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Integrated analytical approaches for the characterization of Spirulina and Chlorella microalgae

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Abstract

Microalgae are well-known for their content of bioactive molecules such as pigments, proteins, fatty acids, polysaccharides, vitamins and antioxidants, all of which are of great interest in the preparation of a wide range of products such as food, cosmetics and nutraceuticals. The purpose of this project was the analytical characterization of commercial dry microalgal biomass: four samples of *Chlorella* and five of *Spirulina* were analysed in order to highlight their content in terms of micro/macro nutrients. The research was oriented towards the development and validation of accurate, fast and reproducible methods for the nutritional assessment of algal biomasses, aiming to provide a guiding methodology. The lipid profiles of algal matrixes were analysed for the content of saturated, unsaturated and polyunsaturated fatty acids. The process was divided into two phases: firstly, the extraction and determination of the total lipids and pigment content; secondly, the trans-esterification of the extracted lipid-pigment portion in order to analyse fatty acid methyl esters (FAME) with a GC-MS method. A fingerprinting of MUFAs and PUFAs was obtained regarding microalgae species. The determination of total carotenoids and chlorophylls content in the lipid extracts was evaluated through a fast UV-Vis spectrophotometric analysis, which was validated by a new HPLC-DAD analysis. Furthermore, the total antioxidant activity of each lipid extract was determined along with the determination of the microalgae protein content. Then, with the aid of the principal component analysis (PCA) plots, the two microalgae were clustered in terms of their micro/macro nutrients, for differentiating their properties. *Spirulina*, resulting to have a greater antioxidant activity, supposedly due to a higher content in pigments and higher protein concentration, could be suggested for an appropriate diet for sporting people. *Chlorella*, instead, showed a more balanced profile of PUFAs and MUFAs and its use could be suggested for cosmetics and vegan diets. This paper puts forward an overall analytical approach, sustained by a multivariate analysis, for emphasising content differences and activity of two different microalgae strains, in order to underline specific claims for each class, addressed to defined final users.

Keywords= *Chlorella* and *Spirulina*, unsaturated fatty acids, pigments, gas chromatography-mass spectrometry, HPLC-DAD, antioxidant activity, multivariate analysis

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1.Introduction

In 1898 for the first time the term "nutraceutical" was coined to combine the concepts of "nutrition" and "pharmaceutical". Nowadays, there is not an accepted international definition for nutraceuticals but generally they indicate bioactive compounds extracted from food matrix helpful for improving human health. Nutraceuticals reduce the risks for chronic disease, age-related degeneration and therefore they contribute to increase life expectancy [1]. Nowadays, in the search of nutraceuticals, marine microalgae are gaining attention for their interesting content in high value-added compounds. Indeed, microalgae which include both eukaryotic microorganisms and prokaryotic cyanobacteria (blue-green algae) are now considered as sustainable source for food and energy [2]. Microalgae can be found in both seawater and freshwater, and they have more efficient photosynthetic mechanisms in comparison to land-based plants producing elevated yield of biomass. This could be due to their lower cell structure complexity and in the meantime to the possibility of consuming CO₂ and further nutrients in an easier way since they grow in aquatic environments. This is associated to further positive aspects: their way of cultivation avoids the soils competition with the traditional crops and less fertilizers and pesticides are required. Finally, fixing atmospheric CO₂ with higher efficiency than terrestrial plants and being potentially grown exploiting wastewater from industry, the microalgae cultivation contribute to reduce environmental pollution [3]. Microalgae are characterized by higher protein level than the one determined e. g. in soybean, corn, and wheat, containing all essential amino acids. Concerning the lipid portion, fatty acids (FAs) are bound to glycerolipids (mainly phospholipids, glycolipids and triacylglycerols) and they are different depending on the species. From the nutraceutical point of view, the research is focused on long-chain polyunsaturated fatty acids (PUFAs), in particular on ω -3 and ω -6 classes. The former is responsible of reducing the risk chronic disease and the latter is involved in inflammatory reactions. In particular, concerning ω -6 family of PUFAs, arachidonic acid (AA) is one of the main components of the cell membrane of the gray matter and of retina visual elements. Furthermore, AA is a substrate for the synthesis of eicosanoids, which are lipid-based signaling molecules involved in the communication between innate and adaptive immunities by acting on the cells of both systems [4]. On the other hand, ω 3 fatty acid family is essential in various pathological conditions such as reducing the risk of chronic brain diseases and cardiovascular dysfunctions, lowering blood pressure and heart rate, reducing the amount of serum triglycerides. Since ω -3, such as EPA and DHA, are widely

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present in cold-water fish and seafood, vegans are recommended to assume them with the diet in order to increase endogenous production. Moreover, consuming nuts, seeds, vegetables, and vegetable oils such as soybean oil, safflower oil, and corn oil, vegans assume with the diet more linoleic acid (LA) in comparison to alpha-linolenic acid (ALA) that is rarely present in vegetable foods. LA and ALA are respectively the precursor in the ω -6 and ω -3 metabolic pathways, and they compete for the same synthesizing enzyme Δ 6 desaturase. Therefore, an imbalanced diet intake suppresses DHA synthesis and docosapentenoic acid (22: 5n-6; DPA) production substitutes DHA in the retinal and neural tissues. In addition, the minor microalgae components such as pigments (carotenoids, chlorophylls) have valuable properties that can contribute to human well-being. Among these, β -carotene is recognised for its antioxidant activity and for exerting protective effects towards atherosclerosis. Lutein and Zeaxanthin, identified as yellow pigment found in human retina, are dietary carotenoids, involved in eye health [5]. In addition, Lutein has been recently investigated for its potential anticancer activity [6]. The chemical composition of microalgae is different depending on the species and the cultivation environment. Therefore, the type of cultivation systems is specifically optimized and growth conditions are modulated adding nutrients (nitrogen, magnesium, calcium, selenium, sulphur, glucose), changing the value of culture medium pH and modifying the type of light. In this way it is improved the accumulation of specific biomolecules in microalgae cells, without affecting the grow rate [7,8]. Especially, light intensity, use of pulsed light and/or time of photo exposition, regulate microalgae growth and the accumulation of carotenoids and lipids. Notably, when light is intense the amount of saturated and monounsaturated fatty acids increases, on the contrary the rate of polyunsaturated fatty acids decreases [9]. However, the nutraceutical field lacks analytical approaches to qualitatively, quantitatively and in term of efficacy deeply and fully characterize bioactive components. Since active metabolites contained in complex samples, such as microalgae, belong to several classes, a multivariate approach can be very useful to give an overall and immediate snapshot of the peculiar characteristic of the specific microalgae strain with a quick correlation between several variables and species. The comprehensive clarification of actives' content and secondary metabolites can better address the claims for the specific microalgae strain, tackling more specifically final users' needs. Thus, in this study an integrated analytical approach to profile commercial powders of Spirulina and Chlorella species in terms of content of high-valued compounds is provided. In particular, two different species

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of microalgae are compared for their biochemical composition and their biological properties but also changes in their content are highlighted within the same species. Therefore, these analytical procedures are proposed as a helpful tool for selecting the right growing conditions and performing quality control of the most convenient strain of microalgae in view of future industrial applications [10].

2. Experimental

2.1 Materials

Microalgal samples of *Chlorella Vulgaris* and *Arthrospira Platensis* were commercially available. 2-Propanol ACS reagent $\geq 99.5\%$, Chloroform puriss. p.a. reagent ISO reagent Ph. Eur. 99.0-99.4% (GC), Methanol puriss. p.a. ACS reagent reagent ISO reagent Ph. Eur. $\geq 99.8\%$ (GC), Methanol suitable for HPLC $\geq 99.9\%$, Sulfuric acid 62% for analysis, Hexane puriss. p.a., ACS reagent reagent Ph. Eur. $\geq 99\%$ (GC), Acetonitrile suitable for HPLC gradient grade $\geq 99.9\%$, Water HPLC Plus, Acetone suitable for HPLC $\geq 99.8\%$, Sodium sulfate ACS reagent $\geq 99.0\%$ anhydrous granular and Supelco 37 Component FAME Mix certified reference material TraceCERT[®] in dichloromethane (varied conc.) ampule of 1 mL (FAME Mix), Chlorophyll a analytical standard purity $\geq 95\%$, Chlorophyll b analytical standard purity $\geq 95\%$, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, potassium persulfate, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), sodium dodecyl sulfate, NaOH, Na₂CO₃, CuSO₄ 5H₂O, potassium sodium tartrate tetrahydrate and Folin reagent were purchased from Sigma-Aldrich company (St. Luis, MO, USA).

