Determination of profile of chlorophyll compounds in microalgae species

Determinação do perfil de compostos de clorofila em espécies de microalgas

DOI:10.34117/bjdv7n1-295

Recebimento dos originais: 10/12/2020
Aceitação para publicação: 12/01/2021

Andrêssa S. Fernandes
Mestre em Ciência e Tecnologia de Alimentos pela Universidade Federal de Santa Maria
Instituição: Universidade Federal de Santa Maria - UFSM
Endereço: Avenida Roraima, 1000, 97105-900 - Camobi, Santa Maria –RS, Brasil
E-mail: andressa.asfs@gmail.com

Tatiele C. do Nascimento
Mestre em Ciência e Tecnologia de Alimentos pela Universidade Federal de Santa Maria
Instituição: Universidade Federal de Santa Maria - UFSM
Endereço: Avenida Roraima, 1000, 97105-900 - Camobi, Santa Maria –RS, Brasil
E-mail: tiele.casa@gmail.com

Pricila N. Pinheiro
Mestre em Ciência e Tecnologia de Alimentos pela Universidade Federal de Santa Maria
Instituição: Universidade Federal de Santa Maria - UFSM
Endereço: Avenida Roraima, 1000, 97105-900 - Camobi, Santa Maria –RS, Brasil
E-mail: pricila.nass@gmail.com

Eduardo Jacob-Lopes
Doutor em Engenharia Química pela Universidade Estadual de Campinas
Instituição: Universidade Federal de Santa Maria - UFSM
Endereço: Avenida Roraima, 1000, 97105-900 - Camobi, Santa Maria –RS, Brasil
E-mail: jacoblopes@pq.cnpq.br

Leila Q. Zepka
Doutora em Ciência de Alimentos pela Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas
Instituição: Universidade Federal de Santa Maria –UFSM
Endereço: Avenida Roraima, 1000, 97105-900 - Camobi, Santa Maria –RS, Brasil
E-mail: zepkaleila@yahoo.com.br

RESUMO

Dentre as especialidades químicas subexploradas em microalgas, o perfil das clorofilas em diferentes espécies (*Chlorella vulgaris* e *Aphanothece microscopica Nägeli*) foi caracterizado em detalhes como o objetivo principal deste estudo. A composição das clorofilas e derivados foi determinada por HPLC-PDA-MS (APCI $^{+}$). Os padrões de

fragmentação característicos permitiram identificar oito compostos de clorofila diferentes. Compostos de relevância como espécies de clorofila *a*, clorofila *b*, moléculas derivadas de reações de feofitinização, epimerização e hidroxilação estiveram presentes nas espécies de microalgas. Valores substanciais de 10.734,19 e 9.121,89 $\mu g.g^{-1}$ de peso seco foram obtidos para *C. vulgaris* e *A. microscopica Nägeli*, respectivamente. Assim, a abordagem deste estudo contribui de forma significativa para bancos de dados de composição em constituintes bioativos das espécies avaliadas. Além disso, fornecem informações que elevam a importância desses microrganismos como alternativa para obtenção de componentes de alimentos, enfatizando-os como fontes para atender as necessidades emergentes do mercado de compostos naturais.

Palavras-chave: microalgas, cianobactérias, algas verdes, espectrometria de massa, pigmentos naturais, componentes de alimentos.

ABSTRACT

Among the specialty chemicals sub-exploited in microalgae, the profile of chlorophylls in different species (*Chlorella vulgaris* and *Aphanothece microscopica Nägeli*) was characterized in detail as the main objective of this study. The composition of the chlorophylls and derivatives were determined by HPLC-PDA-MS ($APCI^+$). The characteristic fragmentation patterns have allowed identifying eight different chlorophyll compounds. Compounds of relevance as species of chlorophyll *a*, chlorophyll *b*, molecules derivative from reactions of pheophytinization, epimerization and hydroxylation were present in the species of microalgae. Substantial values of 10,734.19 and 9,121.89 $\mu g.g^{-1}$ dry weight were obtained for *C. vulgaris* and *A. microscopica Nägeli*, respectively. Thus, the approach of this study contributes significantly to composition databases in bioactive constituents of the evaluated species. In addition, provide information that elevates the importance of these microrganisms as alternative for obtaining of food components, emphasizing them as sources to meet the emerging needs the market of natural compounds.

Keywords: Microalgae, cyanobacteria, green algae, mass spectrometry, natural pigments, food components.

1 INTRODUCTION

For decades, research involving biotechnological processes mediated by microalgae has been growing steadily. These microorganisms are considered excellent among all types of biomass sources since they can respond to the future challenges in terms of availability, high rates of growth and production, not competing for arable land. Also, they are considered ideal sources for obtaining valuable co-products for the food industry (Khan et al., 2018; Jacob-Lopes et al., 2019). Within the current market context, approximately 7000 tons of microalgae biomass is produced globally, totaling US$ 32.6 billion. The modeling projects a market of US$ 53.4 billion by 2026 (Rahman, 2020). Of these values, it is estimated that 75% of the production of microalgae-based products is destined for food formulation and obtaining bioactive ingredients (Rumin et al., 2020).

The microalgae biomass has to date found several industrial food applications, since product formulations with functional characteristics and better nutritional conditions, even applications that use pigments extracted from biomass as a dye (Lafarga, 2019). However, nowadays the microalgae biotechnology sector is eminently focused on consolidating microalgae biorefining processes strongly considering the high-value compounds, with the main objective of supporting the sustainable production of functional food compounds (Matos, 2017; Rizwan et al., 2018; Koyande et al., 2019).

