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Clean production of microalgae high-value lipid fraction: Influence of different pretreatments on chemical and cytotoxic profiles of *Chlorella vulgaris* supercritical extracts and life cycle assessment

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ABSTRACT

Microalgae have emerged as a promising natural resource rich in bioactive compounds. Health-beneficial properties of microalgae, coupled with advantageous characteristics such as high biomass productivity, adaptability, robustness, and carbon dioxide mitigation, position them as a viable solution for global sustainable food production. This study explored clean and environmentally friendly processes to enhance the recovery of lipid bioactive fractions. Microwave (MW), enzymatic (ENZ), and ultrasound (US) pretreatments were applied to improve environmentally friendly extraction of lipid-based components using supercritical CO_2. The effects of these pretreatments on extraction yield, chemical profiles, and cytotoxic properties of *Chlorella vulgaris* (Cv) and smooth *C. vulgaris* (sCv) extracts were investigated. Additionally, a Life Cycle Assessment (LCA) was conducted to evaluate environmental impacts. MW pretreatment achieved the highest yield increases, from 2.58 times (Cv) to 3.15 times (sCv). UHPLC-ESI-HRMS analysis revealed shifts in the distribution of pigments and derivatives caused by pretreatments, with ENZ extracts showing the most pronounced changes: pigments increased from 9.24% (control Cv) to 40.92% (Cv) and from 12.52% (control sCv) to 71.12% (sCv). Cv extracts exhibited greater activity against MDA-MB-453 cells, while sCv extracts from US pretreatment demonstrated the strongest effect on HeLa cells. The LCA indicated reduced environmental impacts of the pretreatment-enhanced processes up to 65% compared to the control. A scenario analysis was presented to show further possible impact reduction by recirculating the CO_2 solvent and substituting the energy source. These findings provide valuable insights into sustainable and scalable green processes for recovering microalgal bioactive components.

1. Introduction

According to the United Nations report, the world population is expected to reach 8.6 billion by 2030, 9.8 billion by 2050, and 11.2 billion by 2100 (UN, 2022). This increase in population requires a drastic increase in food production by approximately 70% to meet the needs of the world (Searchinger et al., 2019). Therefore, one of the current global challenges is the provision of sustainable food sources (FAO, 2017).

Microalgae represent a renewable natural source of components with great nutritional and pharmacological potential and can represent a future food source alternative (Mishra et al., 2021). In addition, their production takes place in a more sustainable way compared to

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conventional methods of obtaining food. Moreover, there is less water use, no need for arable land, reduction in the emission of green gases and pollutants, and biomass harvesting daily, avoiding storage. Additionally, they are characterized by rapid growth, high biomass productivity, and tolerance to stress in their environment (Yap et al., 2021).

One of the microalgae with great economic importance, well-known and approved by The European Food Safety Authority (EFSA) is *Chlorella vulgaris*. The importance and significance of this microalga springs from its biochemical profile rich in valuable components with nutritional and biological significance (Mendes et al., 2022). As a result, *C. vulgaris* has the potential of various applications in different fields and production of food ingredients, nutraceuticals, pharmaceuticals, cosmetics, coloring agents, aquaculture fee, biofertilizers, biodiesel (Ru et al., 2020). According to the analysis by Skyquest , in 2019, the global *Chlorella* market was valued at USD 275.21 million, and it is projected to grow from USD 292.55 million in 2023 to USD 506.99 million by 2031, increasing at a 6.30% compound annual growth rate (CARG).

This growing trend of *C. vulgaris* utilization, driven by its versatile applications and potential to align with cleaner production principles, underscores the need for sustainable extraction methods to overcome challenges that limit its optimal implementation. Namely, one of the challenges that must be addressed, to enable the widespread implementation of microalgae, is increasing the availability of components. The hard cell wall of microalgae makes it difficult to release valuable microalgae components. Moreover, due to these structural characteristics, the use of the whole microalgal biomass does not allow the utilization of its components during digestion, because the access to enzymes of the digestive tract is limited (Barkia et al., 2019). A promising solution involves converting biomass into extracts, which isolates bioactive components for further applications.

Modern production processes must adhere to cleaner production principles and align with the Sustainable Development Goals (UN), prioritizing environmentally friendly methods that ensure safe processes and products. Conventional lipid extraction methods rely on organic solvents such as methanol, hexane, and chloroform, which pose environmental and health risks (Zarrinmehr et al., 2022). Also, their application implies extract refinement and removal of these solvents from products, which further implies toxic waste generation, increasing the complexity and cost of the processes. An additional health risk exists due to the possibility of solvent retention in traces.

The application of supercritical carbon dioxide (sCO_2) extraction supports cleaner production principles due to its solvent-free product, reduced waste generation, and low environmental footprint compared to conventional solvent-based extraction. Thanks to the mild conditions under which supercritical conditions are achieved (31.1 °C and 73.8 bar), thermosensitive components can be efficiently extracted with this process (Vladić et al., 2023). Additionally, after extraction, the solvent is easily removed completely by reducing the pressure, providing a solvent-free product (Wrona et al., 2019). By varying the process conditions, the selectivity of the extraction is enabled, which, along with other characteristics such as the low price and availability of CO_2 (Wrona et al., 2019), and the possibility of recirculation after extraction, makes sCO_2 an industrially relevant technology (Vladić et al., 2023). Wetterwald et al. (2023) and Khorramdashti et al. (2021) demonstrated the application of sCO_2 extraction for the recovery of *C. vulgaris* lipids, primarily for biodiesel production. Mendes et al. (2003) further showed the capability of sCO_2 to selectively extract bioactive components from microalgae, underscoring the flexibility and tunability of this technique. Also, the effects of temperature, pressure, and solvent flow rate on extraction yield, total phenolic, chlorophyll and carotenoid content, and antioxidant activity of *C. vulgaris* extracts were investigated.

However, the rigid cell wall of microalgae (Shivakumar et al., 2024) poses a significant barrier to efficient sCO_2 extraction, restricting access to bioactive components. To address this, pretreatments such as microwave (MW), ultrasound (US), and enzymatic (ENZ) methods can be employed to disrupt the cell wall and enhance component release.

Pretreatments such as MW, US, and ENZ methods improve the efficiency of sCO_2 extraction by breaking down the microalgae cell wall and increasing the release of bioactive components. MW and US pretreatments are advantageous for their fast processing times, which significantly reduce the overall extraction duration. However, MW may degrade thermosensitive compounds because of rapid heating (Nour et al., 2021), and US cavitation effects can generate localized high pressures and temperatures that also risk compound degradation (Shen et al., 2023). ENZ pretreatment, in contrast, offers a more selective approach with minimal risk of compound degradation, but requires specific enzymes and incurs higher processing costs, which may limit scalability (Das et al., 2021).

In few studies, the possibility of improving lipid extraction from *C. vulgaris* using supercritical fluids with MW pretreatment has been investigated (Dejoye et al., 2011). Ma et al. (2014) explored the impact of US and MW pretreatments on lipid extraction from microalgae. However, the combination of pretreatments with sCO_2 extraction remains insufficiently explored, presenting a significant research gap. Bearing in mind that the application of pretreatment can significantly alter the quantitative and qualitative chemical profiles of microalgae extracts obtained, and their bioactivity, it is very important to evaluate their adequacy.

The aim of this work was to screen various methodologies for their impact on the release of lipid-based components, chemical profile, and cytotoxic activity of extracts from two microalgae species, *Chlorella vulgaris* (Cv) and smooth *C. vulgaris* (sCv), which will undergo further extraction using supercritical CO_2 (sCO_2). Pretreatments, including microwave (MW), ultrasound (US), and enzymatic (ENZ) methods, were evaluated for their effectiveness. Also, to provide valuable insights into the environmental footprint of investigated processes and evaluate their overall sustainability and viability, Life Cycle Assessment (LCA) methodology was employed. *Chlorella vulgaris* (Cv) was cultivated in autotrophic conditions, while the smooth *C. vulgaris* (sCv) was produced in heterotrophic conditions.

2. Materials and methods

A schematic diagram illustrating the overall framework of the study is presented in Fig. 1.

2.1. Material

Microalgae samples used in this work, *Chlorella vulgaris* (Cv) and smooth *Chlorella vulgaris* (sCv) were purchased from ALLMICROALGAE - Natural Products, SA, Pataias, Portugal. Cv is grown in a sustainable autotrophic manner, using sunlight and CO_2, while sCv is produced in the dark, inside a fermenter vessel, in a heterotrophic cultivation process.

2.2. Pretreatment and supercritical carbon dioxide extraction

Microalgae biomass was used in the pretreatments according to the following procedures outlined by Vladic et al. (2021) with modifications as detailed below:

Enzymatic pretreatment (ENZ) – mixing biomass and enzymatic solution of enzyme mixture Viscozyme (Sigma-Aldrich, United States) at ratio solid material/solution 1:5 (g/mL) and incubation at 45 °C for 60 min. Viscozyme solution which contains a wide range of carbohydrases, including arabanase, cellulase, β-glucanase, hemicellulase, and xylanase was prepared with acetate buffer pH 4.9 at concentration 8% with respect to the solid material.

Ultrasound pretreatment (US) – mixing biomass material and H_2O in 1:5 (g/mL) ratio and exposure to sonification in water bath (EUP540A, EU instruments, France), temperature of 60 °C, time of 5 min, and frequency of 40 kHz.