Lutein analytical standard provided with UV assay for quantitative titration, Zeaxanthin analytical standard provided with UV assay for quantitative titration and β -carotene analytical standard provided with UV assay for quantitative titration were purchased from Extrasynthese, Z.I Lyon Nord - Impasse Jacquard - CS 30062 69727 Genay Cedex France.

2.2 Lipids extraction: sample preparation

Commercial microalgal samples were firstly cryo-lyophilised at -60 °C for 24 hours (Alpha 1-4 LD PLUS, cryo-lyophilizer, Christ). Lipid and pigment contents were evaluated with a gravimetric procedure. In a 50 mL centrifuge tube, a quantitative amount of lyophilised microalgal powder, in the range of 5 g, was exactly weighed. The gravimetric analysis was carried out through sequential extractions. 40 mL of isopropanol were added to the powder. The suspension was vortexed for 3

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minutes and sonicated for 20 minutes. Then, the sample was centrifuged for 5 minutes at 4000 rpm and the solvent was collected in a round bottom flask. Then, following the same procedure, sequential extractions were performed with a volume 30 mL of a mixture of chloroform/methanol prepared in different proportion (2:1, 2:1, 1:1, 1:2 (v/v)). The collected extracts were dried with rotavapor. Then, the crude oil was subjected to the modified Folch wash procedure [10] using 40 mL of a mixture of chloroform, methanol and water (8:4:3). The collected organic solvent was filtered with a PTFE filter disk of 0.45 µm and it was evaporated by rotavapor at 45 °C. Finally, it was dried with a vacuum pump for 5 h. The dried oil represented the gravimetric extract.

The percentages of lipids and pigments present in the algal powders were calculated considering the following formula:

$$\text{\% of lipid and pigment} = (\text{weight of obtained algal oil} / \text{weight of dried microalgal powder}) * 100$$

The lipids and pigment gravimetric evaluation was performed twice for each sample.

2.3 Sample preparation for GC-MS analysis of fatty acids

The characterization of fatty acids was performed through the transesterification of the lipid component. The procedure for transesterification of the lipid components and extraction of FAMES is reported in SI (par.2.1).

The relative percentage of FAMES present in the lipid extract was calculated considering the following formula:

$$\text{\% of FAMES} = (\text{weight of obtained FAME algal oil} / \text{weight of dried lipid and pigment extract before esterification}) * 100$$

The relative percentages of FAMES present in the algal powders were calculated considering the following formula:

$$\text{\% of FAME in total powder} = (\text{\% of FAME in lipid and pigment extract} / \text{\% of lipid and pigment content}) * 100$$

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The lipids methylation was performed twice for each sample.

The obtained extracts of FAMES were solubilised with 3 mL of hexane and filtered in a vial using a PTFE syringe filter of 0.20 µm. Then, 1 µL of the solution was analysed with GC-MS

2.4 GC-MS analysis of FAMES

The chromatographic method was optimized with an Agilent GC 7820A GC System coupled to a single quadrupole selective mass detector Agilent 5977E MSD in electron ionization (EI) mode (70 eV) under a temperature gradient elution using a DBWAXtre capillary column (30 m × 0.25 mm × 0.25 µm; Agilent). Helium, the gas carrier, had a flow rate of 1 mL min⁻¹. 1 µL of the sample was injected in split ratio of 50:1.

The MS source temperature was at 250 °C, the MS quad temperature was at 150 °C, the AUX 1 temperature was at 250 °C and the inlet F temperature was at 250 °C. MS scanned from: 45 to 650 Da each 0.5 s. The GC oven temperature program started at 45 °C to reach 165 °C with rate of 5 °C min⁻¹ and hold time 15 minutes then, the temperature was increased to 215 °C with a rate 3 °C min⁻¹ and hold time of 16 min. Finally, the temperature was increased to 260 °C with a rate of 10 °C min⁻¹ and hold time of 2 minutes. The total run time was 78.17 minutes. Data were acquired with MassHunter GC/MS Acquisition B.07.00, 2013, processed with MassHunter Workstation Software Qualitative Analysis B.06.00, 2012 and compared and identified with NIST Mass Spectral Search Program, 2012 and with Sulpeco 37 component FAME Mix reference standard.

Samples were analysed twice and the percentage of each FAME present in the analysed algal oils, was calculated considering the following formula:

$$\text{\% of FAME of interest} = (\text{area of peak of interest} / \text{total sum of chromatographic peak areas}) * 100$$

2.5 UV-Vis-spectrophotometric pigment analysis

The pigment content was evaluated with a Jasco spectrophotometer UV-Vis V-630 bio. Standard solutions of β-carotene and Chlorophyll a were prepared according to the following the procedure. About 10 mg of β-carotene accurately weighted were solubilised in a 250 mL volumetric flask with a solution of 70:30 dichloromethane/methanol. The stock solution was then diluted 1:8 in methanol. An aliquot of about 1 mg of Chlorophyll a accurately weighted was solubilised in 1 mL of chloroform.

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The stock solution was diluted 1:100 with methanol. An aliquot of about 1 mg of Chlorophyll b accurately weighed was solubilised in 1 mL of chloroform. The stock solution was diluted 1:100 with methanol. The UV-Vis spectra of standards were registered by scanning between 300 to 700 nm. In order to determine the pigment content of the microalgal sample about 30-40 mg, accurately weighed of lipid and pigment extract were solubilized in 5 mL of a 3:2 methanol/dichloromethane mixture. 100 µL of this solution was made up to volume in a 10 mL flask with methanol (dil. 1:100). Using methanol as blank, the UV-Vis spectrum of the obtained solution was recorded in the range 300-700 nm to evaluate the absorption profiles and the maximum wavelengths (λ_{\max}).

The absorbance at 477 nm of the lipid and pigment extracts was compared with that of β -carotene. Since the spectra of β -carotene and Chlorophyll b were overlapped at this wavelength, the contribute of Chlorophyll b in every lipid extract at 477 nm was subtracted taking in consideration the absorbance of Chlorophyll b at 651 nm where there was no interference. The following formula was used:

$$\text{abs } \lambda_{477} \text{ Total carotenoid} = \text{abs } \lambda_{477} \text{ of the extract} - [((\text{abs } \lambda_{651} \text{ Chlorophyll b in the extract}) * (\text{abs } \lambda_{477} \text{ Chlorophyll b STD})) / (\text{abs } \lambda_{651} \text{ Chlorophyll b STD})]$$

The values obtained at 477 nm of total carotenoid and at 664 nm of the lipid pigment extracts were compared with those at the respective λ_{\max} of β -carotene and Chlorophyll a. The total content of the pigment in the lipid extracts was calculated with the following formula:

$$\text{Conc_PE } (\mu\text{g mL}^{-1}) = (\text{Conc_STD_P} * \text{abs_LE}) / \text{abs_STD_P}$$

where Conc_PE= concentration of the pigment in the extract; Conc_P_STD = concentration of the pigment standard solution; ABS_LE= abs of the lipid extract; ABS_STD_P= abs of the pigment standard solution

The percentage of the pigment in the extract was calculated with the formula:

$$\%PE = \text{Conc_PE } (\mu\text{g mL}^{-1}) * 100 / \text{Conc_LE } (\mu\text{g mL}^{-1})$$

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where %PE= percentage of the pigment in the extract; Conc_PE= concentration of the pigment in the extract; Conc_LE= concentration of the lipid extract

Samples were analysed twice. The percentage of the pigment in the dried algal powder was calculated with the formula:

$$\%PAP = (\%LE * \%PE) / 100$$

where %PAP= percentage of the pigment in the algal powder; %LE= percentage of the lipid extract; %PE= percentage of the pigment in the extract

2.6 HPLC-DAD pigment analysis

β -carotene, Zeaxanthin and Lutein were solubilized in 100% acetone in order to obtain concentration of $68.0 \mu\text{g mL}^{-1}$, $28.0 \mu\text{g mL}^{-1}$ and $30.0 \mu\text{g mL}^{-1}$ respectively; Chlorophyll a and Chlorophyll b were solubilized in a mixture of chloroform and methanol (50:50 v/v) in order to obtain $1000.00 \mu\text{g mL}^{-1}$ stock solution for both.