Few species of microalgae have commercial importance. Those that include the genus *Chlorella* (class *Chlorophyceae* or green microalgae) constantly draw attention as commercially valuable sources of a wide spectrum of bioactive compounds (Bhalamurugan et al., 2018). Specifically, the species *C. vulgaris* is successfully used in the food industry, feed industry and also in the pharmaceutical industry (da Silva et al., 2019). Thus, considering the number of different species of microalgae existing (stipulated in more than 50,000) is still very small the number of strains that were studied (Sathasivam et al., 2017). In this respect, looking to the exploring new microalgae species for possible commercial applicability, the microalgae *Aphanothece microscopica Nägeli*, from the *Cyanophyceae* class, has been the subject of intensive research. This microalgae specie has high production rate and synthesis capacity of several valuable compounds, including fatty acids, proteins, and carotenoids (Zepka et al., 2008; Zepka et al., 2010; Patias et al., 2017; Vendruscolo et al., 2018; Maroneze et al., 2019). Nevertheless, there are other important molecules bioactive to be elucidate in the composition of microalgae biomass, one of them being class of chlorophyll pigments. These compounds still little explored in microalgae, are primary pigments in the metabolism these microrganisms due to its "light harvesting'' role in photosynthesis. Also, are considered fundamental molecules of life and probably the most important and widely distributed of all natural pigment (Mulders et al., 2014; Solymosi and Mysliwa-Kurdziel, 2017).

Consists of a class of more than 100 different structures naturally synthesized by oxygenic photosynthetic organisms such as plants, algae, microalgae and cyanobacteria, with five species characterized as chlorophyll *a*, *b*, *c*, *d* and *f* (Perez-Gálvez et al., 2017). Given his sensitivity, the chlorophyll molecules form derivatives compounds, as consequence of natural metabolism, chemical or enzymatic action. Among the chlorophyll derivatives are the oxidized compounds due to a substitution of the H atom at C 13_2 by a hydroxyl group, the so-called hydroxy derivatives. By contrast, when the central magnesium atom of the tetrapyrrol ring is easily replaced by two hydrogen atoms

occurs the formation of pheophytins. On the other hand, the formation of isomers can also occur, originating from decarbomethoxylation at position C-13_2 position (Roca et al., 2016).

Chlorophyll compounds are potentially important molecules not only as a colour pigment but also because of their health benefits. Important and prominent biological activities for human health have been demonstrated for chlorophylls and their derivatives, such as the antimutagenic effect, anti-inflammatory, antigenotoxic properties, and potent antioxidant capacity to eliminate free radicals and prevent lipid oxidation (Lanfer-Marquez et al., 2005; Ferruzzi and Blakeslee, 2007; Pérez-Gálvez et al., 2017).

These compounds are currently produced on industrial scale mainly via higher plants such as spinach, alfalfa, stinging nettle, or corn (Sarkar et al., 2020). However, the growing search for natural food components encourages research aimed at the synthesis of these compounds by alternative biological routes. Accordingly, microalgae are considered attractive sources to be explored, since these microorganisms can synthesize chlorophylls in greater proportions than higher plants (Khanra et al., 2018).

Considering the high concentration of chlorophyll and other compounds present in microalgae, several studies have shown the possibility of application of the biomass of these organisms as the functional and natural green dye in foodstuffs formulations (Gouveia et al., 2006; Gouveia et al., 2007; Gouveia et al., 2008; Fradique et al., 2010; Özyurt et al., 2015; Pool et al., 2016; Batista et al., 2017; Palabiyik et al., 2018; Lafarga, 2019). However, these studies lack exploratory analysis of chlorophyll composition in the microalgae biomass used.

In this sense, aiming to explore potential sources for obtaining natural chlorophylls, a comprehensive analysis of the chlorophylls fraction in *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* was the aim of this study.

2. MATERIAL AND METHODS

2.1 CHEMICALS

Standards of chlorophyll *a*, chlorophyll *b*, (with purities ranging from 95.0% to 99.9%, as determined by HPLC-PDA) were purchased from Sigma-Aldrich (Missouri-MO, USA). The pheophytin *a* standard was obtained in our laboratory through an acid hydrolysis reaction from the standard chlorophyll *a*, where the Mg^{2+} ion is replaced by two hydrogen atoms (Fernandes et al., 2017). Methanol, ethanol, acetone, methyl tert-

butyl ether (MTBE), ethyl acetate, petroleum ether and diethyl ether were purchased from Sigma-Aldrich (St. Louis-MO, USA).

2.2 MICRORGANISMS AND CULTURE MEDIA

Axenic cultures of *Chlorella vulgaris* (CPCC90) were obtained from the Canadian Phycological Culture Center. Axenic cultures of *Aphanothece microscopica Nägeli* (RSMan92) were obtained from the collection of the Cyanobacteria and Phycotoxins Laboratory of the Institute of Oceanography from the Federal University of Rio Grande (www.cianobacterias.furg.br). Stock cultures were propagated and maintained in synthetic BG11 medium (Braun-Grunow medium) (Rippka et al., 1979). The incubation conditions were 30 ºC, photon flux density of 15 μmol.m^{-2}.s^{-} and a photoperiod of 12 h were used.

2.3 MICROALGAE BIOMASS PRODUCTION

The biomass production was carried out in a bubble column photobioreactor (Maroneze et al., 2016) operating in intermittent regime, fed with 2.0 L of BG11 medium (Rippka et al., 1979). The experimental conditions were as follows: initial concentration of inoculum of 100 mg.L^{-1}, temperature of 25 °C, aeration of 1 volume of air per volume of medium per minute, a photon flux density of 150 μmol.m^{-2}.s^{-1}, photoperiod of 12/12 hours light/dark, and a residence time of 168 h.

2.4 BIOMASS CONCENTRATION

The biomasses were separated from the culture medium by centrifugation. It was subsequently freeze dried (Lyophilizer Liotop L101) for 24h at -50 °C above -175 μm Hg, and then stored under refrigeration until the time of analysis.

2.5 CHLOROPHYLL EXTRACTION

The chlorophylls was exhaustively extracted from the freeze-dried samples (0.2 ± 0.02 g) with ethyl acetate and methanol in a mortar with a pestle followed by centrifugation (Hitachi, Tokyo, Japan) for 7 min at 1500 × g. (Mandelli et al, 2012). The extraction procedure was repeated until the supernatant becomes colorless. The homogenized sample suspension was filtered through a 0.22 μm polyethylene membrane, concentrated in a rotary evaporator (T<30 °C), flushed with N_2 and kept at -37 °C in the dark until chromatographic analysis.