Microwave pretreatment (MW) – mixing biomass material and H_2O

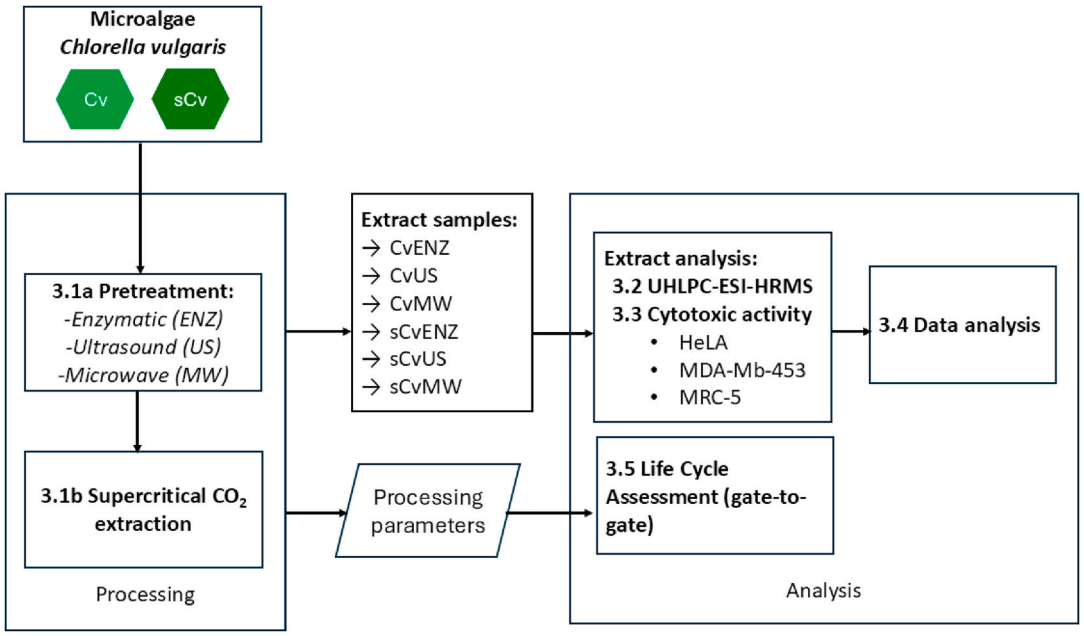

Fig. 1. Schematic diagram of the study (Cv - *Chlorella vulgaris*; sCV - smooth *Chlorella vulgaris*; extracts obtained from Cv biomass: CvENZ (enzymatic pretreatment), CvUS (ultrasound pretreatment), CvMW (microwave pretreatment) and extracts obtained from sCV biomass: sCvENZ (enzymatic pretreatment), sCvUS (ultrasound pretreatment), sCvMW (microwave pretreatment); UHPLC-ESI-HRMS - ultra-high-performance liquid chromatography–high-resolution mass spectrometry; HeLa - human cervix adenocarcinoma cell line; MDA-MB-453 - human breast carcinoma; MRC-5 - normal human fetal lung fibroblast cell line).

in 1:5 (g/mL) ratio and exposure to microwave irradiation power 800 W for 3 min. The process was conducted using a modified commercial microwave oven (NN-E201W, Panasonic, Osaka, Japan) adapted for laboratory use. The modifications included integrating a glass apparatus consisting of a round-bottom flask and an external condenser. The round-bottom flask containing the biomass-water mixture was placed inside the microwave oven's cavity and connected to an external condenser through a heat-resistant glass pipe extending outside the unit. This setup ensured efficient condensation of vapor generated during microwave heating, preventing vapor loss and maintaining a controlled environment. The external condenser played a critical role in retaining volatile components, while the closed-loop design minimized the risk of overheating.

After the pretreatments, the biomass was separated from the liquid phase and dried in a freeze drier (Alpha 1–2 LPlus, Christ, Osterode am Harz, Germany). The dried biomass was further used for the cCO_2 extraction. The cCO_2 extraction process was carried out in a laboratory-scale high-pressure extraction system (HPEP, NOVA, Swiss, Effertikon, Switzerland) using CO_2 with purity >99.98% (w/w) (Messer, Novi Sad, Serbia). The extraction was conducted for 4 h at 300 bar and 40 °C, and a CO_2 flow rate of 0.194 kg/h. The separator conditions were maintained constant at 15 bar and 23 °C. Control extraction was conducted under the same extraction conditions (300 bar, 4 h, and 40 °C), without pretreatment. These extraction conditions were selected based on previous research by Vladić et al. (2022). After the extraction, the extracts were collected into glass vials and stored in a dark place at 4 °C until further analysis. Extracts obtained from Cv biomass used in the work were labeled in the following manner: CvENZ (enzymatic pretreatment), CvUS (ultrasound pretreatment), CvMW (microwave pretreatment) and extracts obtained from biomass sCV were labeled sCvENZ (enzymatic pretreatment), sCvUS (ultrasound pretreatment), sCvMW (microwave pretreatment).

2.3. Ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-ESI-HRMS) analyses of sCO_2 extracts

Ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-ESI-HRMS) analyses of supercritical CO_2 extracts were performed as described by Vladić et al. (2022). An ExionLC AD system (AB Sciex, Canada) equipped with a Q-TOF mass spectrometer (TripleTOF 6600+) was used for the analysis. The chromatographic separations employed an Acquity UPLC CSH Phenyl-Hexyl column (2.1 mm × 100 mm, 1.7 μm; Waters, MA, USA) at 30 °C and a flow rate of 0.4 mL/min, with water and acetonitrile (both containing 0.1% formic acid) as mobile phases. Detection was conducted in positive electrospray ionization (ESI+) mode. The data were processed using ACD/Spectrus Processor 2021.1.0 (ACD/Labs, Canada), enabling elemental composition determination and tentative identification of compounds based on their mass spectra. A full description of the methodology is available in the Supplementary material 1.

2.4. Cytotoxic activity

Human cervix adenocarcinoma cell line (HeLa), human breast carcinoma (MDA-MB-453), and normal human fetal lung fibroblast cell line (MRC-5) were obtained from the American Type Culture Collection (Manassas, VA, USA). The effect of microalgae supercritical CO_2 extracts on cell survival was assessed using the MTT assay. After a 72-h incubation period, absorbance was measured to determine the percentage of viable cells and calculate the IC_{50} values. A full description of the methods is available in the Supplementary material 1.

2.5. Data analysis

Prior to chemometric analysis, the data transformation was carried out so the values of the determined area in the chromatograms were divided by the factor 10^6. Considering the fact all the data are on the same scale, the data normalization was not performed. Pattern recognition chemometric approaches were used for the data treatment and comparison of the extracts in terms of their composition. The pattern recognition methods included hierarchical cluster analysis (HCA) and principal component analysis (PCA).

The HCA was carried out to detect similarities and dissimilarities among the extracts. It was done by using NCSS 2023 NCSS 2023

Statistical Software (2023) applying Ward's minimum variance algorithm and Euclidean distances as distance method and standard deviation as scaling method. The PCA was performed in Statistica v14.0.0.15 software (TIBCO Software Inc, 2020) based on correlations in order to gain an overview of the distribution of the extract in the space of the considered variables (content of the extracted compounds). Also, Wilcoxon matched pairs test (WMPT), as a statistical non-parametric test, was applied in order to compare the pairs of the analyzed extracts in Statistica v14.0.0.15 software. In the WMPT the *p* value was set at 0.05. The results of the UHPLC analysis that include the peak areas of each identified compound in the extracts, were presented in the form of combination (COMBO) charts to analyze the properties of measurements for each extract. The COMBO charts were created in NCSS 2023 software and included the box, density, and dot plots. They present the center and the spread of the data around the central value, extreme values (outliers) and the distribution of the data. The Inter-Quartile Range (IQR) of the box plot includes the whisker boundaries with ±1.5 IQR, while severe outlier boundaries are ± 3.0 IQR from the box edge. Detailed explanation of the chemometric methods used are given elsewhere (Miller and Miller, 2010).

For comparisons of survival of cancer and normal control cells among two different extracts, one-way analysis of variance (ANOVA) and Student's t-test was performed. To analyze the results of cytotoxicity treatment, comparisons between the extracts were performed by employing a two-tailed, unpaired Student's t-test for each cell line. p = 0.05 value was set like a significance threshold. The software GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, U.S.A.) was undertaken for statistical analysis.

2.6. Life cycle assessment (LCA)

The LCA was conducted following the guidelines described by ISO standards 14040:2008 and 14044:2006, consisting of (1) goal and scope definition, (2) life cycle inventory, (3) life cycle impact assessment and result interpretation (ISO, 2008; ISO, 2006). The software OpenLCA 2.0 was used for the analysis (GreenDelta, 2023). The studied system was assumed to be mid-industrial scale with a processing capacity of around 5 kg biomass per month. The initial biomass input to the system was assumed to have 20% dry weight content.

Goal and scope definition: This study was a gate-to-gate LCA with initial gate of microalgae downstream processing and final gate of the extract production (Fig. 2). The functional unit was 1 kg of microalgae extract. The goal of this LCA study was to compare the environmental impacts of three different methodologies (enzymatic, microwave, and ultrasonic) for improving the release of lipid-based components of two microalgae, *C. vulgaris* (Cv) and smooth *C. vulgaris* (sCv). This LCA study also had the objectives to indicate the contribution of each process to the total impacts of the extraction process, determine the hotspots, and evaluate improvement scenarios that can lower the total impacts of the system.

Life cycle inventory: As a gate-to-gate LCA study, the parameters that were considered as inputs in this study were infrastructure/equipment, electricity, and chemicals at the biorefinery facility. The foreground data for the system included the electricity and resource consumption, details of the equipment required, and the projected yields and loss for each step of the extraction process. The background data included the electricity production and transmission, raw material extraction, and end-of-life scenario. For both infrastructure and chemicals aspects, the raw material extraction, the use phase, and end-of-life phase were the main parameters considered while the manufacturing and transportation of the materials were omitted in this study. This decision was made based on the European Union LCA method (Damiani et al., 2022) and on the fact that all scenarios use similar equipment, so adding their transportation and manufacturing would only add uncertainty to the comparison model. Ecoinvent 3.7 was used as the database for background data and OpenLCA 2.0. was used to model the studied system.

Life cycle impact assessment and results interpretation: Recipe 2016 Midpoint (Hierarchist) methodology was used to characterize the impacts of the system. There are 18 impact categories in this method including fine particulate matter formation, fossil resource scarcity, freshwater ecotoxicity, freshwater eutrophication, global warming, human carcinogenic toxicity, human non-carcinogenic toxicity, ionizing radiation, land use, marine ecotoxicity, marine eutrophication, mineral resource scarcity, ozone formation – human health, ozone formation – terrestrial ecosystem, ozone depletion, terrestrial acidification, terrestrial ecotoxicity, and water consumption.

3. Results and discussion

3.1. Total extraction yield

The yield of control sCO_2 extraction was 0.41 ± 0.04% for *C. vulgaris* (Cv) and 0.46 ± 0.08% for smooth *C. vulgaris* (sCv) (Fig. 3a and b and Table S1 - Supplementary material 2). The application of pretreatments had a significant impact on the extraction process, resulting in notable increases in the extraction yield for both biomasses. Among the

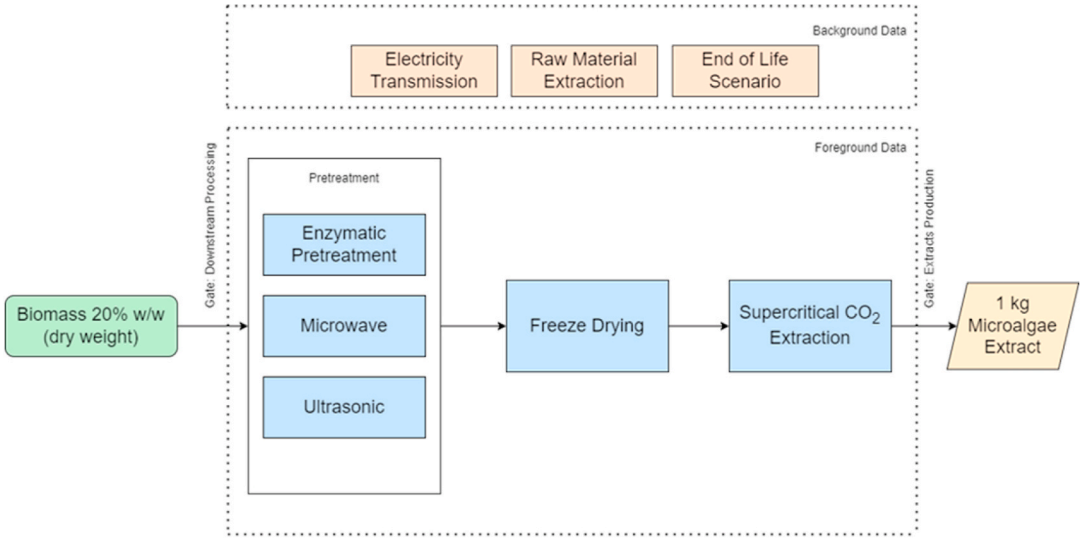

Fig. 2. The boundaries of Life Cycle Assessment (LCA): conceptual process framework for the model used in comparative LCA to screen of 1 kg microalgae extract.