The separation and identification of pigments were performed by HPLC analysis with Agilent Infinity 1260 with a binary pump VL, a Diode Array Detector VL, Infinity II manual injector equipped with a loop of 20 μL and with a Standard Degasser. Before injection, solubilized lipid extracts were filtered through a 0.2 μm PTFE syringe filter. The chromatographic separation of carotenoids in lipid extracts of microalgae samples was performed by using a Develosil[®] RP-Aqueous, Phenomenex chromatographic column (5 μm , C30, 140 Å, 250 x 4.6 mm) at room temperature. The mobile phase (A) consisted of methanol-acetonitrile-water (79:14:7 v/v/v) and mobile phase B consisted of acetone (100%), at a flow rate of 1.3 mL min^{-1} . The optimized gradient solvent system was 0-6 minutes, 100% A; 6-26 minutes, 95% A; 26-36 minutes, 50% A; 36-60 minutes, 20% A; 60-65 minutes 20% A; 65-80 minutes, 100% A for conditioning. The total runtime was 80 minutes. The response was detected at 450 nm (ref: 700nm) for carotenoids and Chlorophyll b and at 660 nm (ref: 700 nm) for Chlorophyll a. The identification of pigments in the lipid microalgae samples was carried out by the comparison of the retention time and UV-vis spectra of sample peaks with those obtained for the pure standards. *Linearity.* Standard calibration curves were obtained by analyzing each standard in different ranges: β -carotene (0.72 - $22.60 \mu\text{g mL}^{-1}$), Lutein (0.29 - $28.00 \mu\text{g mL}^{-1}$), Zeaxanthin (0.31 - $10.00 \mu\text{g mL}^{-1}$),

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Chlorophyll a ($1.95\text{-}125.00\text{ }\mu\text{g mL}^{-1}$) and Chlorophyll b ($1.95\text{-}125.00\text{ }\mu\text{g mL}^{-1}$). Solutions were stored in the dark at -20°C .

Sensitivity. The limit of detection (LoD) and limit of quantitation (LoQ) values were obtained by statistical evaluation considering the standard deviations of the signals ($\text{LoD} = 3 \cdot \text{SE}/m$ and $\text{LoQ} = 10 \cdot \text{SE}/m$; standard error (SE)).

Precision. The intra- and inter-day precisions were evaluated by analysing a mixture of β -carotene, Lutein, Zeaxanthin, Chlorophyll a, Chlorophyll b, standard pigments at fixed concentration (range $2.34\text{-}16.67\text{ }\mu\text{g mL}^{-1}$), during the same day ($n = 3$) and three times a day in three different days during one week ($n = 9$).

Recovery. Before the lipid extraction, recovery determination was carried out twice on sample **S3** by adding a fixed concentration of Lutein to the algal sample in order to obtain a final concentration of added Lutein of $2.5\text{ }\mu\text{g mL}^{-1}$. The recovery values were obtained by the following formula:

$$\% \text{ Recovery} = [(\text{Peak Area of Lutein in spiked sample} - \text{Peak Area of Lutein in unspiked sample}) / (\text{Peak Area Lutein of standard solution})] \cdot 100$$

Accuracy. The accuracy was determined by adding a fixed concentration of Lutein on sample **S3** before its lipid extraction. Then lipid extraction was performed by following the procedure reported in par. 2.2. Hence, the resulting lipid extract was dissolved in chloroform and methanol (50:50 v/v). The obtained solution was injected in the HPLC following the chromatographic conditions previously described. This procedure has been repeated twice. The obtained Lutein peak areas of unspiked **S3** were subtracted to the ones found in spiked **S3**. The resulting Lutein peak area was interpolated in the standard calibration curve to obtain Lutein amount added to unspiked **S3** sample.

Then, accuracy was calculated by using percentage of the deviation between the experimental concentrations of Lutein added in spiked **S3** sample and the nominal one (theoretical final concentration of added Lutein in sample = $2.5\text{ }\mu\text{g mL}^{-1}$). Concerning algal sample preparation, lipid and pigment extracts were solubilized in chloroform and methanol (50:50 v/v) in order to obtain concentration in the range $0.74\text{-}6.02\text{ mg mL}^{-1}$ according to the content of each sample. Then, samples were sonicated for 5 minutes at room temperature and analyzed by HPLC-DAD. Concentrations of unknown peaks were calculated by interpolating with the calibration curve of β -carotene if the UV-Vis spectra were similar to that of carotenoids (named

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unknown carotenoids), conversely, if the UV-Vis spectra were similar to that of chlorophylls, the concentration was calculated considering the calibration curve of Chlorophyll a (named *unknown chlorophylls*).

Percentage of analytes in each lipidic extract was calculated with the following formula:

$$\text{\% of analyte on lipidic extract} = [(\text{mg of analyte})/(\text{weight of the lipidic extract (mg)})]*100$$

Then, the percentage of each analyte present in the dried powder was calculated by using the following formula:

$$\text{\% analyte on dried powder} = [(\text{mg of analyte}/(100\text{g of lipidic fraction})) * \text{\% of lipids}]/100$$

Microalgae samples were extracted and analysed twice daily for carotenoids and chlorophylls determination in order to calculate intra-day precision. Whereas, inter-day precision was assessed by repeating the overall analysis in three different days.

2.7 Antioxidant activity, ABTS cation radical scavenging assay

The ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical scavenging activity was performed by spectrophotometric measurement of the lipid and pigment extracts obtained with the procedure described in par. 2.2, optimized by the method described by Re et al. [11]. The procedure for ABTS cation radical scavenging assay is described in par. 5SI. Samples were analysed twice.

2.8 Protein content

The protein content was evaluated through the colorimetric Lowry method following the procedure described by Lowry et al. [12,13]. The procedure of protein extraction and quantification is reported in par. 6SI. Samples were analysed four times.

3. Results and discussion

Due to their beneficial properties, microalgae *Spirulina* and *Chlorella* have been widely used as food source so far. Therefore today, their biochemical composition consisting of lipids, pigments, vitamins

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and minerals, polysaccharides and proteins is receiving growing interest for applications in food, feed, pharmaceutical and biotechnological fields [14]. Thus, determining the biochemical composition of microalgae is essential to select the best strain for specific applications [15].

In this context, the present work was aimed to provide an integrated analytical approach useful to characterize different commercial samples of microalgae. In particular, four commercial samples of *Chlorella* (**C1, C2, C3, C4**) and five of *Spirulina* (**S1, S2, S3, S4, S5**) were analysed in order to highlight differences in composition among the same species and between the two species. The first step of this work was focused on the research and validation of analytical methodologies for microalgal lipid and pigment characterization. For a raw quantification of lipids and pigments, extraction procedures followed by gravimetric measurements were performed with the aim of improving the yield of extraction and to obtain an extract lacking hydrophilic components. In addition, for the qualitative determination of fatty acids, a gas chromatographic coupled to mass spectrometry method was developed (GC-MS) to study the profile of each sample. The chromatographic peaks were compared with reference standards and identified to cluster the most representative fatty acids of the two species in saturated, mono- and poly- unsaturated fatty acids.

UV-Vis spectroscopy was selected to perform a fast semi-quantitative determination of pigment content, to detect and quantify the presence of chlorophylls and carotenoids in the lipid extract and it was confirmed by analysis conducted with a new validated HPLC method. Finally, the profiles of each microalgae regarding PUFAs and pigments were studied and correlated to the total antioxidant activity of their lipid extracts. Then the total protein content was determined.

A comparison was carried out about the respective analyte content within the same species (intra-species) and among *Chlorella* and *Spirulina* (inter-species) underlying their differences.

3.1 Lipids and pigments gravimetric determination

The evaluation of the lipid and pigment content included the extraction of the entire lipid portion and its gravimetric determination. Many extractive procedures were described [16,17]. In this work, the first part of the extractive method described by Kamel A Harrata et al [17], which is one of the most used, was optimized by maintaining the organic solvents described in literature, but reducing the amount of algal powder (5 g instead of 20 g), volumes (160 mL instead of 1500 mL), and total time (115 minutes instead of 360 minutes), introducing the use of ultrasounds assisted extraction and

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avoiding the use of the homogenizer (table 1SI). Finally, with the modified Folch wash the final lipid sample was gravimetrically determined [17].

The new extraction was validated by independently comparing the method described by Kamel A Harrata et al [17] with the new procedure reported in par 2.2. Each procedure was applied twice. Since the yield of extractions was found almost similar with the two methods (data regarding sample **C1** and sample **S1** are reported in table 1SI), the efficiency of the new proposed method was furthermore demonstrated by a reduced amount of organic solvent required for the extraction of algal sample and a shorter operating time. The new validated extraction was applied to each algal species (four different samples of Chlorella and five different samples of Spirulina).