2.6 HPLC-PDA-MS/MS ANALYSIS

The chlorophylls were analyzed by high performance liquid chromatography HPLC (Shimadzu, Kyoto, Japan) equipped with binary pumps (model LC-20AD), online degasser, and automatic injector (Rheodyne, Rohnert Park-CA, USA). The equipment was connected in series to a PDA detector (model SPD-M20A) and a mass spectrometry was performed with a Shimadzu 8040 triple quadrupole mass spectrometer and atmospheric pressure chemical ionization (APCI) source (Shimadzu America, Columbia, MD, USA). The UV-vis spectra were obtained between 350 and 660 nm, and the chromatograms were processed at 660 nm. Chlorophyll separation was carried out on a C30 YMC column (5 µm, 250 × 4.6 mm) (Waters, Wilmington, DE, USA). Prior to HPLC-PDA-MS/MS analysis, the chlorophylls extract was solubilized in MeOH:MTBE (70:30) and filtered through Millipore membranes (0.22 µm). HPLC-PDA-MS/MS parameters were set as previously described by De Rosso and Mercadante (2007), and Fernandes et al. (2017). The mobile phase consisted in MeOH (solvent A) and MTBE (solvent B) mixture. A linear gradient was applied from 95:5 to 70:30 in 30 min, to 50:50 in 20 min. The flow rate was 0.9 mL.min^{-1} and the column temperature set to 29 °C, and the injection volume was 20 µL.

The MS/MS detection was achieved according to Giuffrida et al., (2017) with adaptations, the APCI interface operated in positive (+) mode; detector voltage: 4.5 kV; interface temperature: 350 ºC; DL temperature: 250 ºC; heat block temperature: 200 ºC; nebulizing gas flow (N_2): 3.0 L.min^{-1}; drying gas flow (N_2): 5.0 L.min^{-1}; collision induced dissociation (CID) gas: 23 kPa (argon); event time: 0.5 s. To improve the quality of identification, the MS was used simultaneously in SIM (Select Ion Monitoring) and MRM (Multiple Reaction Monitoring) modes.

The identification was performed according to the following combined information: elution order on C30 HPLC column, co-chromatography with authentic standards, UV-Visible spectrum (λ máx, spectral fine structure), and mass spectra characteristics (protonated molecule ($[M + H]^+$) and MS/MS fragments), compared with data available in the literature (Chen et al., 2017; Fernandes et al., 2017; Loh et al., 2012; Gauthier-Jaques et al., 2001).

The chlorophylls were quantified by HPLC-PDA using external calibration curves for chlorophyll *a*, chlorophyll *b* and pheophytin *a* with a minimum of five concentration levels. 13^2-hydroxy chlorophyll *a*, 13^2-hydroxy chlorophyll *a*', chlorophyll *a*, and chlorophyll *a′* where quantified using the curve of chlorophyll *a*; the 13^2-hydroxy

pheophytin *a*, pheophytin *a*, and pheophytin *a*' using the curve of pheophytin *a*; and chlorophyll *b* was quantified using the curve of chlorophyll *b*. Total chlorophyll content was calculated considering all identified peak areas.

2.7 STATISTICAL ANALYSIS

Descriptive statistics and Student's t-test ($p < 0.05$) were applied to experimental data. The analyses were performed with the software GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla-CA, USA).

3 RESULTS AND DISCUSSION

Table 1 and Figure 1 shows a qualitative profile of eight chlorophyll derivatives identified in *Chlorella vulgaris* and *Aphanothece microscopica Nägeli*. Because the chlorophyll compounds have low polarity, atmospheric pressure chemical ionization (APCI) was used to facilitate the ionization and production of $[M + H]^+$ ions.

Table 1 Characterization by HPLC-PDA-MS/MS of the profile of chlorophyll compounds present in biomass of *Chlorella vulgaris* and *Aphanothece microscopica Nägeli*.

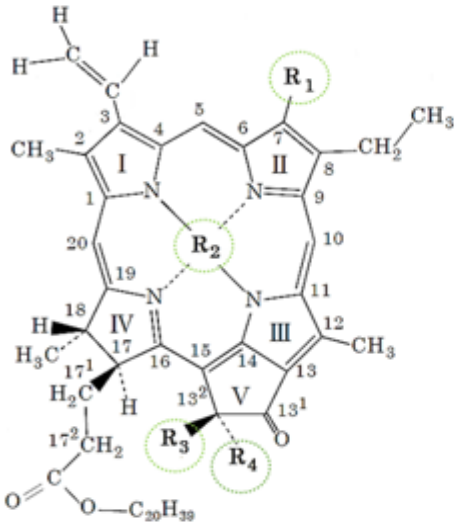
Peak[a]	**Chlorophyll**	**t_R (min)**[b]	**$\lambda_{máx}$ (nm)**[c]	**$[M+H]^+$**	**MS/MS fragment ions (*m/z*)**
1	13^2-hydroxy chlorophyll *a*	9.2	421, 660	909	891$[M+H-18]^+$; 631$[M+H-278]^+$; 613$[M+H-278-18]^+$
2	13^2-hydroxy chlorophyll *a'*	10.2	422, 663	909	891$[M+H-18]^+$; 631$[M+H-278]^+$; 613$[M+H-278-18]^+$
3	Chlorophyll *b*	10.6	468, 659	907	875$[M+H-32]^+$; 629$[M+H-278]^+$; 597$[M+H-278-32]^+$; 569$[M+H-278-60]^+$
4	Chlorophyll *a*	15.1	432, 665	893	615$[M+H-278]^+$; 583$[M+H-278-32]^+$; 555$[M+H-278-60]^+$
5	Chlorophyll *a'*	17.0	431, 665	893	615$[M+H-278]^+$; 583$[M+H-278-32]^+$; 555$[M+H-278-60]^+$
6	13^2-hydroxy pheophytin *a*	24.2	409, 666	887	869$[M+H-18]^+$; 803$[M+H-63]^+$; 609$[M+H-278]^+$; 591$[M+H-278-18]^+$; 531$[M+H-278-18-60]^+$
7	Pheophytin *a*	31.7	408, 666	871	593$[M+H-278]^+$; 533$[M+H-278-60]^+$
8	Pheophytin *a'*	33.0	408, 665	871	593$[M+H-278]^+$; 533$[M+H-278-60]^+$

[a]Numbered according to the elution order on C30 HPLC column.

