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# Sequential valorisation of microalgae biomass grown in pig manure treatment photobioreactors

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ABSTRACT

Sequential valorisation of microalgae biomass grown in pig manure was evaluated to harness the major accumulated components. First, supercritical carbon dioxide extraction (100, 200, and 300 bar; 40 and 60ºC) was applied to solubilise the lipid components. The maximum lipid extraction (75%) was achieved at 300 bar and 60ºC. The extraction kinetic increased with the temperature at all the pressures tested and decreased with pressure only for experiments at 60ºC.

After supercritical $CO_2$ extraction, the exhausted solid fraction was assessed using subcritical water extraction (100, 130, 160, and 190ºC for 10 min) where the carbohydrate components were selectively solubilised. The raffinate solid fractions after subcritical water extraction retained 30 to 46% carbohydrates and 67 to 73% proteins. A 60% monosaccharide recovery yield was achieved, with a maximum carbohydrate degradation rate of 10%.

Subcritical water extraction was also evaluated using the initial raw biomass. Therefore, the solubilisation of carbohydrates and their recovery was lower than in the experiments using the exhausted solid fractions after supercritical $CO_2$ extraction. As a result, supercritical extraction seems to be a promising method for the sequential valorisation, as was confirmed in the scanning electron microscopy analysis. Finally, the composition of raffinate solid fractions after subcritical water extraction was analysed to check their potential use as bio-fertiliser, applying a bio-refinery concept.

**Highlights**

- High pressure and temperature on $CO_2$ extraction improved the lipid solubilisation.
- Supercritical lipid extraction increased the monosaccharide recovery.
- Subcritical extraction caused cell disruption and carbohydrates degradation <10%.

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- Subcritical extraction reached a 60% monosaccharides recovery (190ºC, 10 min).
- Co-solubilisation of proteins (~ 30%) was detected during subcritical extraction.

Keywords: Carbohydrates; Lipids; Proteins; Subcritical water; Supercritical $CO_2$

## 1. Introduction

The application of a bio-refinery concept that can achieve the integral valorisation of biomass has increased substantially in recent years. This concept has the ability to enhance economic benefits, minimise the consumption of raw materials, and mitigate the problems that waste production can cause in sanitation, the environment, and public health [1], [2]. This circular-economy concept is not only focused on low added value products, but in a complete valorisation of the biomass to produce high and medium -value compounds, applying sequential and selective processes.

Among the possible raw materials from bio-based industries, the use of microalgae has increased considerably due to its numerous applications in the healthcare, cosmetics, and pharmaceutical industries, among others [3]. Biofuels and nutraceuticals produced from microalgae have developed significant interest, resulting in intensive research in bio-refinery processes using pure microalgae cultivated in synthetic media as feedstock [4].

Nevertheless, the elevated production cost of pure microalgae biomass jeopardises the economic viability of a microalgae-based industry [5]. In this context, researchers are paying attention to the use of photobioreactors for wastewater treatment and the valorisation of algal-bacteria biomass grown in these treatment plants. Integrating wastewater treatment and the production of added-value products from low-priced biomass (such as those grown in

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wastewater treatment plants) into the bio-refinery process is the principal challenge in microalgae bio-refinery nowadays [6].

Microalgae-bacteria photobioreactors have been successfully used for the treatment of different wastewaters such as pig manure [7] or palm oil mills [8], among others. Different species of microalgae and bacteria grow symbiotically, forming consortia in a mutually beneficial relationship in these wastewater treatment plants. Microalgae generate oxygen that bacteria need to oxidate organic pollution. By degrading organic matter, bacteria produce carbon dioxide – a nutrient necessary for the efficient growing of microalgae. Nitrogen and phosphorus compounds are also transformed by bacteria and used for microalgae growth [9].

Different microalgae species coexist in open photobioreactors that are used for wastewater treatment [7]. However, only robust microalgae species with a rigid, homogenous, and multi-layered cellular wall (*Scenedesmus, Chlorella, Tetraselmis, Nannochloropsis*…) are able to compete with bacteria and grow in the high stress environment of a wastewater treatment photobioreactor.