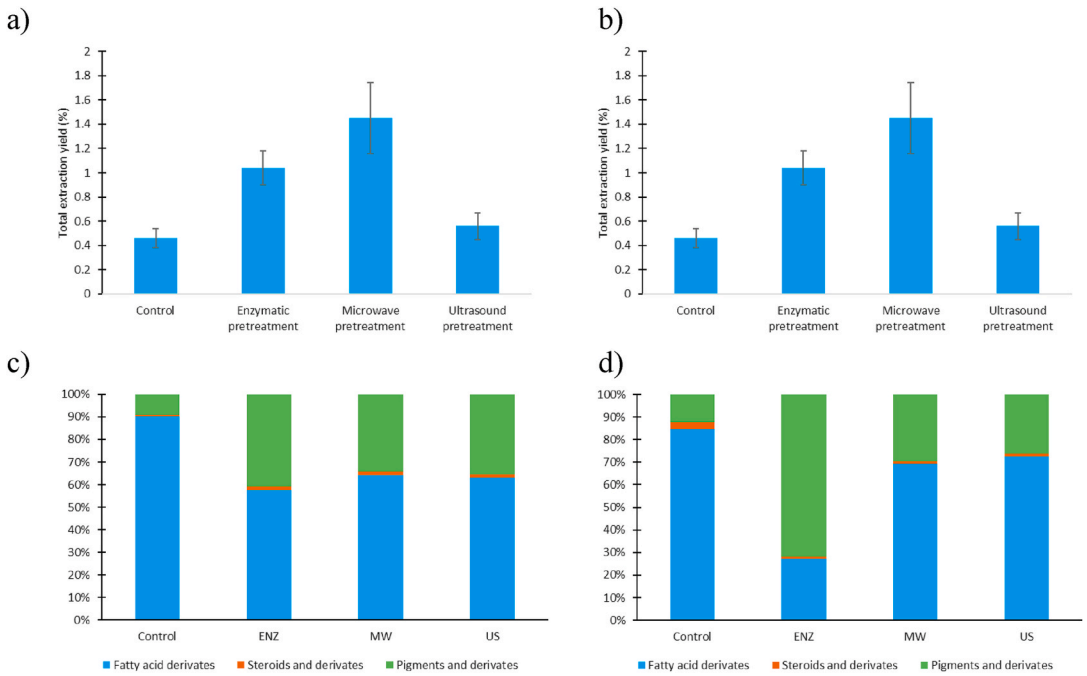

Fig. 3. Total lipid extraction yield (%, w/w) from supercritical CO_2 extraction (pressure 300 bar, temperature 40 °C, extraction time 4 h) of (a) *Chlorela vulgaris* and b) smooth *Chlorella vulgaris* and UPLC-HRMS analysis of c) *Chlorella vulgaris* d) smooth *Chlorella vulgaris* extracts (ENZ - enzymatic pretreatment, US - ultrasound pretreatment, MW - microwave pretreatment).

pretreatments, MW showed the most substantial impact, leading to the highest increase in extraction yield, followed by ENZ and US. For Cv and sCv, the MW treatment resulted in 2.58 and 3.15 times higher yields compared to the control, respectively. This increase can be attributed to the internal heating mechanism induced by MW, where mass and heat gradients move from the inside of the microalgae cells to the surrounding medium. The pressure generated within the cell walls causes their rupture, facilitating the release of lipid components (Ansari et al., 2018). This finding is consistent with the results reported by Dejoye et al. (2011), where MW pretreatment achieved a 2.6-fold increase in lipid yield compared to sCO_2 extraction without pretreatment.

Enzymatic pretreatment also substantially improved extraction yields, increasing the sCvENZ yield by 2.26 times and the CvENZ yield by 1.63 times compared to the control. Enzymatic pretreatment facilitates the degradation of the cell structure through exposure to hydrolytic enzymes, enhancing solvent penetration and lipid release. Ultrasound pretreatment resulted in moderate improvements, increasing extraction yields by 41.46% for Cv and 21.74% for sCv. When exposed to ultrasound waves, cavitation bubbles are formed that grow and collapse. This collapse causes the release of a large amount of energy that exerts its effect on the material. Namely, there is a sudden increase in pressure and temperature on the surface of the solid material, which causes the destruction of biomass structures. Therefore, with US treatment, damage to structures occurs due to external influences on the structure (Ma et al., 2014).

The variations in effectiveness between the pretreatments can be attributed to their distinct mechanisms of action. MW pretreatment's ability to generate intense internal pressure within cells explains its superior performance, as evidenced by the highest yield increases for both Cv and sCv. The lower effectiveness of US pretreatment compared to MW has been previously established (Ma et al., 2014), with MW providing a more uniform and intense disruption of biomass structures.

Overall, the results demonstrate that pretreatments are critical for enhancing the release of lipid-based bioactive components from *C. vulgaris*. The observed improvements in extraction yield align with findings from the literature (Dejoye et al., 2011; Ansari et al., 2018). Furthermore, these results highlight the significant potential of combining green solvent, sCO_2, with pretreatments to improve the efficiency and sustainability of lipid extraction processes. The improved yields obtained with MW, ENZ, and US pretreatments contribute to the broader goal of optimizing microalgae-based bioprocesses for industrial applications, supporting the development of sustainable food and nutraceutical products.

3.2. Chemical profile of C. vulgaris extracts

The chemical profiles of Cv and sCv extracts obtained using various pretreatment methods, MW, US, and ENZ pretreatment, were analyzed. The primary objective was to evaluate changes in the composition of bioactive compounds and assess the potential of these methods for enhancing bioactive compound extraction efficiency. The chemical profile of the obtained supercritical extracts was examined by UHPLC-ESI(+)-HRMS analysis (Fig. 3 c and 3d, Tables 1 and 2), with components tentatively identified on the basis of their elemental compositions and tandem mass spectra. A total of 32 components were identified in Cv extracts, while 29 components were identified in sCv extracts, belonging to the different classes - fatty acid derivatives, steroids and derivatives, and pigments and derivatives.

The application of pretreatments significantly influenced the chemical profiles of the extracts, altering the distribution of compound classes (Fig. 3c and d). In the control samples, fatty acid derivatives were predominantly present with 90.29% (Cv) and 84.46% (sCv), while carotenoid components accounted for 9.24% (Cv) and 12.52% (sCv). However, the application of pretreatments reduced fatty acid derivatives presence while increasing the proportion of pigment and steroid derivatives by a multifold. Due to the application of pretreatment, the porosity of the cell structure and the passage of solvents were increased, so the release of carotenoids, which are usually stored in lipid droplets, was facilitated. In MW and US extracts, the presence of pigments and derivates was 34.17–35.43% and 26.39–29.49% in Cv and sCv, respectively. However, the most pronounced alterations of the chemical profile compared to the control were obtained from ENZ pretreatment. The percentage of pigments and derivatives in ENZ extracts was 40.92% (Cv), and 71.12% (sCv) (control Cv 9.24% and control sCv

Table 1
Chemical composition (UHPLC-ESI(+)-HRMS analysis) of *Chlorella vulgaris* extracts obtained using supercritical CO_2 (pressure 300 bar, temperature 40 °C, extraction time 4 h).