[b]t_R: Retention time on the C30 column.

[c]Linear gradient Methanol:MTBE.

Figure 1. Structures and nomenclature of chlorophylls and their derivatives identified by HPLC-PDA-MS/MS in *Chlorella vulgaris* and *Aphanothece microscopica nageli*.



Peak	Compound	Molecular formula	R_1	R_2	R_3	R_4
1	13^2-hydroxy chlorophyll *a*	$C_{55}H_{73}MgN_4O_6$	CH_3	Mg	OH	$COOCH_3$
2	13^2-hydroxy chlorophyll *a'*	$C_{55}H_{73}MgN_4O_6$	CH_3	Mg	$COOCH_3$	OH
3	Chlorophyll *b*	$C_{55}H_{70}MgN_4O_6$	CHO	Mg	H	$COOCH_3$
4	Chlorophyll *a*	$C_{55}H_{72}MgN_4O_5$	CH_3	Mg	H	$COOCH_3$
5	Chlorophyll *a'*	$C_{55}H_{72}MgN_4O_5$	CH_3	Mg	$COOCH_3$	H
6	13^2-hydroxy pheophytin *a*	$C_{55}H_{74}N_4O_6$	CH_3	2H	OH	$COOCH_3$
7	Pheophytin *a*	$C_{55}H_{74}N_4O_5$	CH_3	2H	H	$COOCH_3$
8	Pheophytin *a'*	$C_{55}H_{74}N_4O_5$	CH_3	2H	$COOCH_3$	H

The characteristics and chemical structure of the compounds separated in the microalgae analyzed are presented in Table 1 and Figure 1, and the identification is discussed according to the elution order. Once a detailed description of chlorophylls identification using chromatographic information has already been reported by our research group (Fernandes et al., 2017), only chromatographic considerations about chlorophyll compounds not previously identified were discussed below.

Peak 1 was identified as the 13^2-hydroxy chlorophyll *a* (molecular formula $C_{55}H_{73}MgN_4O_6$) on the basis of the characteristic UV-visible spectra (421, 660), and protonated molecule at *m/z* 909, similar to the data from the literature (Kao et al., 2011; Loh et al., 2012). In this molecular structure, the subsequent loss of the hydroxyl group ($C13_2$; Fig. 1) and diterpene alcohol phytol correspond to the fragments *m/z* 891 $[M+H-18]^+$ and m/z 631 $[M+H-278]^+$, respectively.

Chlorophyll *a'* (peak 5) was identified, based on UV/visible ($\lambda_{máx}$), retention time of peak and confirmed by HPLC-MS. The maximal absorbance in the UV/visible spectrum, were located at 431 and 665 nm. The protonated molecule was identified as *m/z* 893 and the fragment ions were 615$[M+H-278]^+$, 583$[M+H-278-32]^+$, 555$[M+H-278-60]^+$. The fragment *m/z* 615 $[M+H-278]^+$ corresponds to the characteristic loss of the diterpene alcohol phytol from the C17 propionic substituent (numbering scheme in Fig.1); while the fragment at *m/z* 583 $[M+H-278-32]^+$ represent the loss of CH_3O group; and *m/z* 555 $[M+H-278-60]^+$ corresponds to loss of CH_3COO group, formed from the cleavage of the ester bond (substituent C17). As previously reported in the literature, the MS/MS fragmentation patterns of *a*/*a'* isomers are basically identical in the APCI-HPLC/MS/MS conditions (Gauthier-Jaques et al., 2001). However, a significant difference in the intensity of the main fragment ($[M+H]^+$) allows the differentiation between them. Chlorophyll *a* is higher intense in the main fragment (*m/z* 893), while in chlorophyll *a'* isomer, the intensity is lower.

In addition to the chlorophyll pigments specified above, it was possible to identify chlorophyll *b* (peak 3), chlorophyll *a* (peak 4), its Mg-free derivative pheophytin *a* (peak 7), as well as hydroxyl-containing derivatives were identified as 13^2-hydroxy chlorophyll *a'* (peak 2), 13^2-hydroxy pheophytin *a* (peak 6), and the pheophytin *a'* isomer (peak 8).

The contents of chlorophyll and their derivatives in chlorophyll extract from *Chlorella vulgaris* and *Aphanothece microscopica Nägeli* are shown in Table 2. The total chlorophylls contents from biomass were 1,0734.19 $\mu g.g^{-1}$ and 9,121.89 $\mu g.g^{-1}$, as dry weight, respectively.

Although the use of microalgae of *Chlorella* genus is consolidated in the market of natural products, as far as we know, the literature lacks information about of the chlorophylls detailed profile in the microalgae *Chlorella vulgaris*. A total of six chlorophyll compounds were identified in the *Chlorella vulgaris* extract (Table 2). This microalgae presented a notably superior quantitative profile (10,734.19 $ug.g^{-1}$) when compared to another microalgae under study, considering values of 1.17 fold higher than the concentration of chlorophyll total in *Aphanothece microscopica Nägeli*. The major chlorophyll compounds in *Chlorella vulgaris* were chlorophyll *a* (57.43%), chlorophyll *a'* (15.04%), pheophytin *a'* (13.73%) and 13^2-hydroxy chlorophyll *a'* (8.22%). In parallel, pheophytin *a* and chlorophyll *b* were identified as minor compounds, representing 5.56 % of the total content of chlorophylls.