The extraction of components from these species with hard cell walls generally requires the application of pretreatments or severe extraction conditions [10], [11]. In general, pretreatments (chemical, physical, or biological) are non-selective processes, which increase the solubilisation of all the components from microalgae, as reported by Solé-Bundó et al. [12] who used a thermal pretreatment with lime (CaO) on mixed microalgae-bacteria biomass (*Chlorella* sp. and *Scenedesmus* sp.), Kavitha et al. [13] who applied a biological pretreatment on *Chlorella vulgaris*, and Postma et al. [14] who performed bead milling on three distinct microalgae biomasses.

Consequently, the search for adequate techniques is essential for the selective extraction and sequential recovery of components. Previous studies applying the most

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common chemical or mechanical pretreatments for cell breakthrough of microalgae biomass showed at least a 5% of lipids in the pretreated solid fraction, independent of the initial lipid content [15]. These residual lipids are co-solubilised during further steps in which other components, mainly proteins, are recovered, remarkably reducing their purity and value [16].

As an alternative to conventional pretreatments, supercritical $CO_2$ (SC-$CO_2$) extraction is a very attractive technology in order to increase the lipid extraction yield and to preserve the rest of components of biomass. SC-$CO_2$ is effective for the extraction of low molecular weight lipids and for biomasses containing a low percentage of lipids, such as biomass from wastewater treatment plants [17]. Additionally, the use of SC-$CO_2$ extraction as the first step of sequential valorisation processes prevents the presence of toxic solvents or residues in other valuable component extracts. For example, Bahadar et al. [18] studied the effects of the operational parameters on SC-$CO_2$ lipid extraction from *Chlorella vulgaris* (18% lipid content). They found the maximum lipid extraction (almost complete) at the extreme conditions of pressure (620 bar) and temperature (80ºC) in their experiments. Crampon et al. [19] investigated SC-$CO_2$ lipid extraction from *Spirulina platensis* (14% lipid content) through response surface methodology (RSM). The maximum extraction yield (92%) was achieved at the highest assayed pressure (300 bar) and temperature (60ºC).

After lipid extraction, subcritical water extraction (SWE) is a promising technique to solubilise the polar components of the biomass [20]. Water is a non-toxic solvent that can be easily recovered and recirculated into the process [21], [22]. The operation conditions applied in SWE processes provoke the cleavage of hydrogen bonding, decreasing the dielectric constant and polarity of water and increasing extraction efficiency [23].

However, there is limited research on subcritical water extraction from microalgae and the few published studies worked with pure species. Awaluddin et al. [24] and Phusunti et al.

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[25] optimised the subcritical water extraction on two *Chlorella vulgaris* biomasses with distinct compositions. The co-solubilisation of the carbohydrate and protein components was detected in both research works.

Therefore, the integral valorisation of biomass requires research about the best technique for recovery for each specific component but minimizing the effect on the rest of components [26]. Furthermore, it is essential to avoid the degradation of the biomass components because such a degradation would decrease of the quantity and the quality of the final products [27].

The aim of the present work is to study the valorisation of microalgae biomass grown in pig manure wastewater, involving a sequential extraction, based on to a bio-refinery concept. As a first step, SC-$CO_2$ extraction was applied to the microalgae-based biomass in order to extract the lipids. The effect of the two main operational parameters (pressure and temperature) on the lipid extraction yield and kinetic was analysed. As a second step of the sequential process, the application of subcritical water to extract the carbohydrate components from the exhausted solid fraction after the SC-$CO_2$ extraction was studied. Subcritical water extraction was also performed using the initial raw material (the material not exposed to SC-$CO_2$ extraction) in order to determine the feasibility of the SC-$CO_2$ extraction and the solubilisation of the carbohydrate and protein components. An SEM analysis was used to examine the surface changes of the biomass after each step. Finally, for total recovery, the raffinate solid fractions were analysed after the subcritical water step in order to determine their possible use as a bio-fertiliser.