Name	Monoisotopic Mass (Da)	[M+H]+	MF	t_R(min)	Mass Difference (ppm)	Area (counts)			
						Control	ENZ	MW	US
FATTY ACID DERIVATIVES									
Loliolide	196.11	197.11722	$C_{11}H_{16}O_3$	5.86	4.0	1.20E+06	1.48E+04	3.57E+05	1.37E+04
Tetradecanamide	227.225	228.23219	$C_{14}H_{29}NO$	12.50	3.2	1.93E+07	1.16E+06	1.91E+06	1.52E+06
Palmitoleamide	253.241	254.24784	$C_{16}H_{31}NO$	12.96	4.2	5.77E+07	3.87E+06	6.34E+06	5.20E+06
Palmitamide	255.256	256.26349	$C_{16}H_{33}NO$	13.71	3.4	7.94E+07	6.23E+06	9.00E+06	7.69E+06
Hexadecasphinganine	273.267	274.27406	$C_{16}H_{35}NO_2$	7.63	3.3	1.47E+08	4.74E+06	4.60E+06	4.40E+06
Linoleamide	279.256	280.26349	$C_{18}H_{33}NO$	13.43	4.1	7.11E+07	5.37E+06	8.10E+06	6.71E+06
Oleamide	281.272	282.27914	$C_{18}H_{35}NO$	14.12	3.5	3.54E+08	5.92E+07	8.62E+07	7.35E+07
Octadecanamide	283.288	284.29479	$C_{18}H_{37}NO$	14.81	4.1	2.67E+07	1.78E+06	2.63E+06	2.39E+06
Palmitoylethanolamide	299.282	300.28971	$C_{18}H_{37}NO_2$	10.75	4.2	3.94E+05	3.98E+05	4.50E+05	4.46E+05
Arachidonic acid	304.24	305.24751	$C_{20}H_{32}O_2$	15.39	1.1	4.07E+04	5.89E+04	1.33E+05	1.26E+05
cis-11-Eicosenamide	309.303	310.31044	$C_{20}H_{39}NO$	15.10	4.2	1.34E+07	8.18E+05	1.45E+06	1.11E+06
Glycerol palmitate	330.277	331.28429	$C_{19}H_{38}O_4$	14.02	3.3	1.41E+05	7.12E+04	7.52E+04	7.63E+04
Erucamide	337.334	338.34174	$C_{22}H_{43}NO$	16.00	4.3	1.72E+07	3.03E+06	4.47E+06	3.21E+06
Glycerol monooleate	356.293	357.29994	$C_{21}H_{40}O_4$	14.37	5.2	1.08E+05	6.32E+04	6.92E+04	5.45E+04
Glycerol monostearate	358.308	359.31559	$C_{21}H_{42}O_4$	15.08	4.6	3.64E+04	2.75E+04	4.01E+04	4.36E+04
1,3-Dihydroxy-2-propanyl 5,8,11,14-icosatetraenoate	378.277	379.28429	$C_{23}H_{38}O_4$	13.77	0.4	2.40E+04	3.73E+04	1.80E+04	1.59E+04
2-Hydroxy-3-(-L-talopyranosyloxy)propyl (9Z,12Z,15Z)-octadeca-9,12,15-trienoate	514.314	515.32146	$C_{27}H_{46}O_9$	11.72	5.0	1.67E+04	1.56E+04	3.57E+04	6.81E+04
3-(-D-Galactopyranosyloxy)-2-[(7Z,10Z,13Z)-7,10,13-hexadecatrienoyloxy]propyl (9Z,12Z,15Z)-octadeca-9,12,15-trienoate	746.497	747.50417	$C_{43}H_{70}O_{10}$	16.47	4.8	9.59E+03	2.29E+05	4.23E+05	9.49E+05
4-{[6-{[5-({[3-Carboxy-3-(dodecylamino) propanoyl]oxy}methyl)-3,4-dihydroxy-2-(hydroxymethyl)tetrahydro-2-furanyl] oxy}-3,4,5-trihydroxytetrahydro-2Hpyran-2-yl]methoxy}-2-(dodecylamino)-4-oxobutanoic acid	908.546	909.55298	$C_{44}H_{80}N_2O_{17}$	19.95	1.7	9.19E+05	6.46E+05	9.49E+05	4.29E+04
STEROIDS AND DERIVATIVES									
(22E)-Chola-5,22-dien-3-ol	342.292	343.29954	$C_{24}H_{38}O$	9.19	3.3	3.51E+05	3.00E+05	6.57E+05	5.63E+05
(3β,20E)-24-Norchola-5,20(22)-diene-3,23-diol	344.272	345.27881	$C_{23}H_{36}O_2$	9.05	3.6	2.78E+04	2.50E+04	5.64E+04	5.46E+04
(3β,20R,22E,24S)-Stigmasta-5,22-dien-3-ol (β-Stigmasterol)	394.36	395.36723	$C_{29}H_{46}$	16.02	2.4	4.28E+05	4.78E+04	1.80E+05	1.89E+05
(3β)-3-Hydroxystigmast-5-en-7-one	428.365	429.37271	$C_{29}H_{48}O_2$	17.42	4.8	7.47E+05	1.14E+06	1.13E+06	6.81E+05
(3β,6α)-14-Methylergosta-8,24(28)-diene-3,6-diol	428.365	429.37271	$C_{29}H_{48}O_2$	16.33	5.0	2.36E+06	6.16E+05	8.87E+05	8.58E+05
cholesteryl (4Z,7Z,10Z,13Z,16Z,19Z)-docosahexaenoate	696.585	697.59181	$C_{49}H_{76}O_2$	19.19	3.2	6.94E+04	6.41E+04	3.55E+04	4.21E+04
N-(2-hydroxynonadecanoyl)-1-O-β-D-glucosyl-15-methylhexadecasphing-4-enine	743.591	744.59841	$C_{42}H_{81}NO_9$	20.04	0.7	1.04E+05	2.15E+05	3.56E+05	2.58E+05
PIGMENTS AND DERIVATIVES									
Pheophorbide a	592.269	593.27585	$C_{35}H_{36}N_4O_5$	15.41	4.9	1.49E+05	2.78E+05	6.78E+05	7.71E+05
3-Phorbinepropanoic acid, 3,4-didehydro-9-ethenyl-14-ethyl-24,25-dihydro-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-, (2E)-3,7,11,15-tetramethyl-2-hexadecen-1-yl ester	868.55	869.55755	$C_{55}H_{72}N_4O_5$	19.85	4.9	5.69E+05	3.45E+05	5.79E+05	6.37E+05
Pheophytin a	870.566	871.5732	$C_{55}H_{74}N_4O_5$	20.09	4.1	5.05E+07	3.70E+07	4.15E+07	4.34E+07
Pheophytin b	884.545	885.55246	$C_{55}H_{72}N_4O_6$	19.76	4.9	5.32E+05	3.84E+05	5.91E+05	5.56E+05
3-Phorbinepropanoic acid, 9-acetyl-14-ethylidene-13,14-dihydro-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-, 3,7,11,15-tetramethyl-2-hexadecen-1-yl ester	886.561	887.56811	$C_{55}H_{74}N_4O_6$	19.96	4.4	2.17E+07	2.11E+07	1.92E+07	1.23E+07
Methyl (3R,10Z,14Z,20Z,22S,23S)-12-ethyl-3-hydroxy-13,18,22,27-tetramethyl-5-oxo-23-(3-oxo-3-{[(2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen-1-yl]oxy}propyl)-17-vinyl-4-oxa-8,24,25,26-tetraazahexacycl; o [19.2.1.16,9.111,14.116,19.02,7] heptacosa-1(24),2(7),6 (27),8,10,12,14,16,18,20-decaene-3-carboxylate	902.556	903.56303	$C_{55}H_{74}N_4O_7$	19.97	4.2	7.27E+06	3.46E+06	5.20E+06	2.77E+06

Note: The analysis and tentative identification of components were performed using UHPLC-ESI-HRMS. The method is described in Section 2.3. (ENZ - enzymatic pretreatment, US - ultrasound pretreatment, MW - microwave pretreatment).

Table 2
Chemical composition (UHPLC-ESI(+)-HRMS analysis) of smooth *Chlorella vulgaris* extracts obtained using supercritical CO_2 (pressure 300 bar, temperature 40 °C, extraction time 4 h).

Name	Monoisotopic Mass (Da)	[M+H]+	MF	t_R(min)	Mass Difference (ppm)	Area (counts)			
						Control	ENZ	MW	US
FATTY ACID DERIVATIVES									
Loliolide	196.11	197.11722	$C_{11}H_{16}O_3$	6.2	3.1	1.19E+06	5.27E+05	5.05E+05	3.61E+04
Tetradecanamide	227.225	228.23219	$C_{14}H_{29}NO$	12.5	2.7	1.40E+06	1.63E+06	2.01E+06	7.59E+06
Palmitoleamide	253.241	254.24784	$C_{16}H_{31}NO$	13.0	2.4	4.69E+06	4.21E+06	6.83E+06	2.43E+07
Palmitamide	255.256	256.26349	$C_{16}H_{33}NO$	13.7	1.7	9.71E+06	8.77E+06	9.86E+06	3.16E+07
Hexadecasphinganine	273.267	274.27406	$C_{16}H_{35}NO_2$	7.6	1.3	4.71E+06	5.07E+06	4.20E+06	9.71E+07
Linoleamide	279.256	280.26349	$C_{18}H_{33}NO$	13.4	2.1	6.17E+06	3.67E+06	8.52E+06	2.83E+07
Oleamide	281.272	282.27914	$C_{18}H_{35}NO$	14.1	2.4	6.79E+07	7.24E+07	8.96E+07	2.11E+06
Octadecanamide	283.288	284.29479	$C_{18}H_{37}NO$	14.8	1.5	2.60E+06	2.10E+06	2.82E+06	1.01E+07
Palmitoylethanolamide	299.282	300.28971	$C_{18}H_{37}NO_2$	10.7	0.4	4.86E+05	4.54E+05	4.65E+05	4.52E+05
Arachidonic acid	304.24	305.24751	$C_{20}H_{32}O_2$	15.4	5.0	9.85E+04	1.22E+05	7.39E+04	1.34E+05
cis-11-Eicosenamide	309.303	310.31044	$C_{20}H_{39}NO$	15.1	3.1	2.12E+06	1.61E+06	1.49E+06	5.68E+06
Glycerol palmitate	330.277	331.28429	$C_{19}H_{38}O_4$	14.0	0.8	1.30E+05	1.37E+05	8.21E+04	1.64E+05
Erucamide	337.334	338.34174	$C_{22}H_{43}NO$	16.0	2.4	3.17E+06	4.09E+06	2.22E+06	1.01E+07
Glycerol monooleate	356.293	357.29994	$C_{21}H_{40}O_4$	14.4	0.9	2.08E+05	2.36E+05	1.32E+05	2.18E+05
Glycerol monostearate	358.308	359.31559	$C_{21}H_{42}O_4$	15.1	0.3	6.14E+04	5.16E+04	2.85E+04	6.79E+04
1,3-Dihydroxy-2-propanyl 5,8,11,14-icosatetraenoate	378.277	379.28429	$C_{23}H_{38}O_4$	14.4	2.0	7.63E+04	6.31E+04	3.03E+04	6.61E+04
3-(-D-Galactopyranosyloxy)-2-[(7Z,10Z,13Z)-7,10,13-hexadecatrienoyloxy]propyl (9Z,12Z,15Z)-octadeca-9,12,15-trienoate	746.497	747.50417	$C_{43}H_{70}O_{10}$	17.2	0.0	2.19E+04	4.47E+04	6.91E+04	2.80E+05
4-{[6-{[5-({[3-Carboxy-3-(dodecylamino) propanoyl]oxy}methyl)-3,4-dihydroxy-2-(hydroxymethyl)tetrahydro-2-furanyl] oxy}-3,4,5-trihydroxytetrahydro-2Hpyran-2-yl]methoxy}-2-(dodecylamino)-4-oxobutanoic acid	908.546	909.55298	$C_{44}H_{80}N_2O_{17}$	20.0	1.6	5.91E+05	1.02E+06	1.12E+06	1.50E+06
STEROIDS AND DERIVATIVES									
(3β,20E)-24-Norchola-5,20(22)-diene-3,23-diol	344.272	345.27881	$C_{23}H_{36}O_2$	9.0	1.6	3.65E+04	3.47E+04	4.82E+04	3.73E+04
(3β)-3-Hydroxystigmast-5-en-7-one	428.365	429.37271	$C_{29}H_{48}O_2$	17.4	2.7	3.20E+06	3.76E+06	2.35E+06	2.17E+06
(3β,6α)-14-Methylergosta-8,24(28)-diene-3,6-diol	428.365	429.37271	$C_{29}H_{48}O_2$	16.3	1.7	4.32E+05	3.31E+05	1.95E+05	5.43E+05
24-hydroperoxy-24-vinyl-cholesterol	444.36	445.36762	$C_{29}H_{48}O_3$	17.0	1.7	9.64E+04	1.15E+05	1.19E+05	2.87E+05
PIGMENTS AND DERIVATIVES									
Pheophorbide a	592.269	593.27585	$C_{35}H_{36}N_4O_5$	15.4	2.5	5.31E+04	2.17E+05	2.67E+05	6.66E+05
Fucoxanthin	658.423	659.43062	$C_{42}H_{58}O_6$	14.8	3.7	1.88E+04	1.16E+04	7.95E+03	1.83E+04
3-Phorbinepropanoic acid, 3,4-didehydro-9-ethenyl-14-ethyl-24,25-dihydro-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-, (2E)-3,7,11,15-tetramethyl-2-hexadecen-1-yl ester	868.55	869.55755	$C_{55}H_{72}N_4O_5$	19.8	2.2	1.29E+05	1.42E+05	1.64E+05	1.97E+05
Pheophytin a	870.566	871.5732	$C_{55}H_{74}N_4O_5$	20.1	2.0	1.28E+07	2.52E+08	2.87E+07	3.71E+07
Pheophytin b	884.545	885.55246	$C_{55}H_{72}N_4O_6$	19.7	2.8	1.52E+05	3.34E+05	5.38E+05	7.22E+05
3-Phorbinepropanoic acid, 9-acetyl-14-ethylidene-13,14-dihydro-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-, 3,7,11,15-tetramethyl-2-hexadecen-1-yl ester	886.561	887.56811	$C_{55}H_{74}N_4O_6$	20.0	2.0	8.15E+05	2.46E+07	2.33E+07	3.56E+07
Methyl (3R,10Z,14Z,20Z,22S,23S)-12-ethyl-3-hydroxy-13,18,22,27-tetramethyl-5-oxo-23-(3-oxo-3-{[(2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen-1-yl]oxy}propyl)-17-vinyl-4-oxa-8,24,25,26-tetraazahexacycl; o [19.2.1.16,9.111,14.116,19.02,7] heptacosa-1(24),2(7),6 (27),8,10,12,14,16,18,20-decaene-3-carboxylate	902.556	903.56303	$C_{55}H_{74}N_4O_7$	20.0	2.5	1.67E+06	2.67E+06	2.54E+06	5.62E+06