For chlorophyll *b* series, *C. vulgaris* presented a total of 277.98 $ug.g^{-1}$, represented only by the parental chlorophyll *b*. In contrast, we detected a higher concentration of series *a* compounds, which represents a total of 10,456.21$ug.g^{-1}$. The fraction of compounds without central magnesium ion in their structure (Figure 1) represents approximately 1,793.68 $ug.g^{-1}$ of the total quantified chlorophylls and is constituted of the compounds pheophytin *a* and its

Table 2 Quantitative characterization of chlorophyll compounds in microalgae extracts (μg.g^{-1} dry weight).

Peak	Compound	*Chlorella vulgaris*	*Aphanothece microscopica Nägeli*
1	13^2-hydroxy chlorophyll *a*	nd	7.14 ± 0.17
2	13^2-hydroxy chlorophyll *a'*	881.74^b ± 2.82	1,616.81^a ± 5.61
3	Chlorophyll *b*	277.98 ± 0.89	nd
4	Chlorophyll *a*	6,165.45^a ± 3.21	2,528.59^b± 4.32
5	Chlorophyll *a'*	1,615.31^a ± 5.17	723.09^b ± 2.78
6	13^2-hydroxy pheophytin *a*	nd	279.94 ± 1.24
7	Pheophytin *a*	319.33^b ± 1.02	568.34^a ± 2.08
8	Pheophytin *a'*	1,474.34^b ± 4.72	3,397.94^a ± 2.21
	Chlorophyll *a* series	10,456.21^a ± 2.72	9,121.89^b ± 6.19
	Chlorophyll *b* series	277.98 ± 1.89	nd
	Total pheophytins	1,793.68^b ± 2.74	3,966.29^a ± 3.12
	Total 13^2-hydroxy derivatives	881.74^b ± 2.82	1,903.91^a ± 1.12
	Total chlorophyll	**10,734.19^a ± 5.39**	**9,121.89^b ± 6.19**

nd: not detected.
Values are average and standard deviation of triplicates.
Different letters in the same line differ significantly by Student's t-test (α = 0.05).

pheophytin *a'* isomer. In this microalgae species, only one hydroxylated compound was detected (13^2-hydroxy chlorophyll *a'*), with a quantitative value of 881.74 ug.g^{-1}.

The composition of chlorophyll compounds in *Aphanothece microscopica Nägeli* can be seen in Table 2. A profile of seven compounds was detected in this microalgae. The pheophytin *a'* (3,397.94 µg.g^{-1}) (37.25%) was quantitatively dominant in chlorophyll profile of microalgae, followed by chlorophyll *a* (2,528.59 µg.g^{-1}) (27.72%), 13^2-hydroxy chlorophyll *a'* (1,616.81 µg.g^{-1}) (17.72%) and chlorophyll *a'* (723.09 µg.g^{-1}) (7.92%). The three minor compounds (peak 1, 6, and 7) represented about 9% of the total content.

As expected, only chlorophyll derivatives of series *a* were detected for cyanobacteria *Aphanothece microscopica Nägeli.* Hydroxylated compounds, epimers, and without the central magnesium ion constituted the profile of this species. Of these, Mg-free compounds (peak 7 and 8) represent 3,966.29 µg.g^{-1} of the total chlorophyll content. Among 13^2-hydroxy derivatives, the compounds 13^2-hydroxy chlorophyll *a* (peak 1), 13^2-hydroxy chlorophyll *a'* (peak 2), and 13^2-hydroxy pheophytin *a* (peak 6) were identified and quantified with a total of 1,903.91 µg.g^{-1}. Besides that, the 13^2-hydroxy chlorophyll *a* and 13^2-hydroxy pheophytin *a* were only identified in this species of microalgae.

The total content of pheophytins was relatively abundant in *Aphanothece microscopica Nägeli* (43.48%) which corresponds to approximately 2 times more than that found in the green microalgae under study (16.70%). This can be hypothetically explained by cell morphology, in which the synthesis and storage of chlorophylls in cyanobacteria occur dispersed in the hyaloplasm, which causes less protection of these pigments the action of enzymes or chemistry action in the displacement of the magnesium ion, formation thus, oxidized compounds. On the other hand, smaller amounts of pheophytins were evidenced in *Chlorella vulgaris* (1,793.68 µg.g^{-1}), which is probably attributed to chlorophyll being confined in chloroplasts and also protected by a hydrophobic membrane, which provides greater stability to these compounds.

Regarding the total content of 13^2-hydroxy derivatives, *Aphanothece microscopica Nägeli* also showed a higher concentration, reaching values of 1,903.91 µg.g^{-1}, while in *Chlorella vulgaris* practically half was found (881.74 µg.g^{-1}). According to literature data, the formation of these compounds is probably caused by a hydroxylation formed by the enzyme chlorophyll oxidase (Huang et al., 2008).

Additionally, the chlorophyll *a'* isomer had a relatively lower quantitative profile in the two microalgae when compared to the parental chlorophyll *a*. These results thus demonstrate a concordance with the study by Nakamura et al., (2003) that reports low concentrations of the

compound in photosynthetic microrganisms. Indeed, it has been well established that in addition to degradation, chlorophylls can be susceptible to epimerization at $C13^2$ for chlorophyll *a′* formation (Kao et al., 2011).

In terms of ratio chlorophylls *a/b*, according to Kang et al., (2018), photosynthetic microrganisms present chlorophyll *a* and chlorophyll *b* in a ratio of 3:1, however in our study we found a higher ratio, corresponding to 22:1 in *Chlorella vulgaris*. This high chlorophyll *a/b* the ratio can probably be attributed to the low enzymatic activity of oxygenase since this enzyme catalyzes the conversion of the methyl group bound to ring II (Figure 1) to aldehyde (Xu et al., 2001; Harada et al., 2012; Yen et al., 2013).