## 2. Materials and Methods

### *2.1. Microalgae biomass*

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The microalgae-based biomass used for the experiments was cultivated in a thin-layer 1200L volume photobioreactor [28]. The photobioreactor was fed with centrifuged pig manure (Total Organic Carbon: 17100mg/L, Inorganic Carbon:1870 mg/L, Total Nitrogen: 5500 mg/L, Total Phosphorus:54 mg/L) diluted at 10%. The Total Organic Carbon, Inorganic Carbon, and Total Nitrogen concentrations were determined using a TOC-V CSH analyser with a TNM-1 module (Shimadzu, Japan). The Total Phosphorus concentration was determined according to Standard Methods [7].

The biomass was a microalgae-bacteria consortium. The microalgae species within the consortium included *Scenedesmus obliquus* (39%), *Scenedesmus lagerheimii* (33%), *Scenedesmus opoliensis* (13%), *Scenedesmus magnus* (4%), and others in lesser amounts. The biomass was supplied by the Cajamar Foundation (Almeria, Spain) and freeze-dried and refrigerated at 4ºC prior to use. The identification and quantification measurements of the microalgae species were performed by microscopic examination (OLYMPUS IX70) for at least three different samples using a counting chamber according to Sournia [29]. Biomass samples were fixed with lugol acid at 5% and stored at 4 °C prior to analysis.

The chemical composition of the microalgae-based biomass (93.68% of the total solids) was 5.12% lipids, 44.03% proteins, 31.16% carbohydrates, and 14.33% ash content, all on a dry basis. The analytical procedure for biomass characterisation is explained in the Section 2.4.

### *2.2. Supercritical $CO_2$ extraction*

Figure 1 displays the general diagram of the experiments carried out in this research. First, SC-$CO_2$ extraction was performed using high-pressure extraction equipment (HPEP, NOVA, Swiss, Efferikon, Switzerland) constructed for work at laboratory scale. As was previously explained in the work of Vladić et al. [30], the main components of the plant

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included a gas cylinder supplied with $CO_2$, a diaphragm type compressor (with a pressure range up to 1000 bar), an extractor with a heating jacket (containing an internal volume of 200 mL and a maximum operating pressure of 700 bar), a separator with a heating jacket (containing an internal volume of 200 mL and a maximum operating pressure of 250 bar), pressure control valves, a temperature regulation system, and a handling system for taking samples.

The raw biomass - RB - (17.0 g of initial freeze-dried microalgae-based biomass) was placed in the extractor vessel, and the extraction step was carried out applying different operation parameters to study the yield dynamics and kinetics of the SC-$CO_2$ extraction. Based on previous results and economic considerations, experiments were carried out at pressures of 100, 200, and 300 bar and temperatures of 40 and 60°C. $CO_2$ flow (0.194kg/h) and all other SC-$CO_2$ extraction parameters were held constant [30], [31]. Previous research evidenced thermal degradation of valuable compounds working with temperatures higher than 60ºC, and co-extraction of waxes at pressures greater than 300 bar. The experiments were performed in replicate for each sample.

Samples of the exhausted solid fraction and the extracted semi-solid fraction were weighed after SC-$CO_2$ extraction and the total solids were measured to verify the mass balances. The total SC-$CO_2$ extraction yields, SC-$CO_2$ Y, were calculated as grams of the SC-$CO_2$ extracted semi-solid fraction per 100 g of initial dry raw biomass (g/100 g). In order to evaluate the co-extraction of different components, carbohydrates, proteins, and lipids were quantified in both fractions after SC-$CO_2$ extraction, and SC-$CO_2$ extraction yields for each component were calculated using Equation (1):

$$\text{Component SC} - \text{CO}_2 \text{ extraction yield} = \frac{\text{g component in the SC}-\text{CO}_2 \text{ semi}-\text{solid extracted fraction}}{\text{g component in initial dry microalgae}-\text{based biomass}} \cdot 100 \quad \text{Eq. (1)}$$

where component refers to carbohydrates, proteins, and lipids.

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Additionally, scanning electron microscopy analysis was carried out in these SC-$CO_2$ exhausted solid fractions.

