid concentration in the samples.

Under the same culture conditions, it is possible to determine the lipid concentration of each sample using the chemometric models generated in this work, being necessary only 15 to 30 min for the preparation and quantification of each sample. This time is significantly smaller than the time required for the extraction by the Bligh and Dyer method, which takes on average 3 hours. The results obtained in this work present better results with an average R^2 of 0.998 using our methodology for the selection of fluorescence excitation/emission wavelength pairs.

CONCLUSION

This study demonstrates that the combination of fluorescence spectroscopy and chemometric modeling is capable of quantifying lipids in microalgae *Scenedesmus* sp. Therefore, fluorescence has the potential to provide a fast characterization technique, which may enable lipid content monitoring. In the tests performed,

it was possible to satisfactorily predict the concentration of lipids in microalgae samples using a combination of Nile Red, fluorescence measurements, and the PSCM methodology.

The best models trained with our methodology using four Ex/Em pairs presented a mean absolute error smaller than 5% and R^2 of almost 0.99 for the training and test subsets, both for the search of pairs in the whole spectra and the focused search in the area of greater fluorescence. Once the test metrics were comparable and consistent with the training metrics, the models can be considered robust and able to correctly deal with new information.

Characterizing the concentration of lipids in microalgae samples using Nile Red and fluorescence spectroscopy measurements showed promising results. The proposed methodology is much more time-efficient than the traditional gravimetric method, requiring less than 30 minutes when the traditional methods can take up to 5 hours.

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Conflicts of Interest: The authors declare no conflict of interest.

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