After the identification of chlorophyll profile from microalgae was possible to determine the dominant polarity of compounds as lipophilic, since they have a propionic acid esterified with diterpene phytol alcohol in C17. However, hydroxyl compounds have tendency to polar character. These compounds represent 8.21% in *C. vulgaris*, and 20.85% in *A. microscopica Nägeli*.

Although the different microalgae phylum presents a difference in the chlorophyll fraction, five compounds (peak 2, 4, 5, 7, and 8) are common among the two microalgae investigated. However, all compounds showed a significant difference ($p<0.05$) in the quantitative profile. Accordingly, the green microalgae showed compounds de chlorophyll equivalent to those of the cyanobacteria under study, when considering the qualitative profile. This is probably attributed to the same route of synthesis of chlorophylls, in the two groups of microalgae, to occur along the C5 pathway, in which the first dedicated precursor of the pathway, 5-aminolevulinic acid (ALA), is synthesized from a molecule of glutamate. However, it is still a challenge to understand the specific route of chlorophyll synthesis in different classes of microalgae. This is because these compounds are inherently unstable and reactive in the presence of oxygen and light (Beale, 1999; Lohr et al., 2005; Larkin et al., 2016).

In relation to microalgae culture, Kong et al. (2011) demonstrated higher concentrations of chlorophylls in phototrophic cultures, due to the fact, that the synthesis/formation of photosynthetic pigments highly influenced by the light source (Mohsenpour et al., 2012). Most algae cultured under optimum condition were reported contained about 4% dry weight of chlorophyll from overall cell weight (Kong et al., 2014). On the other hand, our results presented values of 5.3% (*Chlorella vulgaris*) and 4.5% (*Aphanothece microscopica Nägeli*) chlorophyll on a dry weight.

The literature reports scarce data on the complete characterization of chlorophyll pigments in the microalgae species under study (Kong et al., 2011; Plaza et al., 2012), which makes it difficult to compare them with data from the literature. However, our results demonstrate an interesting profile to be explored and considered as an alternative source for obtaining natural chlorophyll pigments.

4 CONCLUSION

The methodology used in HPLC-PDA-MS/MS allowed the complete characterization of the profile of chlorophylls and their derivatives of *Chlorella vulgaris* and *Aphanothece microscopica Nägeli*, determining a total of eight compounds.

Among the compounds identified, chlorophyll *a* was the major pigment *Chlorella vulgaris*, representing values of 57.43% in dry weight. On the other hand, *Aphanothece microscopica Nägeli* presented pheophytin *a'* as higher compound at concentrations of 37.25% in dry weight. Also, it was possible to identify molecules derivatives from chlorophyll as hydroxylated compounds, isomers, and pheophytins in the two microalgae understudy.

Although there is a constant search by the food industry for natural food components and functional, there are still few studies that explore different species of microalgae as a source of natural chlorophyll pigments. Taking these fine chemicals into account, the results obtained here generate new possibilities for the industrial sources of the presented chlorophylls, since the two species of microalgae showed a profile of chlorophylls substantially interesting to be considered for exploration to industrial level.

ACKNOWLEDGEMENTS

This study was financially supported by the PNPD/CAPES (001), FAPERGS (17/2551-878 0000930-4 and 19/2551-0000673-0), and CNPq (306964/2017-1).

REFERENCE

Batista, A. P., Niccolai, A., Fradinho, P., Fragoso, S., Bursic, I., Rodolfi, L., Biondi, N., Tredici, M. R., Sousa, I., & Raymundo, A. (2017). Microalgae biomass as an alternative ingredient in cookies: sensory, physical and chemical properties, antioxidant activity and in vitro digestibility. *Algal research*, 26, 161-171.

Beale, S. I. (1999). Enzymes of chlorophyll biosynthesis. *Photosynthesis research*, 60(1), 43-73.

Bhalamurugan, G. L., Valerie, O., & Mark, L. (2018). Valuable bioproducts obtained from microalgal biomass and their commercial applications: A review. *Environmental Engineering Research*, 23(3), 229-241.

Chen, K., Ríos, J. J., Pérez-Gálvez, A., & Roca, M. (2017). Comprehensive chlorophyll composition in the main edible seaweeds. *Food Chemistry*, 228, 625-633.

da Silva, J. W. A., Maia, H. D., de Lima, R. L., Gomes, I. G. R. F., de Araújo, A. L. A. C., da Cruz Coelho, A. A., ... & Costa, F. H. F. (2019). Effect of sodium nitrate concentration on the lipid content of *Chlorella vulgaris*/Efeito da concentração de nitrato de sódio no conteúdo lipídico de *Chlorella vulgaris*. *Brazilian Journal of Development*, 5(12), 33506-33524.

De Rosso, V. V., & Mercadante, A. Z. (2007). Identification and quantification of carotenoids, by HPLC-PDA-MS/MS, from Amazonian fruits. *Journal of Agricultural and Food Chemistry*, 55, 5062-5072.

Fernandes, A. S., Nogara, G. P., Menezes, C. R., Cichoski, A. J., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2017). Identification of chlorophyll molecules with peroxyl radical scavenger capacity in microalgae *Phormidium autumnale* using ultrasound-assisted extraction. *Food Research International*, 99, 1036-1041.

Ferruzzi, M. G., & Blakeslee, J. (2007). Digestion, absorption, and cancer preventative activity of dietary chlorophyll derivatives. *Nutrition Research*, 27(1), 1-12.

Fradique, M., Batista, A. P., Nunes, M. C., Gouveia, L., Bandarra, N. M., & Raymundo, A. (2010). Incorporation of *Chlorella vulgaris* and *Spirulina maxima* biomass in pasta products. Part 1: Preparation and evaluation. *Journal of the Science of Food and Agriculture*, 90(10), 1656-1664.

Gauthier-Jaques, A., Bortlik, K., Hau, J., & Fay, L. B. (2001). Improved method to track chlorophyll degradation. *Journal of Agricultural and Food Chemistry*, 49, 1117-1122.