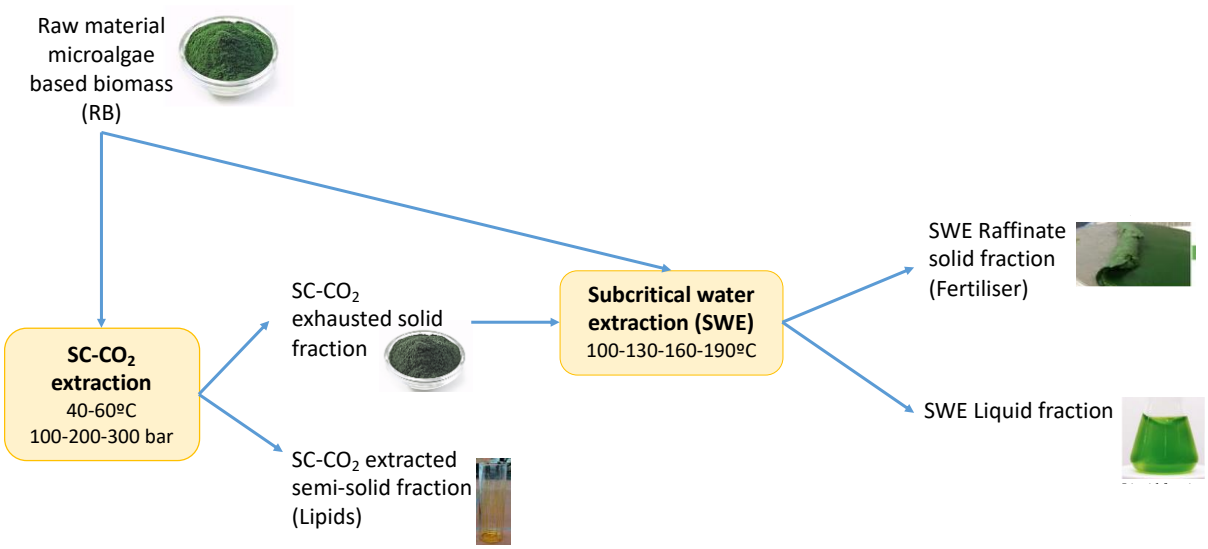


**Figure 1.** Global Scheme of Experiments of Sequential Valorisation of Microalgae-Bacteria Biomass carried out in this work. RB: raw material microalgae-based biomass; SC-$CO_2$ extracted semi-solid fraction: extracted fraction with the lipid content; SC-$CO_2$ exhausted solid fraction: solid fraction used for the SWE; SWE Raffinate solid fraction: solid fraction after SWE; SWE Liquid fraction: liquid fraction after SWE.

The cumulative extracted biomass was plotted against the time of the experiment. The supercritical fluid extraction step was modelled using Modified Brunner's equation (Eq. (2)) [31]. Moreover, the model parameters were calculated by minimising the least square difference between observed and predicted values.

$$SC-CO_2\ Y = SC-CO_2\ Y_0 \cdot (1 - e^{-k \cdot t}) \quad \text{(Eq. 2)}$$

where SC-$CO_2$ Y represents the total SC-$CO_2$ extraction yield (%), SC-$CO_2$ $Y_0$ is the total potential SC-$CO_2$ extraction yield at infinite extraction time and is specific for each pressure and temperature conditions, k is the rate coefficient ($min^{-1}$) and t is the extraction time (min).

*2.3. Subcritical water extraction*

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Subcritical water extraction was performed with the initial raw freeze-dried microalgae-based biomass and the exhausted solid fraction from SC-$CO_2$ extraction at the selected experimental conditions. Subcritical water extraction was performed in a batch-type high-pressure extractor with an internal volume of 450 mL and a maximum operating pressure of 200 bar and a maximum operating temperature of 350°C, connected with a temperature controller (4838, Parr Instrument Company, USA). The operational conditions were selected based on previous works including [32], [33], [34]. All extractions were performed with 10g of sample (the initial raw biomass or the exhausted solid fraction after SC-$CO_2$ extraction) and a biomass/water ratio of 1:10 (w/v). Experiments were performed at 100, 130, 160, and 190 °C, in which 10 minutes of extraction time for all the experiments was used. The reactor was magnetically stirred (1000 rpm) to increase the mass and heat transfer and to avoid overheating in the inner walls of reactor. After the SWE extraction time, the reactor was cooled in an ice bath for 10 minutes. The samples were immediately filtered with filter paper (4–12 μm pore size, Schleicher and Schuell, Germany) under vacuum (V-700, Büchi, Switzerland). The experiments were performed in replicate for each sample.