Table 2SI reports the lipid and pigment contents extracted from Chlorella and Spirulina samples. In terms of total amount of lipids, the two species contained a similar percentage average of lipid and pigment content ($11.44 \pm 1.31E-01$ and $11.24 \pm 2.10E-01$, Chlorella and Spirulina respectively). In particular, sample **C2** and sample **S1** are the two richest in terms of lipid and pigment oil.

3.2 FAMES analysis

The composition of the fatty acids in the lipid samples was analysed by GC-MS analysis.

Although, in quantitation, GC-FID is more suited instead of GC-MS, the latter guarantee an unambiguous identification also of the fatty acids not available as standards.

In detail, as described in par. 2.3, the process was consisted in the trans-methylation of lipid esters, in the subsequent FAMES extraction and finally in their determination both in the lipid extracts and in the total powders.

The lipid component was analysed with GC-MS. The chromatographic conditions [17] were optimized with Agilent GC-MS System with a DBWAXtre Agilent capillary column. In particular, the oven conditions (par. 2.4) were adjusted in order to obtain an efficient analyte elution of the commercial standard FAMES mix (10 μ L FAMES mix mixed with 10 μ L of nonadecanoic acid methyl ester, conc.= 418 μ g mL⁻¹, previously esterified as reported in par. 2.3).

The identification of the fatty acids in the analysed algal samples was performed by comparison of the reference standard retention times and their mass spectra matching with NIST 2012 library (Figure 1SI). All the four samples of Chlorella and the five samples of Spirulina were analysed.

In Figure 1 the chromatograms of **C2** and **S3** samples are reported. Different population of FAMES were identified in the two algal species. For instance, the peaks of the polyunsaturated 7,10,13-

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hexadecatrienoic acid (C16:3) and of the linolenic acid (C18:3), are present in C2, but missing in S3 which, instead, contains γ -Linolenic acid (C18:3).

In supporting information (3SI - 11SI) the tables related to the detailed FAMES composition for each algal sample are reported and values are expressed as a relative percent.

The relative percentage of each fatty acid is also showed in base of the individual peak area over the total peak area sum (par. 2.4).

To summarize, (table 12SI) the FAMES determined in each algal sample are grouped in saturated, monounsaturated (MUFAs) and PUFAs. In addition, due to the variability of FAMES content between the two species, only the most common fatty acids are reported. By comparing the two species, the average content of PUFAs is higher in Chlorella samples, Spirulina samples are richer in saturated FAMES, while the content of MUFAs is roughly similar in both species (Figure 2a).

In Figure 2b the most abundant fatty acids that populate the FAMES portion (approximately representing a range between 60 and 80 percent of the total lipid fraction) in analysed microalgae are described. In particular, S2 is the richest in terms of palmitic acid (C16:0) samples contained oleic acid (C18:1) and a precursor of the previous one, the stearic acid that demonstrated the highest content in samples C2 and S4 ($\% = 1.71 \pm 2.95E-02$, $\% = 3.67 \pm 3.90E-02$ respectively). Considering Chlorella species, C2 has the highest content of the omega 7 palmitoleic acid (C16:1). Concerning PUFAs, although the linoleic acid (C18:2) is contained in both species, the essential linolenic fatty acid (C18:3) is present only in the Chlorella species and the semi-essential γ -linolenic acid (C18:3) in the Spirulina species.

3.3 UV-Vis-spectrophotometric pigment content

Due to their lipophilicity, carotenoids and chlorophylls, were extracted together in the lipid sample of microalgae, as described in par. 2.2. To get a preliminary and semiquantitative profile of pigment extracts, a fast UV-Vis spectrophotometric method was applied. Indeed, carotenoids and chlorophylls spectra show sufficiently resolved absorption bands to allow their selective determination.

The UV-Vis profiles of β -carotene, Zeaxanthin and Lutein are mostly overlapped, showing a λ_{\max} at 477 nm, whereas Chlorophyll a absorbs selectively at $\lambda_{\max} = 664$ in a different region of the visible range (Figure 3). Therefore, the UV-Vis spectrum in the range between 300 nm and 700 nm of a mixed standard solution of β -carotene, Zeaxanthin, Lutein, Chlorophyll a and Chlorophyll b (Figure 3a), was registered in order to evaluate the absorption bands and the λ_{\max} by using methanol as blank.

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Since the profiles of β -carotene, Zeaxanthin and Lutein were mostly overlapped, it was considered the λ_{\max} = 477 nm for determining the raw carotenoid content in the microalgal extracts. Instead, the λ_{\max} of 664 nm was chosen to quantify the total amount of chlorophylls in the algal samples since the chlorophylls and carotenoids spectra are overlapped, the absorbance chlorophylls contribute at 477 nm was subtracted to the one of carotenoids. UV-Vis overlapped spectra of sample **C1** and **S3** are reported in Figure 3b.

The estimated concentration of analytes of each algal sample (table 13SI) was calculated with formula reported in par. 2.5. The lipid extract was solubilized in a 3:2 (v/v) methanol/dichloromethane mixture and diluted with methanol to reach the optimal signal in response to the concentration to register the UV-Vis spectra between 300-700 nm.

The carotenoid content, that was less than 0.5% in each algal sample, was found to be higher in **C1** and **S3**, but there was no evidence of interspecies massive differences (Figure 4). Concerning chlorophylls, values were less than 2% and, in particular, *Spirulina* species was found to be the richest one. Among the same species, **C1** and *Spirulina* **S2** had the highest content of chlorophylls.

3.4 HPLC-DAD pigment analysis

To confirm data obtained by the UV-Vis pigments analysis, a HPLC-DAD method was developed and validated, by optimizing the method described by Inbaraj et al. [18] and by Aluç et al. [19] by adding the determination of Chlorophyll a and of Chlorophyll b along with carotenoids.

In order to improve the separation of the analytes (β -carotene, Lutein, Zeaxanthin, Chlorophyll a and Chlorophyll b), instead of a C18, a C30 column was chosen considering the better resolution and efficiency [20].

A gradient elution was carried out with an increased flow rate of 1.3 mL min⁻¹ (instead of 0.6 and 1.0 mL min⁻¹ as previously reported). The mobile phase (A) consisted of methanol-acetonitrile-water (79:14:7 v/v/v) as described before, whereas the mobile phase B consisted of acetone (100%), instead of chloroform (100%) [18,19]. The use of acetone as the new mobile phase B, whose compatibility with the column was assessed in a previous study [21] resulted in a stable efficiency during the chromatographic analyses, allowing a better separation of the selected analytes.

Furthermore, it preserved the components of the HPLC, such as gaskets and O-rings, without the risk of instrumental damages due to the use of chloroform. In addition, the use of acetone instead of chloroform is recommended since the latter is well-known for its acute toxicity (oral, dermal, inhalation). The

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optimized gradient solvent system was 0-6 minutes, 100% A; 6-26 minutes, 95% A; 26-36 minutes, 50% A; 36-60 minutes, 20% A; 60-65 minutes 20% A; 65-80 minutes, 100% A for conditioning, with a total runtime of 80 minutes. The identification of pigments in the lipid fractions of microalgae samples was carried out by the comparison of the retention time and UV-vis spectra of detected peaks with those obtained for the pure standards (Figure 2SI).

Standard calibration curves, obtained by analyzing each standard in different ranges optimized with the common concentration found in the lipid extracts are reported in table 14SI and they demonstrated a satisfactory linearity, since R^2 was higher than 0.9999 for each analyte.

LoD values obtained for carotenoids were in the range of 0.06-0.21 $\mu\text{g mL}^{-1}$ and LoQ values were in the range of 0.21-0.69 $\mu\text{g mL}^{-1}$. For chlorophylls, LoD and LoQ values were in the ranges of 0.54–0.57 $\mu\text{g mL}^{-1}$ and 1.80-1.90 $\mu\text{g mL}^{-1}$ respectively. In order to determine recovery, a fixed concentration of Lutein was added to the algal sample **S3** before the lipid extraction (final concentration of added Lutein= 2.5 $\mu\text{g mL}^{-1}$). A good recovery percentage ($98.32 \pm 8.98\text{E-}01\%$, table 15SI) confirmed the higher efficiency of the reported extractive method than the reference one [17]. The determination of accuracy was determined on sample **S3** by adding a fixed concentration of Lutein to the algal sample before the lipid extraction (theoretical final concentration of added Lutein in sample= 2.5 $\mu\text{g mL}^{-1}$). Accuracy was found to be $98.08 \pm 1.02\text{E+}00\%$ (table 16SI).