Giuffrida, D., Zoccali, M., Giofre, S. V., Dugo, P., & Mondello, L. (2017). Apocarotenoids determination in *Capsicum chinense* Jacq. cv. Habanero, by supercritical fluid chromatography-triple-quadrupole/mass spectrometry. *Food chemistry*, 231, 316-323.

Gouveia, L., Raymundo, A., Batista, A. P., Sousa, & I., Empis, J. (2006). *Chlorella vulgaris* and *Haematococcus pluvialis* biomass as colouring and antioxidant in food emulsions. European *Food Research and Technology*, 222(3-4), 362.

Gouveia, L, Batista, A. P., Miranda, A., Empis, J., & Raymundo, A. (2007). *Chlorella vulgaris* biomass used as colouring source in traditional butter cookies. *Innovative Food Science & Emerging Technologies*, 8(3), 433-436.

Gouveia, L., Coutinho, C., Mendonça, E., Batista, A. P., Sousa, I., Bandarra, N. M., & Raymundo, A. (2008). Functional biscuits with PUFA-ω3 from *Isochrysis galbana*. *Journal of the Science of Food and Agriculture*, 88(5), 891-896.

Harada, J., Mizoguchi, T., Tsukatani, Y., Noguchi, M., & Tamiaki, H. (2012). A seventh bacterial chlorophyll driving a large light-harvesting antenna. *Scientific reports*, 2 (671), 1-5.

Huang, S.C., Hung, C.F., Wu, W.B., & Chen, B.H. (2008). Determination of chlorophylls and their derivatives in *Gynostemma pentaphyllum* Makino by liquid chromatography-mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, 48, 105-112.

Jacob-Lopes, E., Maroneze, M. M., Deprá, M. C., Sartori, R. B., Dias, R. R., & Zepka, L. Q. (2019). Bioactive food compounds from microalgae: An innovative framework on industrial biorefineries. *Current Opinion in Food Science*, 1, 1-7.

Kang, Y. R., Park, J., Jung, S. K., & Chang, Y. H. (2018). Synthesis, characterization, and functional properties of chlorophylls, pheophytins, and Zn-pheophytins. *Food chemistry*, 245, 943-950.

Kao, T. H., Chen, C. J., & Chen, B. H. (2011). An improved high performance liquid chromatography–photodiode array detection–atmospheric pressure chemical ionization–mass spectrometry method for determination of chlorophylls and their derivatives in freeze-dried and hot-air-dried *Rhinacanthus nasutus* (L.) Kurz. *Talanta*, 86, 349-355.

Khan, M. I., Shin, J. H., & Kim, J. D. (2018). The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microbial cell factories*, 17(1), 36.

Khanra, S., Mondal, M., Halder, G., Tiwari, O. N., Gayen, K., & Bhowmick, T. K. (2018). Downstream processing of microalgae for pigments, protein and carbohydrate in industrial application: A review. *Food and bioproducts processing*, 110, 60-84.

Kong, W., Liu, N., Zhang, J., Yang, Q., Hua, S., Song, H., & Xia, C. (2014). Optimization of ultrasound-assisted extraction parameters of chlorophyll from *Chlorella vulgaris* residue after lipid separation using response surface methodology. *Journal of food science and technology*, 51(9), 2006-2013.

Kong, W., Song, H., Cao, Y., Yang, H., Hua, S., & Xia, C. (2011). The characteristics of biomass production, lipid accumulation and chlorophyll biosynthesis of *Chlorella vulgaris* under mixotrophic cultivation. *African Journal of Biotechnology*, 10(55), 11620-11630.

Koyande, A. K., Chew, K. W., Rambabu, K., Tao, Y., Chu, D. T., & Show, P. L. (2019). Microalgae: A potential alternative to health supplementation for humans. *Food Science and Human Wellness*, 8(1), 16-24.

Lafarga, T. (2019). Effect of microalgal biomass incorporation into foods: Nutritional and sensorial attributes of the end products. *Algal Research*, 41, 101566.

Lanfer-Marquez, U. M., Barros, R. M., & Sinnecker, P. (2005). Antioxidant activity of chlorophylls and their derivatives. *Food Research International*, 38(8), 885-891.

Larkin, R. M., Stefano, G., Ruckle, M. E., Stavoe, A. K., Sinkler, C. A., Brandizzi, F., Malmstrom C. M., & Osteryoung, K. W. (2016). Reduced chloroplast coverage genes from *Arabidopsis thaliana* help to establish the size of the chloroplast compartment. *Proceedings of the National Academy of Sciences*, 113(8), E1116-E1125.

Loh, C. H., Inbaraj, B. S., Liu, M. H., Chen, B. H. (2012). Determination of chlorophylls in *Taraxacum formosanum* by high performance liquid chromatography-diode array detection−mass spectrometry and preparation by column chromatography. *Journal of Agricultural and Food Chemistry*, 60, 6108-6115.

Lohr, M., Im, C. S., & Grossman, A. R. (2005). Genome-based examination of chlorophyll and carotenoid biosynthesis in *Chlamydomonas reinhardtii*. *Plant Physiology*, 138(1), 490-515.

Mandelli, F., Miranda, V. S., Rodrigues, E., & Mercadante, A. Z. (2012). Identification of carotenoids with high antioxidant capacity produced by extremophile microorganisms. *World Journal of Microbiology and Biotechnology*, 28(4), 1781-1790.

Maroneze, M. M., Siqueira, S. F., Vendruscolo, R. G., Wagner, R., de Menezes, C. R., Zepka, L. Q., & Jacob-Lopes, E. (2016). The role of photoperiods on photobioreactors–A potential strategy to reduce costs. *Bioresource technology*, 219, 493-499.

Maroneze, M. M., Jacob-Lopes, E., Zepka, L. Q., Roca, M., & Pérez-Gálvez, A. (2019). Esterified carotenoids as new food components in cyanobacteria. *Food chemistry*, 287, 295-302.