After the subcritical water extraction experiments, the raffinate solid fraction and the liquid fraction were weighed, and the total solids were measured to check the mass balances. Samples of both fractions were stored at 4 °C until analysis.

Carbohydrate, protein, and lipid contents were measured in the raffinate solid fractions. The possible use of these solid fractions as a fertiliser was evaluated, analysing the elements (C, N, S, P), amino acids, and heavy metals (Al, As, Ca, Cr, Cu, Fe, K, Mg, Mn, Pb, Zn, Hg). Moreover, scanning electron microscopy analysis was carried out to determine the structural changes after the subcritical water extraction. Monosaccharides, proteins, and the probable by-products were analysed in the SWE liquid fractions.

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Retention, solubilisation and recovery yields in the subcritical water step were quantified using Equations 3 and 4 for the SWE experiments using RB and Equations 5 and 6 for the SWE experiments using the SC-$CO_2$ exhausted solid fraction. The solubilisation yields and the degradation were calculated using Equations 7 and 8 for all the SWE experiments:

$$\text{SWE retained component yield} = \frac{\text{g component in the SWE raffinate solid fraction}}{\text{g component in the RB}} \cdot 100 \qquad \text{Eq. (3)}$$

$$\text{SWE component recovery yield} = \frac{\text{g component in the SWE liquid fraction}}{\text{g component in the RB}} \cdot 100 \qquad \text{Eq. (4)}$$

$$\text{SWE retained component yield} = \frac{\text{g component in the SWE raffinate solid fraction}}{\text{g component in the SC}-\text{CO2 exhausted solid fraction}} \cdot 100 \qquad \text{Eq. (5)}$$

$$\text{SWE component recovery yield} = \frac{\text{g component in the SWE liquid fraction}}{\text{g component in the SC}-\text{CO2 exhausted solid fraction}} \cdot 100 \qquad \text{Eq. (6)}$$

$$\text{SWE solubilised component yield} = 100\text{-SWE retained component yield} \qquad \text{Eq (7)}$$

$$\text{SWE component degradation} = \text{SWE solubilised component yield} - \text{SWE component recovery yield} \qquad \text{Eq (8)}$$

where components refer to carbohydrates (as monosaccharides in the SWE liquid fraction), proteins, and lipids.

*2.4.Analytical methods*

The mass balance was estimated using the total solids analysed according to the NREL protocols [35]. The lipid content was determined in the solid fractions using a modified protocol based on a chloroform-methanol 2:1 extraction applying the Kochert method [36] and the protein content was calculated by multiplying the Kjeldahl Total Nitrogen by a factor of 5.95 [37].

The carbohydrate content was determined as total monosaccharides in the raw material, solid fraction, and liquid fraction by using an NREL procedure [38]. The biomass

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samples (300 mg dry biomass) were subjected to a concentrated acid hydrolysis for 1 h by adding 3 mL of 72% w/w $H_2SO_4$ at 30 ºC. Then, 84 mL of deionised water was added to dilute the acid concentration to 4% w/w and the samples were autoclaved at 121 ºC for 1 h. Then, the solid and liquid fractions were separated by filtration and the resulting liquid fraction was stored at 4 ºC in order to determine the total carbohydrate content using an HPLC-RI.

A Bio-Rad HPX-87H ion-exclusion column installed in a Waters e2695 separation module was used for the quantification of the monosaccharide content and possible by-products in the liquid fractions. A refractive index detector (Waters 2414) was used to determine the monosaccharides and by-products, such as methanol, xylitol, glycerol, ethanol and acetone. Other by-products (oxalic, formic, acetic, lactic, butyric, succinic, levulinic acid, furfural, and HMF) were measured with a photodiode detector (Waters 2998) at 210nm [39]. An aqueous solution of 0.025 M $H_2SO_4$ was eluted at a flow rate of 0.6 mL/min and 50ºC. The external calibration method was used for quantification. Multi-standard calibration solutions were prepared by adequate dilution of individual standards commercially available with a purity >95% (Sigma Aldrich, Spain). All the analyses were carried out in duplicate for each experiment.