The intra- and inter-day precision were evaluated by analysing a mixture of standard pigments at fixed concentration (β -carotene= 3.33 $\mu\text{g mL}^{-1}$; Lutein= 2.34 $\mu\text{g mL}^{-1}$; Zeaxanthin= 2.59 $\mu\text{g mL}^{-1}$; Chlorophyll a= 16.67 $\mu\text{g mL}^{-1}$; Chlorophyll b=16.67 $\mu\text{g mL}^{-1}$) during the same day ($n = 3$) and three times a day in three different days during one week ($n = 9$).

Concerning pigments, data of intra- and inter day reproducibility (tables 17SI and 18SI) demonstrated an RSD lower than 2.85 and 2.44 respectively, confirming also the stability of the mixed solutions for at least five days.

Once the HPLC method was validated, the lipid samples of the two microalgae species were analysed. Chromatograms of sample **C2** and of **S2** are shown in Figure 5 as an example. Regarding the peak identification in this HPLC analysis, besides the retention times, the comparison of the UV-vis spectra acquired at the peak maximum with those of pure standards were used to confirm the identity of pigments in the microalgae samples. Figure reporting HPLC chromatogram of standard and UV-Vis spectra taken at the peak maximum is reported in SI.

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In Figure 5 peaks without Rt correspondence with standards were identified too. In fact, their UV spectra were found corresponding with those ones of chlorophylls and carotenoids. Therefore, we supposed they are cis/trans isomers, which show the same spectroscopic features, but different chromatographic performance. Concentration of these compounds were calculated by interpolating the peak area in the calibration curve of β -carotene or of Chlorophyll a, if they demonstrated a UV-Vis profile similar to carotenoids or chlorophylls respectively. In SI are provided chromatograms and UV-Vis spectra related to each peak of algal sample **C2** and **S2**.

Results concerning the determination of analytes are reported in table 19SI in terms of mg/100 g of dried algal powder. To better visualise the pigment content in algal samples, data are reported in a bar graph (Figure 5SI). Considering the carotenoids and chlorophylls HPLC determination in Chlorella and Spirulina microalgae samples, intra- and inter-day repeatability was found to be less than 5%.

Concerning an inter-species comparison, Spirulina species demonstrated the highest amount of β -carotene, in particular in sample **S5**. Conversely, Lutein is predominant in the Chlorella species, with the maximum level in sample **C3**, if compared to Spirulina that showed a lower content except for sample **S3**. The intraspecies evaluation showed that β -Carotene was higher in sample **C3** and in sample **S5**. The maximum Lutein content was found in **C3** and in **S3** and Zeaxanthin was predominant in **C3** and in **S5**. Chlorophyll a was mainly in sample **C2** and in sample **S3**, and Chlorophyll b had the highest content in sample **C3** and in sample **S3**. Zeaxanthin is mainly contained in Spirulina microalgae (highest content in sample **S1**), differently only traces are detectable in Chlorella species. Chlorophyll a is higher in Spirulina (sample **S3**) and Chlorophyll b does not prevail in any species, in particular the values are very low.

Concerning the average of total carotenoids and chlorophylls content, Spirulina species demonstrated a higher percentage than Chlorella ($123.93 \pm 6.13E+01$ and $99.10 \pm 5.45E+01$) respectively, (table 20SI).

To better describe the trend, in Figure 6 the total carotenoid (Figure 6a) and total chlorophylls contents (Figure 6b) by both the UV-Vis-spectrophotometric method and the HPLC-DAD analysis are reported to better clarify the results obtained.

In general, except some cases where the values deviate although within a limited range, results obtained with the spectrophotometric method can be comparable with those found with HPLC. However, it is necessary to point out that with the spectrophotometric method a semi-quantitative

determination of the total carotenoid and total chlorophylls content were carried out. Conversely, by HPLC analysis the five main pigments (β -Carotene, Lutein, Zeaxanthin, Chlorophyll a and Chlorophyll b) were accurately determined in the microalgae samples and, in addition, analytes with the UV-Vis profile similar to carotenoids or to chlorophylls were quantified too. Therefore, since a similar trend was obtained with the two methods, it indicates that the spectrophotometric method was validated and can be a suitable quick approach to get a preliminary indication of the content of pigments in microalgae species.

3.5 Antioxidant activity, ABTS Cation Radical Scavenging Assay

The ABTS^{•+} radical scavenging assays was applied to assess the antioxidant potential of lipid extracts from the nine different microalgae samples belonging to *Spirulina* and *Chlorella* species. This colorimetric assay is based on the ability of the extracts to reduce the ABTS^{•+} radical, which was previously generated by the oxidation of the ABTS. The obtained results, that are shown in Figure 7, suggested that each lipid microalga extract has a potential antioxidant activity, even if some differences can be highlighted.

The antioxidant activities of the lipid and pigment extracts were assayed in the range 7-600 $\mu\text{g mL}^{-1}$. For each lipid and pigment extract a plot of percentage of inhibition calculated versus concentrations of the sample solution ($\mu\text{g mL}^{-1}$) in ethanol was prepared. Then, a linear regression analysis was performed for each sample.

The tested algal samples concentrations range showed a linear response with inhibition. The sample linear equations were used to interpolate the IC_{50} values. The lipid and pigment extracts of all *Spirulina* samples (**S1**, **S2**, **S3**, **S4**, **S5**) and one of *Chlorella* (**C2**) demonstrated higher potency when compared to *Chlorella* samples (table 21SI). The other samples of *Chlorella* (**C1**, **C3** and **C4**) showed higher IC_{50} than the others, almost the double.

The lowest IC_{50} , higher potency, was registered for lipid and pigment extracted from **S3** ($\text{IC}_{50}= 90 \pm 1.90\text{E}+01 \mu\text{g mL}^{-1}$) followed by the ones of sample **S2** ($\text{IC}_{50}= 160.20 \pm 0.00\text{E}+00 \mu\text{g mL}^{-1}$) and **S5** ($\text{IC}_{50}= 180 \pm 1.00\text{E}+00 \mu\text{g mL}^{-1}$) that belong to *Spirulina* class. The lower antioxidant activity was found in the lipid extract from sample **C4** ($\text{IC}_{50}= 518 \pm 7.90\text{E}+01 \mu\text{g mL}^{-1}$) followed by the ones of samples **C3** ($\text{IC}_{50}= 363 \pm 7.30\text{E}+01 \mu\text{g mL}^{-1}$) and **C1** ($\text{IC}_{50}= 303 \pm 6.00\text{E}+00 \mu\text{g mL}^{-1}$).

3.6 Protein content

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The protein content was determined following the Lowry spectrophotometric procedure [13]. The calibration curve, $y = (4.18 \pm 1.20E-01)x + (0.0668 \pm 2.50E-04)$, $R^2 = 0.9994$, was prepared with the BSA as standard in the range of $0.3\text{--}1\text{ g L}^{-1}$. LoD and LoQ values were found to be 10 and 20 mg L^{-1} respectively. Proteins in algal samples were obtained with 0.5 M NaOH aqueous solution by performing four sequential vortexed and heated extractions, then the collected extracts underwent to the colorimetric reaction described in par 2.8 followed by the spectrophotometric analysis at 750 nm. [22–25]

In table 22SI the percentages of protein content in the dried powder of algal samples are shown. Spirulina samples indicated an average protein content higher in comparison to that of Chlorella ($65.38 \pm 5.59E+00\%$ and $53.78 \pm 13.51E+00\%$ respectively). In details, **C3** and **S1** showed the highest content of proteins.

3.7 Principal component analysis (PCA)

The traditional principal component analysis (PCA) plot presented in Figure 8 shows the biochemical composition and biological activity evaluation obtained by the characterization of the two samples of commercial microalgae powders, Spirulina and Chlorella.

In particular, this PCA analysis correlates Chlorella (**C1–C4**) and Spirulina (**S1–S5**) samples to the analytical and activity results regarding nine variables: the percentage of lipids extracted, the percentage of FAMES in total powder, the percentage of total saturated FAMES, the percentage of total monounsaturated FAMES, the percentage of total polyunsaturated FAMES, the content in terms of $\text{mg}/100\text{ g}$ of dried algal powder of total carotenoids (HPLC method), the content in terms of $\text{mg}/100\text{ g}$ of dried algal powder of total chlorophylls (HPLC method), antioxidant activity (IC_{50}) and the percentage of the protein content.