Note: The analysis and tentative identification of components were performed using HPLC-ESI-HRMS. The method is described in Section 2.3. (ENZ - enzymatic pretreatment, US - ultrasound pretreatment, MW - microwave pretreatment).

12.52%). The ENZ pretreatment likely targeted cell wall components through the action of hydrolytic enzymes, breaking down structural polysaccharides and proteins. This enhanced breakdown resulted in a more efficient release of chlorophyll derivatives and pigments.

Previously, Al-Zuhair et al. (2017) determined that enzymatic (lysozyme) pretreatment increased the amount of extracted pigments from the microalga *Chlorella* sp. Also, the application of different pretreatments that ensure better cell disruption improved the extraction of carotenoids from *Tetradesmus obliquus* (Vladić et al., 2022) and *Botryococcus braunii* (Uquiche et al., 2016).

3.2.1. Chemical profile of Chlorella vulgaris (Cv) extracts

For a more detailed analysis of the results, the chemical profiles of Cv supercritical extracts obtained using different pretreatments were compared using hierarchical cluster analysis (HCA). HCA was conducted based on all the identified components and the components'

abbreviations are given in Supplementary material, Table S2 (Supplementary material 2). Cv extracts obtained by applying different pretreatments are compared with the control extract (the extract that was not subjected to any pretreatment) and the resulting dendrograms are given in Fig. 4. These dendrograms confirm that the control is significantly different from other extracts, and it does not belong to any cluster (Fig. 4a). The distinctiveness of the control is attributed to the lack of cell disruption, limiting the release of intracellular components. Also, by comparing Cv extracts obtained by pretreatment (Fig. 4b), it is observed that the extracts obtained by MW and US pretreatments were the most similar (Fig. 4b), likely due to their shared mechanisms of thermal and mechanical disruption.

Oleamide (FAD7) and pheophytin (PID3) were compounds that were present in the highest abundance in Cv extracts, therefore, they are placed in the separate cluster of the vertical dendrogram of the clustered heat map (Fig. 4b). Oleamides and other amides of fatty acids such as palmitamide, palmitoleamide, erucamide, tetradecanamide, linoleamide were the most represented components of the group of fatty acid derivatives.

In the steroid group, the most dominant presence of (3β,6α)-14-methylergosta-8,24(28)-diene-3,6-diol was identified in the control sample, while the proportion of this component was reduced by pretreatments. The most abundant steroid derivative in pretreated samples was (3β)-3-hydroxystigmast-5-en-7-one, reflecting the enhanced extraction of bioactive steroids following cell disruption.

The group of pigments and derivatives includes pheophytins and pheophorbides, metabolites derived from chlorophyll. Chlorophyll was not identified in extracts, but the dominant presence of its derivatives in

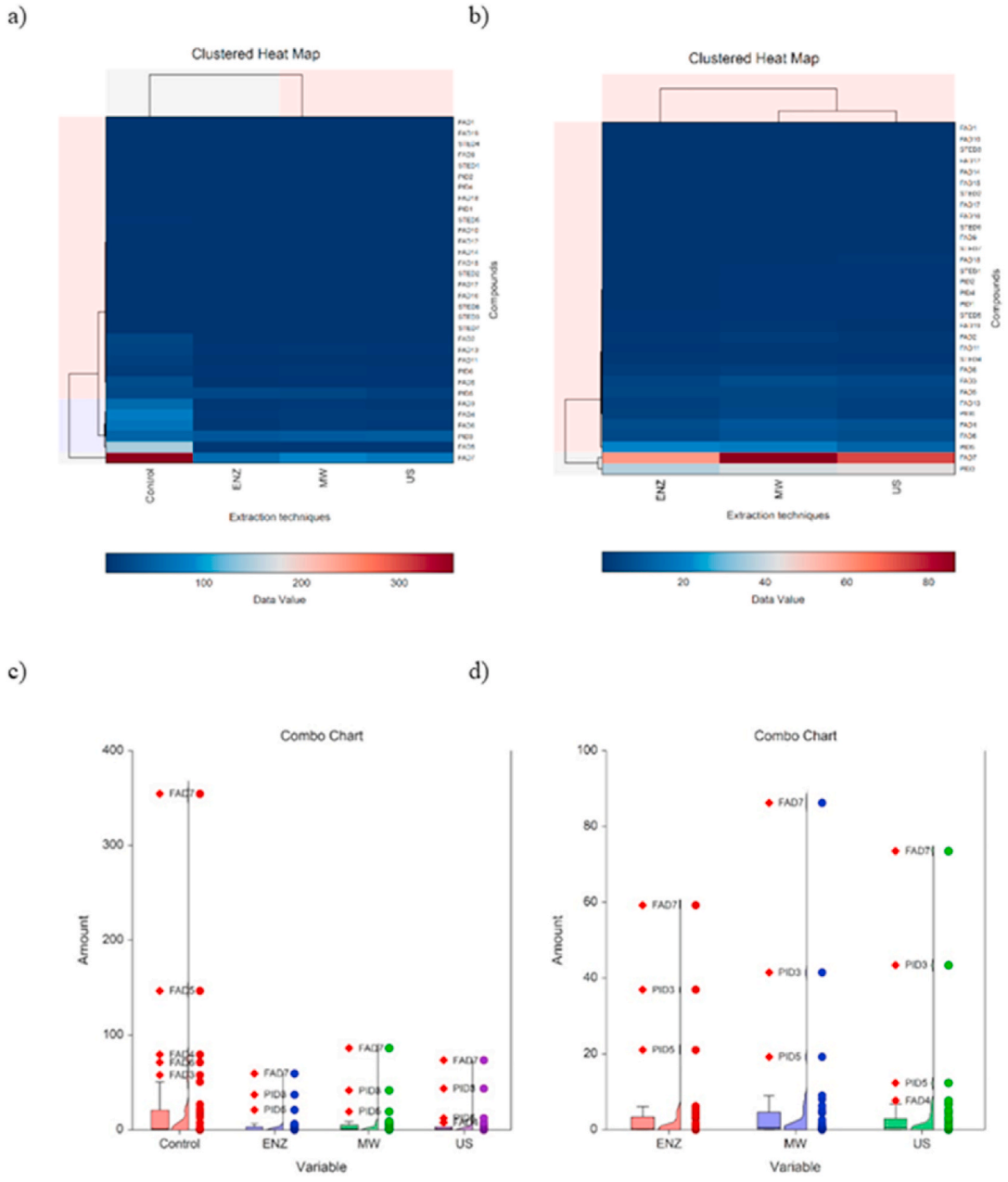

Fig. 4. The clustered heat maps of identified compounds in *Chlorella vulgaris* (a) control and extracts obtained using enzymatic (ENZ), microwave (MW) and ultrasound (US) pretreatment and (b) extracts obtained using ENZ, MW, and US pretreatment. The COMBO charts of the data distribution in the extracts with identified compounds in *Chlorella vulgaris* (c) control and extracts obtained using enzymatic, microwave, and ultrasound pretreatment, (d) extracts obtained using enzymatic, microwave and ultrasound pretreatment. The labeled compounds are considered outliers.

the extracts confirms the initially high content of chlorophyll in the biomass. These derivatives are formed due to the degradation of chlorophyll, which is converted into pheophytin due to the loss of magnesium ions. This conversion can be assisted by different environmental conditions, such as heat and low pH (Daood, 2003). Pretreatments helped the alteration of chlorophyll and the percentage representation of its metabolites in the pretreated samples increased.

The spread and distribution of the data around the central value and extreme values or outliers of the extracts are presented in the form of COMBO charts (Fig. 4c and d). The COMBO chart (Fig. 4c) indicates that the control sample possesses significantly different data distribution and quite wider box and whisker boundaries than pretreated samples, which is attributed to the limited extraction efficiency of untreated biomass. Indeed, the Wilcoxon matched pairs test (WMPT) did show significant differences among control, ENZ, US, and MW extracts based on their composition (Table S3, Supplementary material 2). ENZ-treated extracts exhibited the highest variability in pigment content due to enzymatic hydrolysis targeting cell wall structures.

Further, all three pretreated extracts have similar data distribution, with an observation that the MW extract has the highest oleamide (FAD7) content and a slightly wider whisker boundaries and a slightly higher IQR than in ENZ and US extracts. The compounds FAD7, PID3, and PID5 are marked as outliers in all three extracts considering the fact that those compounds are present in the highest concentrations.