Matos, Â. P. (2017). The impact of microalgae in food science and technology. *Journal of the American Oil Chemists' Society*, 94(11), 1333-1350.
Mohsenpour, S. F., Richards, B., & Willoughby, N. (2012). Spectral conversion of light for enhanced microalgae growth rates and photosynthetic pigment production. *Bioresource technology*, 125, 75-81.
Mulders, K. J., Lamers, P. P., Martens, D. E., & Wijffels, R. H. (2014). Phototrophic pigment production with microalgae: biological constraints and opportunities. *Journal of phycology*, 50(2), 229-242.
Nakamura, A., Akai, M., Yoshida, E., Taki, Y., & Watanabe, T. (2003). Reversed-phase HPLC determination of chlorophyll a and phylloquinone in Photosystem I of oxygenic photosynthetic organisms. *European Journal of Biochemistry*, 270, 2446–2458.
Özyurt, G., Uslu, L., Yuvka, I., Gökdoğan, S., Atci, G., Ak, B., & Işik, O. (2015). Evaluation of the cooking quality characteristics of pasta enriched with *Spirulina platensis*. *Journal of Food Quality*, 38(4), 268-272.
Palabiyik, I., Durmaz, Y., Öner, B., Toker, O. S., Coksari, G., Konar, N., & Tamtürk, F. (2018). Using spray-dried microalgae as a natural coloring agent in chewing gum: effects on color, sensory, and textural properties. *Journal of applied phycology*, 2 (2013), 1-9.
Patias, L. D., Fernandes, A. S., Petry, F. C., Mercadante, A. Z., Jacob-Lopes, E., & Zepka, L. Q. (2017). Carotenoid profile of three microalgae/cyanobacteria species with peroxyl radical scavenger capacity. *Food research international*, 100, 260-266.
Pérez-Gálvez, A., Viera, I., & Roca, M. (2017). Chemistry in the bioactivity of chlorophylls: An overview. *Current medicinal chemistry*, 24(40), 4515-4536.
Plaza, M., Santoyo, S., Jaime, L., Avalo, B., Cifuentes, A., Reglero, G., Reina, G. G., Reina, F. J., & Ibáñez, E. (2012). Comprehensive characterization of the functional activities of pressurized liquid and ultrasound-assisted extracts from *Chlorella vulgaris*. *LWT-Food Science and Technology*, 46(1), 245-253.
Pool, E. K., Shahidi, F., Mortazavi, S. A., Azizpour, M., & Daneshzad, E. (2016). Examination of the effect of *Spirulina platensis* microalgae on drying kinetics and the color change of kiwifruit pastille. *Journal of Food Measurement and Characterization*, 10(3), 634-642.
Rahman, K. M. (2020). Food and High Value Products from Microalgae: Market Opportunities and Challenges. In *Microalgae Biotechnology for Food, Health and High Value Products* (pp. 3-27). Springer, Singapore.
Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M., & Stanier, R. Y. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology*, 111, 1-61.
Rizwan, M., Mujtaba, G., Memon, S. A., Lee, K., & Rashid, N. (2018). Exploring the potential of microalgae for new biotechnology applications and beyond: a review. *Renewable and Sustainable Energy Reviews*, 92, 394-404.
Roca, M.; Chen, K.; Pérez-Gálvez, A. Chlorophylls, In: Carle R., Schweiggert R. (Eds.), *Handbook on natural pigments in food and beverages: industrial applications for improving food color*. Woodhead Publishing: Cambridge, UK, 2016, pp. 125-158.
Rumin, J., Nicolau, E., Junior, R. G. D. O., Fuentes-Grünewald, C., & Picot, L. (2020). Analysis of Scientific Research Driving Microalgae Market Opportunities in Europe. *Marine Drugs*, 18(5), 264.
Sarkar, S., Manna, M. S., Bhowmick, T. K., & Gayen, K. (2020). Extraction of chlorophylls and carotenoids from dry and wet biomass of isolated *Chlorella Thermophila*: Optimization of process parameters and modelling by artificial neural network. *Process Biochemistry*, 96, 58-72.

Sathasivam, R., Radhakrishnan, R., Hashem, A., & Abd_Allah, E. F. (2017). Microalgae metabolites: A rich source for food and medicine. *Saudi journal of biological sciences*, 26(4), 709-722.

Solymosi, K., & Mysliwa-Kurdziel, B. (2017). Chlorophylls and their derivatives used in food industry and medicine. *Mini reviews in medicinal chemistry*, 17(13), 1194-1222.

Vendruscolo, R. G., Facchi, M. M. X., Maroneze, M. M., Fagundes, M. B., Cichoski, A. J., Zepka, L. Q., Barin, J. S., Jacob-Lopes, E., & Wagner, R. (2018). Polar and non-polar intracellular compounds from microalgae: Methods of simultaneous extraction, gas chromatography determination and comparative analysis. *Food research international*, 109, 204-212.

Xu, W., Tang, H., Wang, Y., & Chitnis, P. R. (2001). Proteins of the cyanobacterial photosystem I. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 1507(1), 32-40.

Yen, H. W., Hu, I. C., Chen, C. Y., Ho, S. H., Lee, D. J., & Chang, J. S. (2013). Microalgae-based biorefinery from biofuels to natural products. *Bioresource technology*, 135, 166-174.

Zepka, L. Q., Jacob-Lopes, E., Goldbeck, R., & Queiroz, M. I. (2008). Production and biochemical profile of the microalgae *Aphanothece microscopica Nägeli* submitted to different drying conditions. *Chemical Engineering and Processing: Process Intensification*, 47(8), 1305-1310.

Zepka, L. Q., Jacob-Lopes, E., Goldbeck, R., Souza-Soares, L. A., & Queiroz, M. I. (2010). Nutritional evaluation of single-cell protein produced by *Aphanothece microscopica Nägeli*. *Bioresource Technology*, 101(18), 7107-7111.