Data obtained revealed clearly detectable correlations intra-species. Therefore, all Chlorella (**C1–C4**) samples are located in the opposites side of the graph compared to Spirulina (**S1–S5**) species. Among the nine major contributors to discrimination, the protein content plays a key role. Spirulina has a higher protein percentage than Chlorella. So, this green-blue algae is the richest protein source of microbial origin ($460\text{--}630\text{ g kg}^{-1}$, dry matter basis), that shows a similar protein amount of meat and soybeans [26]. Thus, Spirulina specie confirms to represent an interesting source for protein supplement. The most relevant proteins found in Spirulina reported for food application are phycobiliproteins (phycocyanin, allophycocyanin, and phycoerythrin). These proteins could be

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commercialized not only in food industries or for a nutraceutical purpose but also in cosmetics and pharmaceuticals [27,28].

Spirulina species are characterized by a high content of saturated fatty acid, among these by palmitoleic acid. The palmitoleic acid exerts several fundamental biological functions in cells and tissues. The palmitoleic acid body concentration is regulated by its endogenous biosynthesis by the DNL (de novo lipogenesis). However, in long term this metabolic pathway is not sufficient. For this reason, the dietary intake of palmitoleic acid in association with a certain amount of unsaturated fatty acids, in particular PUFAs, both n-6 and n-3 results crucial. Indeed, an optimal PUFAs/SAFAs (saturated fatty acids) ratio in tissues is important to maintain the FA phospholipids balance of the membrane. Cell membrane fluidity and cell–cell interactions are maintained by a good balance between saturated and unsaturated fatty acids. The FAs imbalance seems involved in different physiological and disease states such as diabetes, cardiovascular disease, obesity, hypertension, neurological diseases, immune disorders, cancer and aging [29].

Chlorella, as showed in Figure 8, stands out for the FAME content mainly composed by MUFAs and PUFAs, useful both in cosmetic and nutraceutical formulations. Among these, LA belonging to ω -6 class and oleic acid are the most widely present. Linoleic acid is an essential nutrient, added in infant formulas and food. It is a component of ceramides, therefore it is involved in the transdermal water barrier regulation in the epidermis. For this reason, it is often used to treat skin-related disorders [30]. In addition, oleic acid is useful in the prevention of cardiovascular disease. Furthermore, as it is evident from PCA plot, Spirulina species can be distinguished by the Chlorella for the higher content in terms of pigments: carotenoids and chlorophylls. Carotenoids are naturally synthesized by microalgae, and they act as provitamins A to be then converted in the human body into vitamin A. Vitamin A daily intake that for 30 % is guaranteed by carotenoid assumption and it is essential during the growth and for allowing some vital processes as proper visual function, regulation of differentiation of epithelial tissues, and embryonic development [31].

Identified carotenoids in Spirulina includes Zeaxanthin, Lutein and β -carotene. Lutein and Zeaxanthin have been studied for their potential protective function against visual disturbances and cognitive diseases, such as age-related macular degeneration (AMD), age-related cataracts (ARC), cognitive diseases, retinopathy ischemic/induced by hypoxia, slight retinal damage, retinitis pigmentosa, retinal detachment, uveitis and diabetic retinopathy. Regarding the mechanism of action carried out at the ocular level, it seems to be related to their physical properties of filtering blue light

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and local antioxidant activity. In addition to the protective effect against light-induced oxidative damage, recent results confirm that these two pigments can also improve normal ocular function by increasing contrast sensitivity and reducing the disability of glare. Studies conducted after the intake of supplements based on Lutein and Zeaxanthin showed a reduction in the risk of AMD and less visual damage [32].

Finally, another valuable pigment present in Spirulina is chlorophyll, characterized by the classical green color that can be exploited both as a dye and also for its potential health activities. Recently it has been reported that chlorophylls can have valuable antimutagen, chemoprotective, antioxidant, anti-inflammatory and antimicrobial properties that, however, must be confirmed [33].

The increased Spirulina activity in terms of antioxidant properties in comparison with Chlorella is probably due to the higher pigment content that allow Spirulina to reach values of IC₅₀ in the lower range of concentrations.

4. Conclusions

The present research was focused on the development of an integrated analytical approach able to characterize, from a nutraceutical point of view, the dried biomass obtained from four Chlorella (C1-C4) and five Spirulina (S1-S5) commercial samples. A modified more efficient extraction method for lipids has been optimized and validated in terms of microalgae sample amount, volumes of extraction solvents and time. Lipid and pigment amount, gravimetrically determined, resulted to be almost comparable in both species. In order to qualify the microalgae lipid composition, the GC-MS method was improved in terms of selectivity allowing the identification of all the fatty acids contained in the microalgae lipid fraction. It was found that Spirulina specie is the richest in saturated fatty acids while the Chlorella ones showed a more interesting content in terms of MUFAs and PUFAs. Therefore, Chlorella species could be exploited for PUFAs content given their increasing nutraceutical and cosmetic interest. Among PUFAs, ω -3 and ω -6 fatty acids have to be mentioned for their important health protection. From the obtained PUFAs profiles, linoleic acid emerged as a peculiar component of the Chlorella species in comparison to Spirulina. Therefore, the use of Chlorella microalgae is relevant in vegan diets [34].

However, following protein quantification achieved by the modified Lowry's colorimetric essay, Spirulina resulted to have the highest content and to be the most suitable species for the preparation of protein supplements. Besides applying methods to characterize the lipid fraction, the determination

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of the total content of carotenoids and chlorophylls in the lipid extracts was obtained by a fast UV-Vis spectrophotometric method, validated by an HPLC-DAD method for a detailed characterization of both categories of pigments in a single analysis. These analyses highlighted a higher percentage of pigments in *Spirulina* samples. Pigments (carotenoids and chlorophylls), in particular β -carotene, Lutein and Zeaxanthin demonstrated important valuable properties in human health prevention and the market of food supplements can take benefit from them [5,6]. In agreement, the application of the ABTS colorimetric assay showed a greater antioxidant activity for the *Spirulina* species. Finally, the use of a multivariate analysis of the data by PCA allowed to correlate the peculiar characteristics that emerged from the analysis of the two species and to note that *Spirulina* antioxidant activity can be closely associated to the content of pigments, confirming their potential use in the nutraceutical and cosmetic field.

Therefore, this integrated analytical approach was found useful to trace a profile of the intrinsic characteristics of each of the two species that can be distinguished for different possible uses. In conclusion, this paper puts forward a very useful overall analytical approach, sustained by a multivariate analysis, for emphasizing different contents of bio-active components of diverse microalgae strains for a more accurate use indication.

Author statement

I hereby certify that all authors have seen and approved the final version of the manuscript being submitted. The article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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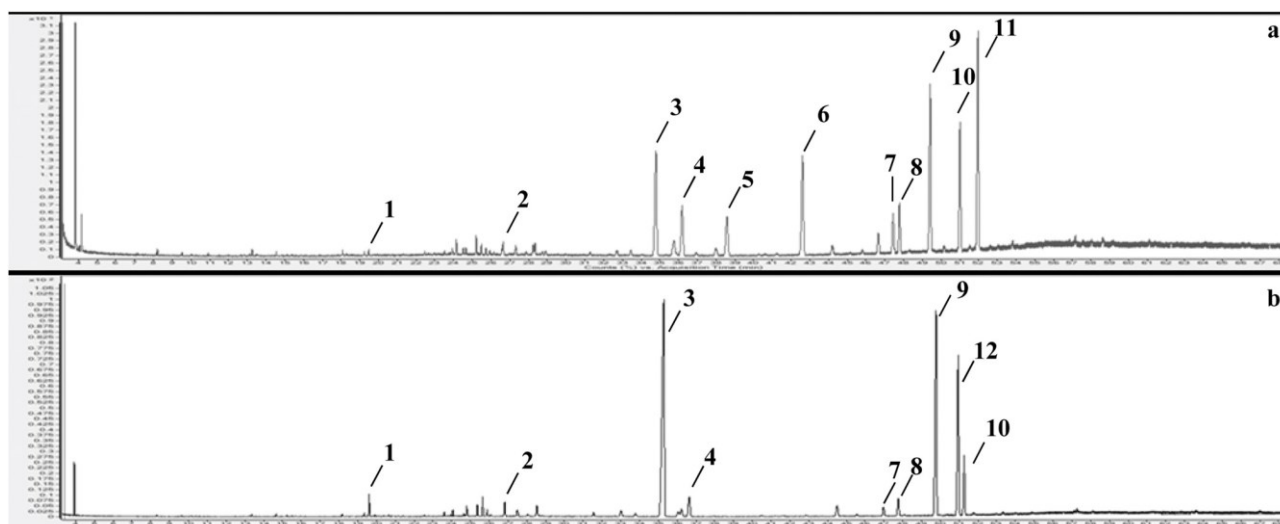