3.2.2. *Chemical profile of smooth Chlorella vulgaris (sCv) extracts*

The comparison of the sCv extracts was carried out in the same way as Cv extracts. The results are presented in the form of clustered heat

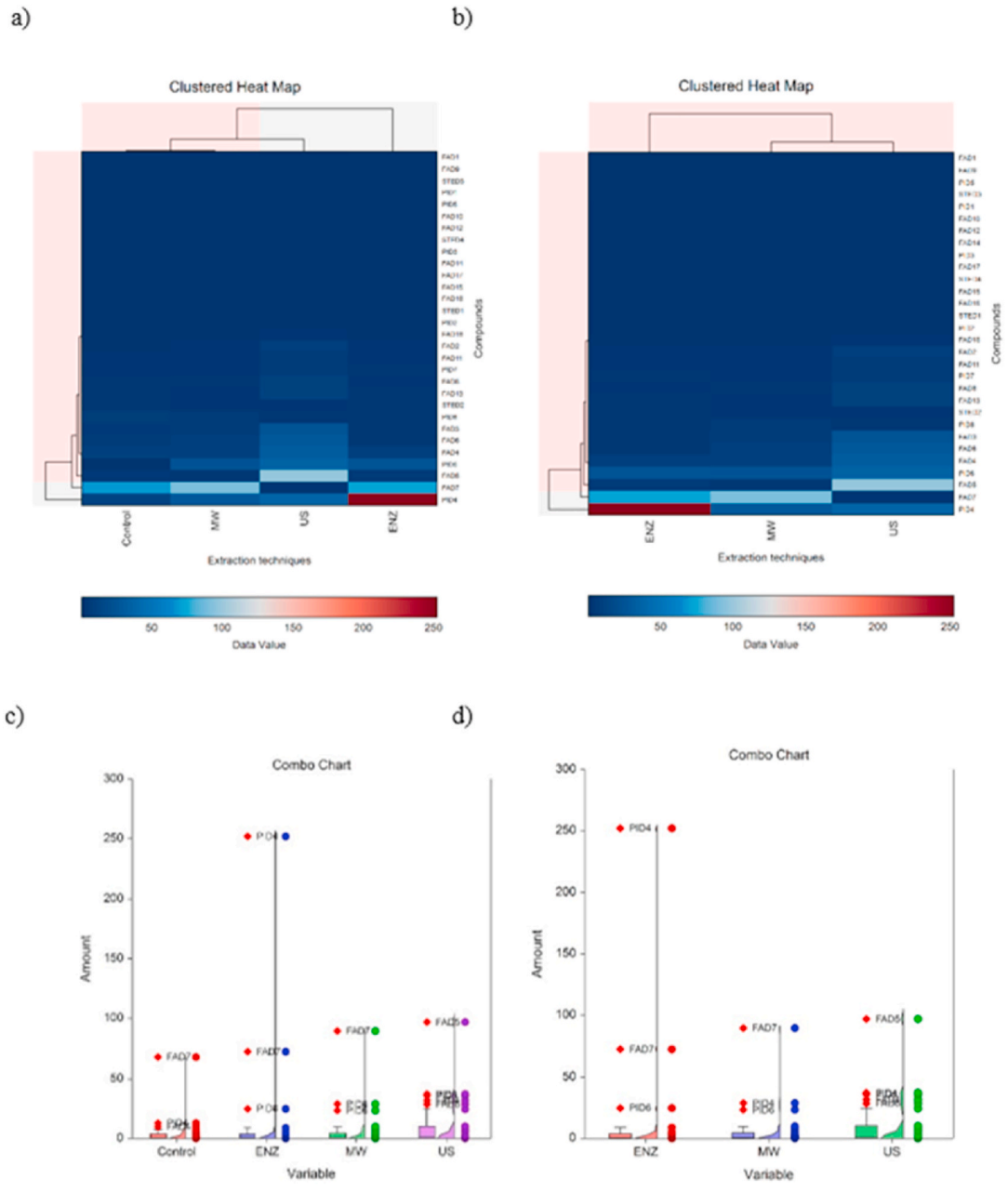

Fig. 5. The clustered heat maps of identified compounds in smooth *Chlorella vulgaris* (a) control and extracts obtained using enzymatic (ENZ), microwave (MW), and ultrasound (US) pretreatment and (b) extracts obtained using ENZ, MW, and US pretreatment. The COMBO charts of the data distribution in the smooth *Chlorella vulgaris* extracts with identified compounds in (c) control and extracts obtained using ENZ, MW, and US pretreatment, (d) extracts obtained using ENZ, MW, and US pretreatment. The labeled compounds are considered outliers.

maps (Fig. 5a and b). The results point out the similarities between the control and MW extract (they are placed in the same cluster). The ENZ extract does not belong to any cluster and can be considered an outlier, due to the most significant change in the percentage of components, an increase in pigment components and a reduction in the presence of fatty acid derivatives. Also, comparing the profiles of pretreated samples without control (Fig. 5b), the extract with ENZ treatment was outside the cluster, while the extracts obtained by MW and US extraction pretreatments were similar.

Furthermore, the US extract had the highest concentration of hexadecasphinganine (FAD5) compared to others in which oleamide was the most abundant among fatty acid derivatives. (3*β*)-3-Hydroxystigmast-5-en-7-one had the highest percentage in the group of steroids in all extracts of sCv. In the group of pigments, pheophytin a was the dominant in all extracts, with ENZ extract having the highest amount. Moreover, in US and MW extracts, it was determined that along with pheophytin a, 3-phorbinepropanoic acid, 9-acetyl-14-ethylidene-13,14-dihydro-21-(methoxycarbonyl)-4,8,13, 18-tetramethyl-20-oxo-, 3,7,11, 15-tetramethyl-2-hexadecen-1-yl ester were dominantly abundant. A potential cause could be the higher intensity and temperature during these pretreatments compared to ENZ, so the conversion of chlorophyll and derivatives was aided by that.

The COMBO charts (Fig. 5c and d) also show that ENZ extract significantly differs from the others. It can also be seen (Fig. 5d) that the ENZ extract has the highest IQR and whisker boundaries, while the other extracts have the data located close to the zero amount. The results of the WMPT (Table S3, Supplementary material 2) indicate the similarities between the control and ENZ and MW extracts, ENZ and MW extracts, while the US extract is significantly different from control and other extracts.

3.2.3. Comparison of *Chlorella vulgaris* vs. smooth *Chlorella vulgaris* extracts

The chemical composition of the extracts indicates that Cv extracts represent valuable products due to bioactive components that have various applications. The extracts contained bioactive fatty acid amides, such as oleamide, which has neuropharmacological effects, such as an antidepressant-like property (Akanmu et al., 2007). Also, it was suggested that thanks to the antioxidative activity of oleamide, radish extract has a protective role against oxidative stress induced neuronal toxicity (Choi et al., 2020). Apart from oleamide, there was also a significant presence of other bioactive derivatives: erucamide, which exhibits antidepressant and anxiolytic effects in mice (Li et al., 2017), can regulate water imbalance and angiogenesis (Hamberger and Stenhagen, 2003), and inhibits the cleavage of glycine to anti-stress liver injury in rats (Cuiying et al., 2021); sleep-inducing linoleamide can cause significant increases in Ca^{2+} in renal tubular cells (Huang and Jan, 2001). The pigments, whose share was significantly increased after the application of pretreatment, represent a class of components for which the global interest is constantly growing. Due to their numerous health-promoting properties, they are highly important in the production of animal feed, human food, dietary supplements, nutraceuticals, cosmetics, and textile (Meléndez-Martínez et al., 2022).

The comparison between all Cv and sCv extracts was performed applying the PCA approach. The PCA resulted in a 5-PC model covering 95.1% of the total variance. The first two PCs cover 68.7% of total variance of which PC1 covers 43.49% and PC2 25.24%. The PCA was performed on the extracts based on their mutual components (the component present in all extracts). Their abbreviations are given in Supplementary material (Supplementary material 2; Table S4). The PCA results are presented in Fig. 6 in the form of the score (a) and the loadings plot (b).

Along the PC1 axis, there is a separation of CvControl extract from the rest of the extracts. This extract is placed in a significant distance from other extracts whose projections are located on the positive end of the PC1 axis, while the projection of CvControl extract is placed on the negative end of this axis. Therefore, there is no separation of the Cv and sCv samples on the PC1 axis. Also, sCvUS extract is located on the negative part of PC1 axis, however unlike CvControl, it is closer to the rest of the extracts.

The compounds F2 (tetradecanamide), F3 (palmitoleamide), F4 (palmitamide), F5 (hexadecasphinganine), F6 (linoleamide), F8 (octadecanamide), F11 (*cis*-11-eicosenamide) and F12 (glycerol palmitate) have strongest influence on the data distribution along the PC1 axis. On the other hand, the compounds F14 (glycerol monooleate) and F16 (1,3-dihydroxy-2-propanyl-5,8,11,14-icosatetraenoate) have the strongest positive influence and the compound P2 (3-phorbinepropanoic acid, 3,4-didehydro-9-ethenyl-14-ethyl-24,25-dihydro-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-, (2E)-3,7,11,15-tetramethyl-2-hexadecen-1-yl ester) the strongest negative influence on the distribution of the data along the PC2 axis. The data projection on the PC2 axis reveals the separation of Cv and sCv extracts, so the sCv extracts belong to the quadrants with positive part of the PC2 axis while the Cv extracts are in the quadrants with negative part of the PC2 axis. This separation is

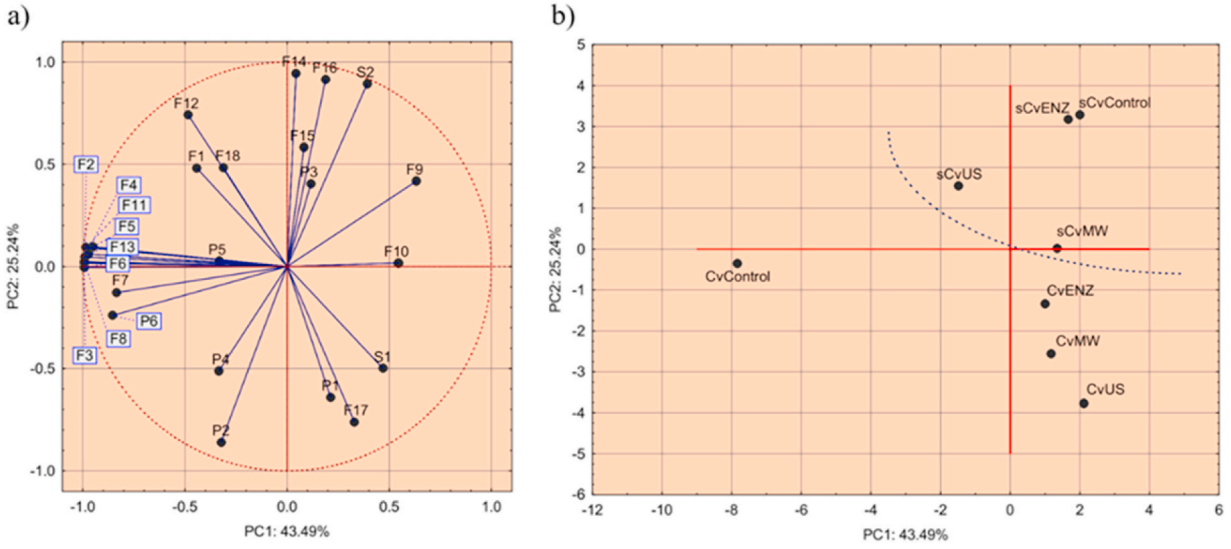

Fig. 6. The score (a) and loadings plot (b) of the PCA based on components identified in the *Chlorella vulgaris* (Cv) and smooth *Chlorella vulgaris* (sCv) extracts (ENZ - enzymatic pretreatment, US - ultrasound pretreatment, MW - microwave pretreatment).

based on the content of F14, F16 and P2 compounds.