Figure 1: a= GC-MS chromatogram of sample **C2**; b= GC-MS chromatogram of sample **S3**; 1= Methyl undecanoate, 2= Methyl myristate, 3= Methyl palmitate, 4= Methyl palmitoleate, 5= 7,10Hexadecadienoic acid methyl ester, 6= 7,10,13-Hexadecatrienoic acid methyl ester, 7= Methyl stearate, 8= Oleic acid methyl ester, 9= Methyl linoleate, 10= Nonadecanoic acid methyl ester, 11= Methyl linolenate, 12= γ -Linolenic acid methyl ester

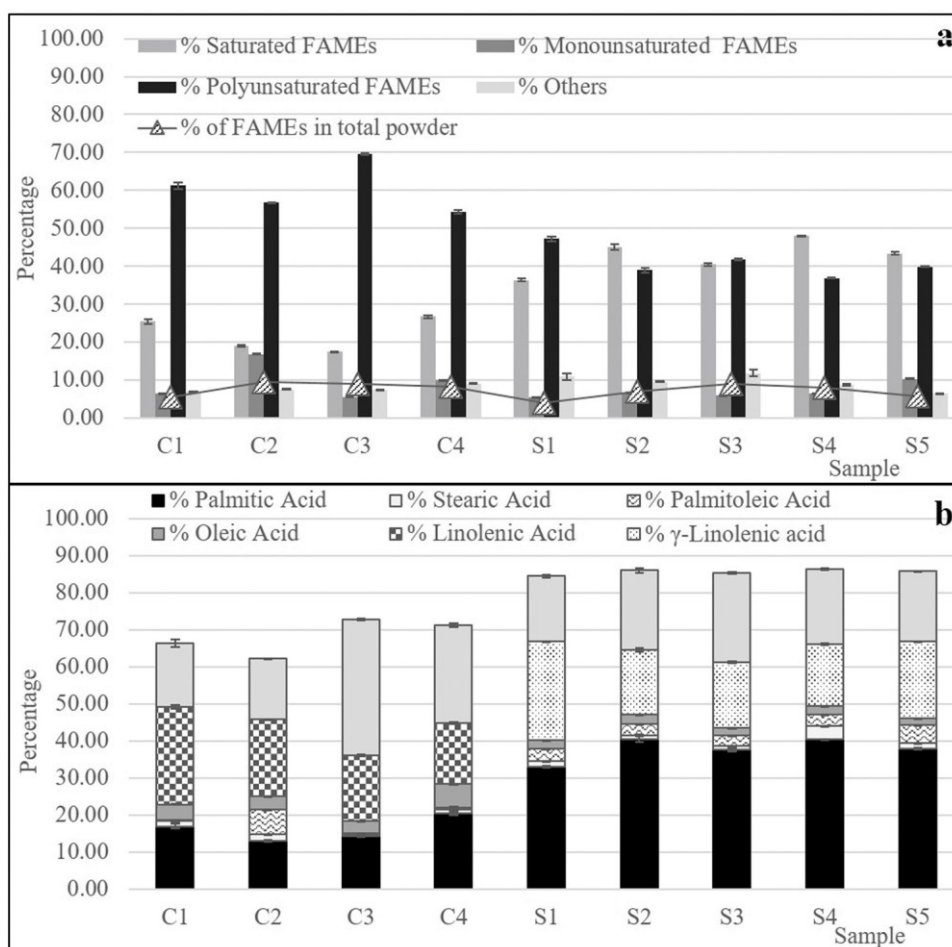


Figure 2: a) FAMES content in algal samples; b) comparison of Chlorella and Spirulina samples regarding the most representative fatty acids.

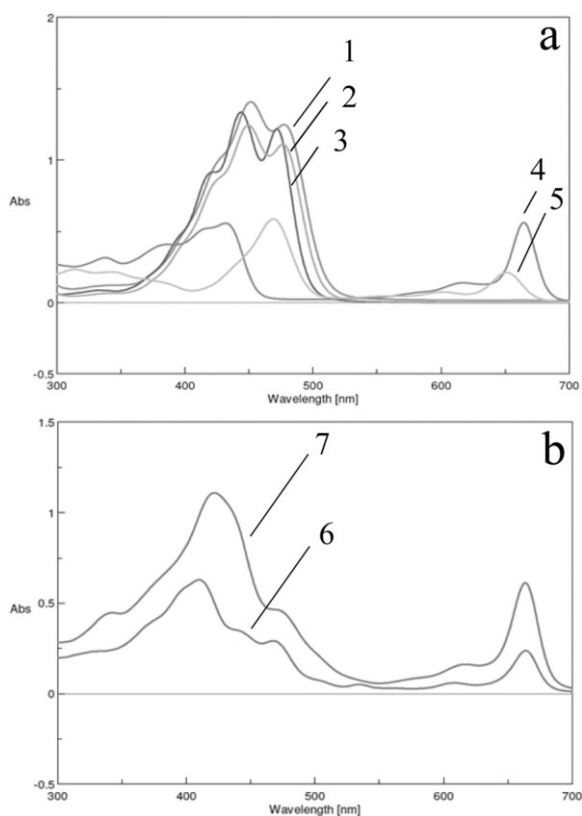


Figure 3: a) UV-Vis spectrum of standards pigment b) overlapped UV-Vis spectra of algal samples **C1** and **S3**; 1= β-carotene; 2= Zeaxanthin; 3= Lutein; 4= Chlorophyll a; 5= Chlorophyll b; 6= sample **C1**; 7= sample **S3**.

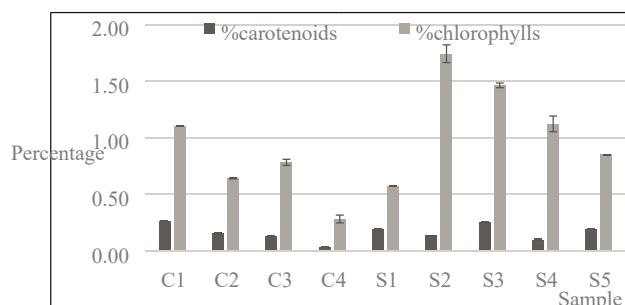


Figure 4: bar graph reporting the percentage of total carotenoid and chlorophyll content in algal sample calculated by UV-Vis-spectrophotometric analysis

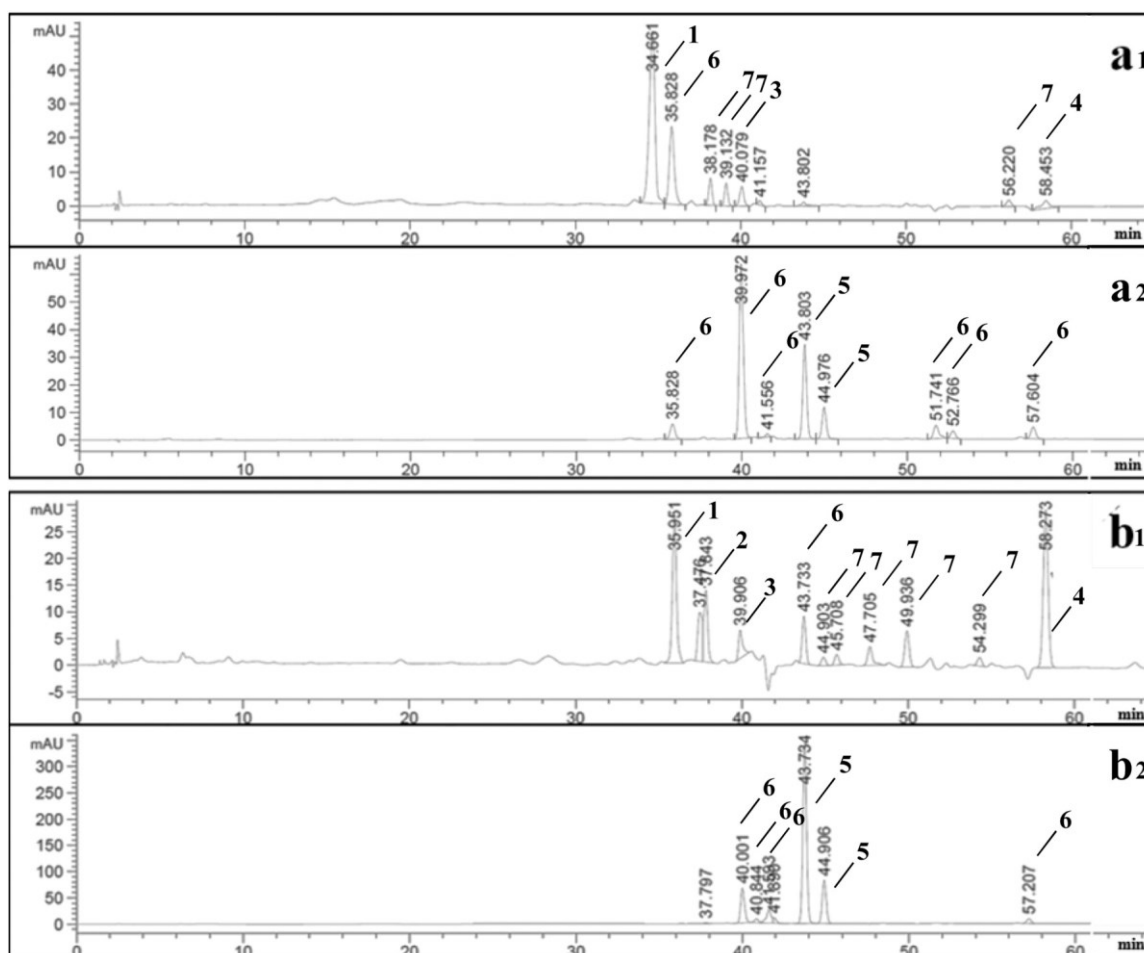


Figure 5: a) sample **C2**, chromatogram **a1** is registered at $\lambda=450$, chromatogram **a2** at $\lambda=660$; b) sample **S2**, chromatogram **b1** is at $\lambda=450$, chromatogram **b2** at $\lambda=660$; 1= Lutein; 2= Zeaxanthin; 3= Chlorophyll b; 4= β -carotene; 5= Chlorophyll a; 6= UV-Vis profile similar to chlorophylls; 7= UVVis profile similar to carotenoids

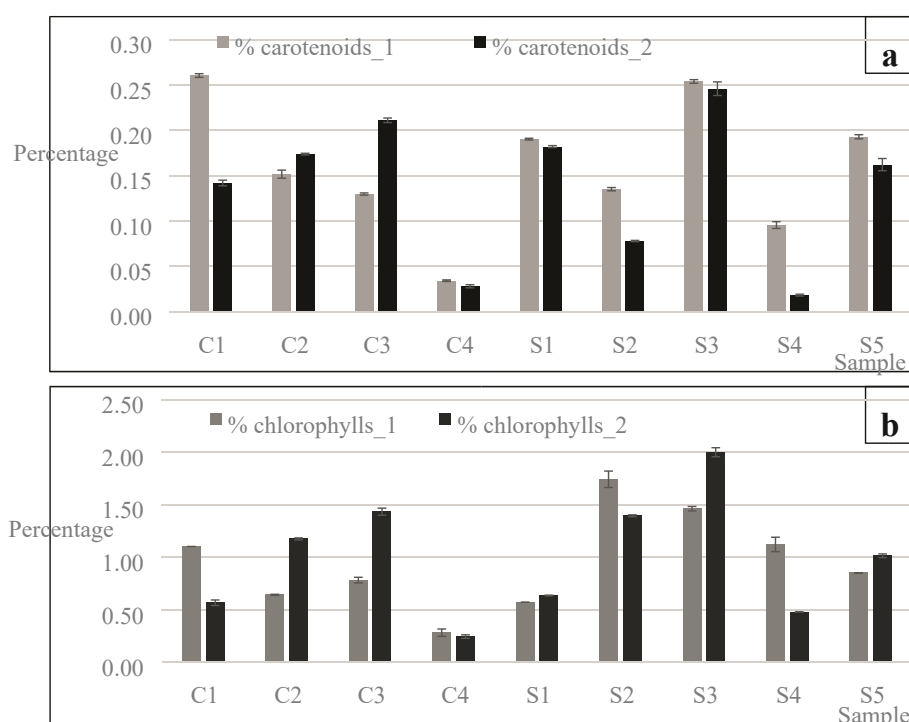


Figure 6: a) Carotenoids contents in microalgae samples obtained by UV-Vis method (grey) and HPLC (black); b) Chlorophylls contents in microalgae samples obtained by UV-Vis method (grey) and HPLC (black).

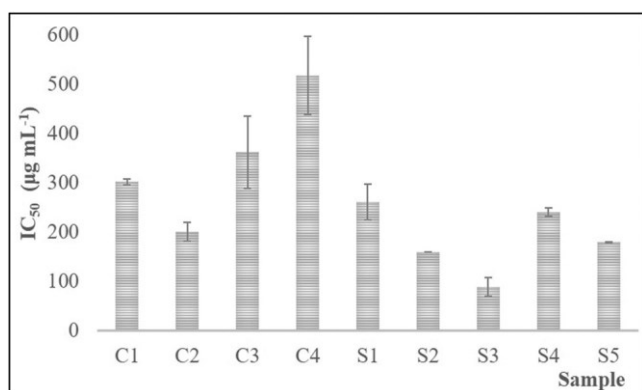


Figure 7: evaluation of the antioxidant activity of lipid extracts from different microalgae. Concentration of lipid extract ($\mu\text{g mL}^{-1}$) that provided 50% inhibition of the ABTS \bullet radical; values are displayed as the mean ($n = 2$) \pm standard deviation

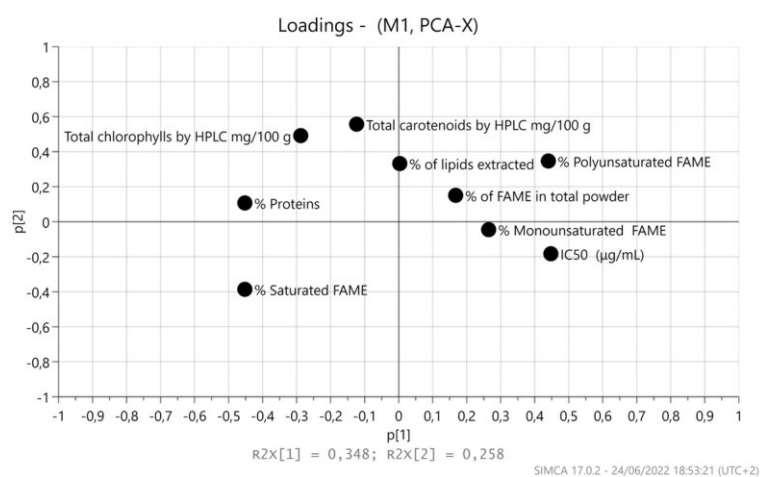
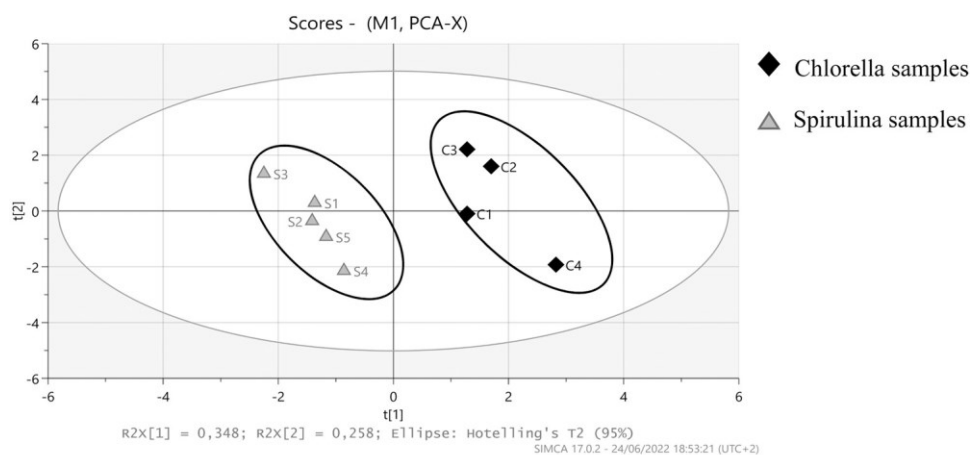


Figure 8: PCA score plot showing biochemical composition of sets of four samples of Chlorella (C1-C4) and 5 samples of Spirulina microalgae (S1-S5)