PCA was based on components identified in both microalgae. It is important to note that fucoxanthin, a pigment with extremely important bioactive properties, such as anticancer, antiobesity, antifibrotic, anti-inflammatory was identified in sCv extracts. In addition, the presence of several components belonging to fatty acids derivatives and steroid derivatives and not identified in sCv was identified in Cv (Xiao et al., 2020).

3.3. Cytotoxic activity of C. vulgaris extracts

Assessing cytotoxic activity toward cancer cell lines enables the identification of extracts with selective anticancer potential, broadening their scope of use, while evaluating cytotoxicity against normal cells ensures the safety of these extracts for various applications. The cytotoxic activity of the extracts was tested against human cervix (HeLa) and breast cancer (MDA-MB-453) cell lines. Also, the activity was tested against normal fibroblast cells (MRC-5). Table 3 shows the activity expressed as IC_{50}, concentrations that induced 50% decrease in cell survival. The activity of the extracts obtained by pretreatment was analyzed, while the control extract was not analyzed due to a very low amount insufficient for the cytotoxic activity analysis. The IC_{50} values against HeLa cancer cells were in the range from 166 ± 14 to 334 ± 32 μg/mL, where samples sCvUS and CvENZ exhibited the most pronounced activity. Activity against MDA-MB-453 cells was 178 ± 2–333 ± 22 μg/mL, and Cv extracts showed more pronounced activity compared to sCv samples. Comparing the activity of the extracts, it can be concluded that there is no strict separation of the Cv and sCv extracts based on their cytotoxic activity.

Furthermore, to determine the selectivity of extract, coefficients of selectivity were calculated as the ratios between the IC_{50} values obtained on MRC-5 and malignant cells. CvMW showed the highest selectivity towards HeLa cells, while CvMW and CvUS extracts showed the most pronounced selectivity towards MDA-MB-453.

The analysis of variance (ANOVA) showed that there was a significant difference in cytotoxicity for *C. vulgaris* and smooth *C. vulgaris* extracts against control cells ($p < 0.05$). The results of the *t*-Test showed statistically significant differences in cytotoxicity of CvENZ vs. sCvENZ as well as CvUS vs. sCvUS against control MRC-5 cells (Fig. S1, Supplementary material 2).

The anticancer activity of *Chlorella* against different types of cancer has been investigated before. However, according to the available literature, the potential of supercritical extracts of *C. vulgaris* was investigated in only one study. In the study, *C. vulgaris* C-C extract obtained by sCO_2 with 50% aqueous ethanol as a co-solvent, reduced the lung cancer cell growth and migration of tumor cells (Wang et al., 2010). The obtained extract was analyzed for total phenolic and flavonoid content, but a more detailed chemical profile was not investigated.

Regarding the examination of the activity against breast cancer, an *in vivo* study was conducted where Ehrlich ascites tumor-bearing mice were treated with dry *C. vulgaris* dissolved in distilled water. It was noted that the protective antitumor effect, since the application of the extract prolonged the survival of mice with the Ehrlich ascites tumor (Justo et al., 2001). El-Fayoumy et al. (2021) reported the anticancer activity of extracts and fractions of *C. vulgaris*, according to HeLa cells. In the aforementioned study, *C. vulgaris* was cultivated under various copper stress conditions and the extract was obtained by extraction with organic solvents, methanol and methylene chloride. Further fractionation was performed with hexane, chloroform, ethyl acetate and the fractions with the most pronounced anticancer activity had IC_{50} values of 162.30, 154.7, 84.2, 40.0 μg/mL (El-Fayoumy et al., 2021). The values obtained in our work, where the extracts were obtained using a green solvent and the activity of the whole extract was measured, are in accordance with the reported values. The activity of *C. vulgaris* against MDA-MB-453 cancer cells has not been previously reported in the literature.

Correlation analysis was conducted, based on the examination of the dependence of cytotoxic activity towards MRC-5, HeLa, and MDA-MB-453 cell lines in terms of presence of components identified in the extracts. Those components were identified based on correlation matrix (Fig. 7) and linear relationship graphs (Figs. S2 and S3, Supplementary material 2).

Considering the Cv extracts, the extracts with higher abundance of hexadecasphinganine (HDS) and 1,3-dihydroxy-2-propanyl 5,8,11,14-icosatetraenoate (DPIT) possessed stronger cytotoxic activities. In Fig. 8 it can be seen how much the IC_{50} value decreased by increasing the presence of HDS and DPIT in the extracts. Recently, it was indicated that many sphingolipid-containing nanotherapeutics have potential for application in cancer treatments (Choi and Song, 2020). Although the mechanism is unclear, it is suggested that components with sphingoid bases may have anticancer potential as they can suppress transformation of normal cells induced by irradiation, cause differentiation of transformed cells, and inhibit growth of different cancer cells (Symolon et al., 2011).

In the case of sCv extracts, the most significant relationships were detected between the cytotoxic activity and the concentration of 3β,6α-14-methylergosta-8,24(28)-diene-3,6-diol (MDD) and fucoxanthin (FUC). Higher abundances of these two compounds were related to the higher cytotoxic activity, as can be seen in Fig. 8. The most significant decrease of IC_{50} values was noticeable towards HeLa and MDA-MB-453 cell lines when MDD and FUC concentrations increased.

(3β,6α)-14-Methylergosta-8,24(28)-diene-3,6-dio belongs to the group of phytosterols, which are reported to cause inhibition of tumor growth, through the reduction of cell cycle progression, induction of apoptosis, and inhibition of metastasis (Shahzad et al., 2017). Fucoxanthin is also recognized as a natural bioactive anticancer compound (Méresse et al., 2020). Jin et al. (2018) indicated that fucoxanthin and tumor necrosis factor-related apoptosis-inducing ligand can synergistically increase the apoptosis of cervical cancer cell lines, including HeLa cell lines. Also, its anticancer activity against breast cancer was reported, due to the inhibition of tumor-induced lymphangiogenesis *in vitro* and *in vivo* (Wang et al., 2019).

Table 3
Cytotoxic activity of *C. vulgaris* extracts - average IC_{50} values ± standard deviations - SD, and 95% confidence intervals (CI) based on MTT assay for the action of investigated extracts.

Sample	Pretreatment	IC_{50} (μg/mL)		
		HeLa	MDA-MB-453	MRC-5
Chlorella vulgaris	ENZ	208 ± 22 (166, 252)	178 ± 2 (174, 182)	250 ± 8 (232, 266)
	MW	224 ± 32 (160, 288)	184 ± 0 (184, 186)	341 ± 11 (319, 363)
	US	232 ± 24 (186, 280)	190 ± 2 (186, 192)	348 ± 0 (348, 350)
Smooth *Chlorella vulgaris*	ENZ	268 ± 26 (216, 320)	276 ± 14 (248, 302)	374 ± 0 (372, 374)
	MW	334 ± 32 (277, 396)	333 ± 22 (289, 377)	436 ± 20 (396, 474)
	US	166 ± 14 (138, 192)	196 ± 8 (180, 212)	213 ± 7 (199, 227)

Enzymatic pretreatment (ENZ), microwave pretreatment (MW), ultrasound pretreatment (US).

3.4. Life cycle assessment

3.4.1. Environmental impacts of sCO_2 extraction from control and pretreated biomass

The environmental impacts of 1 kg microalgae extract with pretreatments prior to sCO_2 were compared to the untreated extraction. It was shown that in most categories, the microalgae extract with pretreatment has lower environmental impacts compared to the untreated

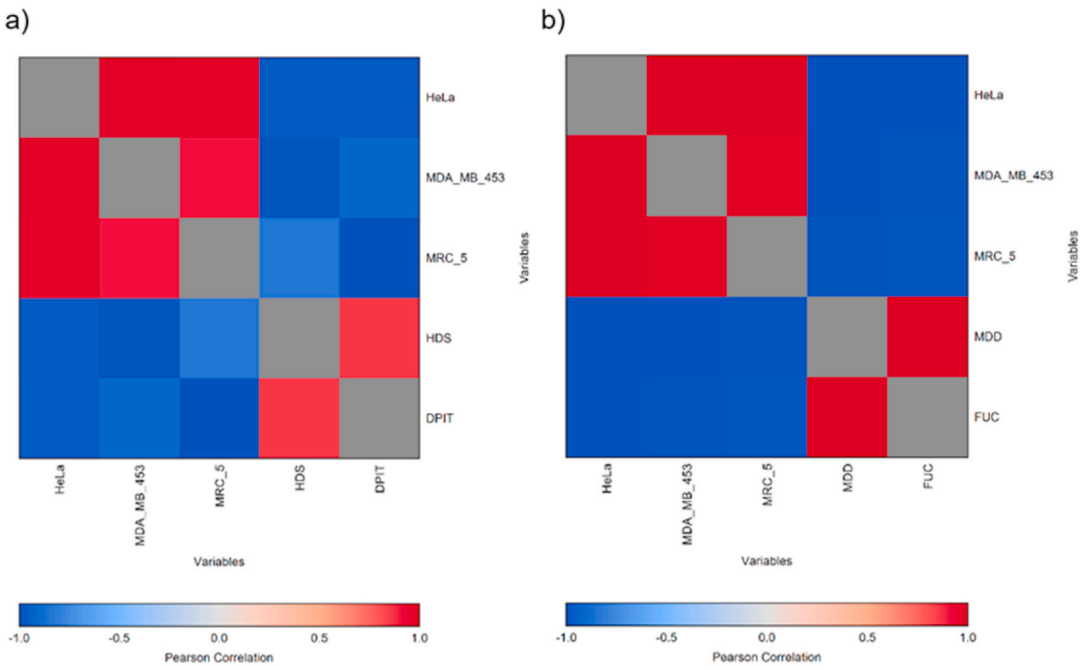

Fig. 7. Heat maps of Pearson's correlation coefficients between cytotoxic activity of the analyzed extracts towards HeLa (human cervix adenocarcinoma cell line), MDA-MB-453 (human breast carcinoma cell line), and MRC-5 (normal human fetal lung fibroblast cell line) and: a) HDS (hexadecasphinganine) and DPIT (1,3-dihydroxy-2-propanyl 5,8,11,14-icosatetraenoate) of *Chlorella vulgaris* extracts; b) MDD (3β,6α-14-methylergosta-8,24(28)-diene-3,6-diol) and FUC (fucoxanthin) of smooth *Chlorella vulgaris* extracts.

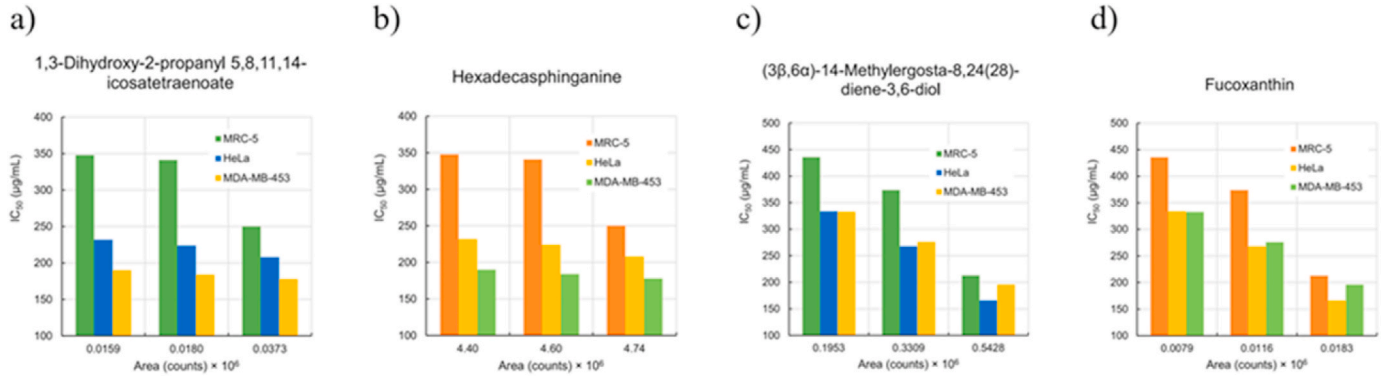

Fig. 8. Comparison of IC_{50} (μg/mL) values of cytotoxic activity and the area on the chromatogram of components well correlated with IC_{50} from *Chlorella vulgaris* extracts (a and b) and smooth *Chlorella vulgaris* extracts (c and d). (HeLa - human cervix adenocarcinoma cell line, MDA-MB-453 - human breast carcinoma cell line, MRC-5 - normal human fetal lung fibroblast cell line).

control (Fig. 9a). For Cv, the environmental impacts of ENZ, MW, and US pretreatment were on average around 22%, 57%, and 22% respectively lower than the control. For sCv, the impacts of ENZ, MW, and US were on average around 45%, 65%, and 9% respectively lower than the control. However, for some impact categories, the impacts of extract from ENZ pretreatment were slightly higher than control, i.e., 29% and 3% higher than control for land use and marine eutrophication respectively for Cv biomass. This was caused by the use of enzymes in ENZ pretreatment. In this LCA model, the impact for enzyme production was extracted from Ecoinvent database which assumed plant-based enzymes. Due to the high usage of the enzymatic solution in ENZ pretreatment, the impacts from the enzyme became significant. Similarly, the impacts of ENZ pretreatment of sCv for these impact categories, despite being lower than control were higher than the average impacts in other impact categories. The impacts from enzyme production were compensated by the higher total extraction yields for sCvENZ (2.26 times more than the control). It is important to highlight that the enzymes also can have a high cost which can be a challenge for the scale-up of the technology, therefore when using this technology route, a complete techno-economic analysis need to be performed to assess the feasibility of it.

Of all pretreatment alternatives evaluated in this study for both Cv and sCv, MW pretreatment was shown to have the least environmental impacts. These impacts were highly affected by the extraction yields from each extraction routes. The notably increased extraction yields from the applied pretreatments reduced the environmental impacts of the extraction process. The total extraction yields from MW pretreatment were the highest (Section 3.1), thus the impacts from MW were the least among others. This aligns with the objective of the study on identifying the best pre-route for extraction, but results can change if the functional unit is modified, as the impacts are calculated based on the extract mass (1 kg of microalgae extract) as functional unit.

Fig. 9. a) Environmental impacts of the supercritical carbon dioxide extraction of *Chlorella vulgaris* (Cv) and smooth *Chlorella vulgaris* (sCv) with pretreatments (bars): enzymatic, microwave, and ultrasound, compared to the untreated biomass (control – dashed line); **b)** process contribution analysis for steps in microalgae supercritical carbon dioxide extraction with enzymatic, microwave, ultrasound pretreatments, and the untreated biomass (control); **c)** hotspot analysis of different input categories (infrastructure/equipment, electricity, and chemicals) for microalgae supercritical carbon dioxide extraction with enzymatic, microwave, and ultrasound pretreatments, and the untreated biomass (control); **d)** Impact reduction of the improvement scenario from baseline for control, enzymatic, microwave and ultrasonic pretreatments.

c)

d)

Fig. 9. (*continued*).

3.4.2. *Process contribution and hotspot analysis*

The impacts from each process step were analyzed to see the contribution of each step to the total impacts (Fig. 9b). The profiles for process contribution for both microalgae Cv and sCv were observed similar, hence no distinctions were made in the analysis. From the process contribution analysis, it was shown that sCO_2 extraction process was the most impactful. The contribution of pretreatment process was observed negligible (0.01–0.16%) for MW and US, and up to 47% for

ENZ. The impacts for MW and US pretreatment process were lower compared to the freeze drying and extraction process. This can be explained by the significantly shorter duration of these pretreatment processes (3–5 min) compared to the freeze drying (48 h) and the sCO_2 extraction (4 h). The contribution of pretreatment process was observed slightly more visible for ENZ. Not only was the pretreatment time longer (60 min), but the use of enzymes also increased the total impact in some categories such as land use and marine eutrophication.

For the hotspot analysis, the contributions of infrastructure (production of equipment), electricity, and chemicals (e.g., enzymes, CO_2) were distinguished and analyzed (Fig. 9c). Similar to the process contribution analysis, the profiles for Cv and sCv were non-distinctive. The impacts from the infrastructure in all impact categories were observed negligible (0.01–0.75% of the total impacts) while chemical materials were the major contributors to the total impacts (44–95%). Liquid CO_2 that was used for the supercritical extraction process was found to be highly impactful and thus, improving the use of CO_2. For instance, recirculating used gas can theoretically lower the environmental impacts of the system. Another input parameter that contributed to the total impacts of the extraction system was electricity. Improving the electricity use or substituting the energy source with a more renewable energy provider are strategies that could be implemented to lower the total impacts of the system.

3.4.3. Scenarios for process improvement

A sensitivity analysis was performed with a scenario developed to improve the environmental performance of the extraction processes. From process contribution and hotspot analysis, it was recognized that the use of liquid CO_2 during supercritical extraction and electricity consumption were the most impactful factors. Therefore, in the improvement scenario, the CO_2 was set to be recirculated and the source for electricity was set to be from a more renewable source e.g., photovoltaic panels. The impacts of this improvement strategy, considering 90% CO2 recirculation and 100% solar electricity, were then compared to the baseline scenario. It was observed that for all impact categories, the improvement strategy reduced impacts from around −20% to −80% (Fig. 9d).

Recirculating the CO_2 and changing the electricity source affected all impact categories but most notably the ionizing radiation and toxicity categories. These categories are related to the emission of radionuclides from nuclear fuel cycle or from the conventional energy generation e.g., burning the coal (Huijbregts et al., 2016). Hence, substituting the energy source into a more renewable source will reduce the impacts in these categories (UNECE, 2021; World Energy Council, 2004). Moreover, the reduced impacts also come from solvent (CO_2) recirculation that can save up to 90% of the solvent use. The reduction from recirculation strategy was still effective despite the slight increase in the electricity consumption due to additional processes for creating the supercritical phase of the solvent, e.g., additional filtration, drying, and pressurizing.

The land use was observed to be the least affected impact category by the proposed improvement strategy ranging from −20% to −40% reduction from the baseline. If the energy source is substituted into solar energy, the land cover change from the installation of the solar panels may occupy 0.5–5% of total land (Van de Ven et al., 2021). Therefore, in this scenario analysis, despite being lower than the baseline scenario, the impact reduction was not as significant as in other impact categories. Especially for ENZ pretreatment, since the impacts for land use were also affected by other factors (e.g., enzyme use). The reduction was observed even less (−22%) than in other pretreatments (MW, US) and control.

This analysis highlights that the impact of the system is highly dependent of the circularity of the systems and where the facility is located in terms of consumables inputs availability and electricity sources. Therefore, the LCA is a useful tool for a comparative analysis and more detailed analysis might be performed in order to achieve specific results.

4. Conclusions

Different pretreatments to sCO_2 extraction were applied to analyze their influence on yield, chemical and pharmacological properties of *Chlorella vulgaris* (Cv) and *smooth C. vulgaris* (sCv) extracts. Application of microwave pretreatment provided the highest extraction yields, increasing 2.58 times for Cv and 3.15 times for sCv compared to the control. Pretreatments significantly affected the chemical composition of the extracts, so the presence of the most represented class component in the control, fatty acid derivatives, was significantly reduced due to the application of pretreatments, with a multiple increase in the presence of pigment and steroid derivatives. These compositional enhancements demonstrate the potential for targeting bioactive compounds through pretreatment methods. In terms of cytotoxic activity, the sCvUS extract exhibited the strongest effect against HeLa cells, while Cv extracts showed greater activity against MDA-MB-453 cells. These findings highlight the potential of pretreated extracts for pharmaceutical applications. The LCA revealed significant reductions in environmental impacts, with pretreatment-enhanced processes achieving up to 65% lower impacts compared to control. Furthermore, the scenario analysis also indicated the potential for up to 80% more impact reduction from the current set up by recirculating the CO_2 solvent and substituting into a more renewable energy source. The results of this work demonstrate that combining innovative pretreatments with sCO_2 extraction aligns with cleaner production principles by enhancing efficiency and reducing environmental impacts. This study supports the transition toward more sustainable production and consumption of microalgae, directly addressing the goals of sustainability and cleaner production. In the further stages of research, it is a priority to optimize the operating conditions according to the target products and/or activities. This will enable greater selectivity and maximum efficiency of the process along with its optimal environmental parameters.

CRediT authorship contribution statement

Jelena Vladic: Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Sanja Radman:** Software, Investigation, Formal analysis. **Zeljko Zizak:** Writing – review & editing, Investigation, Funding acquisition. **Irina Besu:** Formal analysis, Data curation. **Igor Jerkovic:** Writing – review & editing, Validation, Supervision, Funding acquisition. **Lais Galileu Speranza:** Writing – review & editing, Software, Methodology, Conceptualization. **Ahmad Furqan Hala:** Writing – original draft, Software, Data curation. **Strahinja Kovacevic:** Writing – original draft, Visualization, Software, Data curation. **Hugo Perreira:** Resources, Funding acquisition, Conceptualization. **Luisa Gouveia:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2025.144823.

Data availability

No data was used for the research described in the article.

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