The determination of the carbon and nitrogen contents on the exhausted solids was performed using a LECO CHNS932 analyser, while phosphorus, sulphur, and all the heavy metal analyses were carried out spectrophotometrically after acid digestion in a microwave according to the internal protocol of the Laboratory of Instrumental Analysis (University of Valladolid). The amino acid content was measured by HPLC-UV after acid digestion in a microwave. A Zorbax Eclipse (AAA 4.6x150 mm 3.5 micron) column was used coupled with

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a derivatisation pre-column of OPA and FMOC using as the mobile phase: buffer $NaH_2PO_4 \cdot H_2O$ (pH 7.8) and acetonitrile:methanol:milliQ water (45:45:10).

*2.5. Scanning Electron Microscopy*

Scanning Electron Microscopy (SEM) was performed according to internal procedures, for magnifications from 1000 to 5000 [16].

## 3. Results and discussion

*3.1. Supercritical $CO_2$ extraction and kinetics*

Supercritical $CO_2$ extraction solubilised only the lipid components from the raw biomass. The global mass balance was equal to the individual mass balance of lipids. This fact demonstrated the unique but incomplete extraction of the lipid components during the supercritical carbon dioxide step. There was no variation of the carbohydrate and protein quantities in the exhausted solid fraction after SC-$CO_2$ extraction with respect to the untreated biomass, which also corroborated the result of the lipid extraction [19].

Figure 2 displays the total SC-$CO_2$ extraction yield calculated with respect to the initial amount of microalgae inside the supercritical reactor, at different time experiments. The total SC-$CO_2$ extraction yields obtained at 150 min ranged from 0.41 to 3.84 g/100g algae, corresponding to 8 and 75% of SC-$CO_2$ lipid extraction yields, respectively. These values were in agreement with the experiment done by Safi et al. [40] who obtained a lipid extraction yield of 69% using *Chlorella vulgaris* at 600 bar and 60ºC, and the experiment

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done by Nobre et al. [41] who obtained a lipid extraction yield of 78% using *Nannochloropsis* sp. at 300 bar and 40ºC. However, Beaufils et al. [42] found both low and very constant lipid extraction yields when applying supercritical carbon dioxide (150 min, 50ºC, 1.5kg $CO_2$/h) to *Nannochloropsis oculate* with a high lipid content (40%). They reported lipid extraction yields of 12.5% at 250 bar and 16% at 450 and 750 bar, but using a biomass grown under conditions of nitrogen depletion to induce lipids accumulation.

The temperature parameter was closely related to the pressure parameter. At low pressure (100 bar), a slight deviation was observed among the two studied temperatures (40 and 60ºC). Bahadar et al. [18] reported the same effect but in a different pressure range on *Chlorella vulgaris* (18% of lipid content). At 275 and 344 bar, the temperatures provided no variation or even a decrement on the lipid extraction yields from 72 and 77% at 40ºC to 66 and 77% at 60ºC, respectively.

Nevertheless, the temperature had a remarkable effect on the SC-$CO_2$ lipid extraction yield during those experiments that used 200 and 300 bars of pressure. The lipid extraction yields for the experiments at 150 minutes increased from 30% at 40ºC to 66% at 60ºC for 200 bar; and from 50% at 40ºC to 75% at 60ºC for 300 bar. Taher et al. [43] noted a similar result in an experiment with *Scenedesmus* sp., which was attributed to the crossover phenomenon of $CO_2$ solvation and lipid volatility; $CO_2$ density decreases when temperature increases while the volatility increases at the same time. Bahadar et al. [18] also reported the same enhancement of lipid extraction yields from 50% at 40ºC to 100% at 60ºC but at 620 bars and 180 min. The trend detected in this study was the same as observed by Crampon et al. [19] using *Spirulina platensis* with 14% of lipid content. They also reported no variation in lipid extraction yields (28%) at 100 bar for both temperatures (40 and 60ºC) at 150 min. However,

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they found a two-fold increase in lipid extraction yields at 200 bar with 28% at 40ºC and 55% at 60ºC at 180 min.

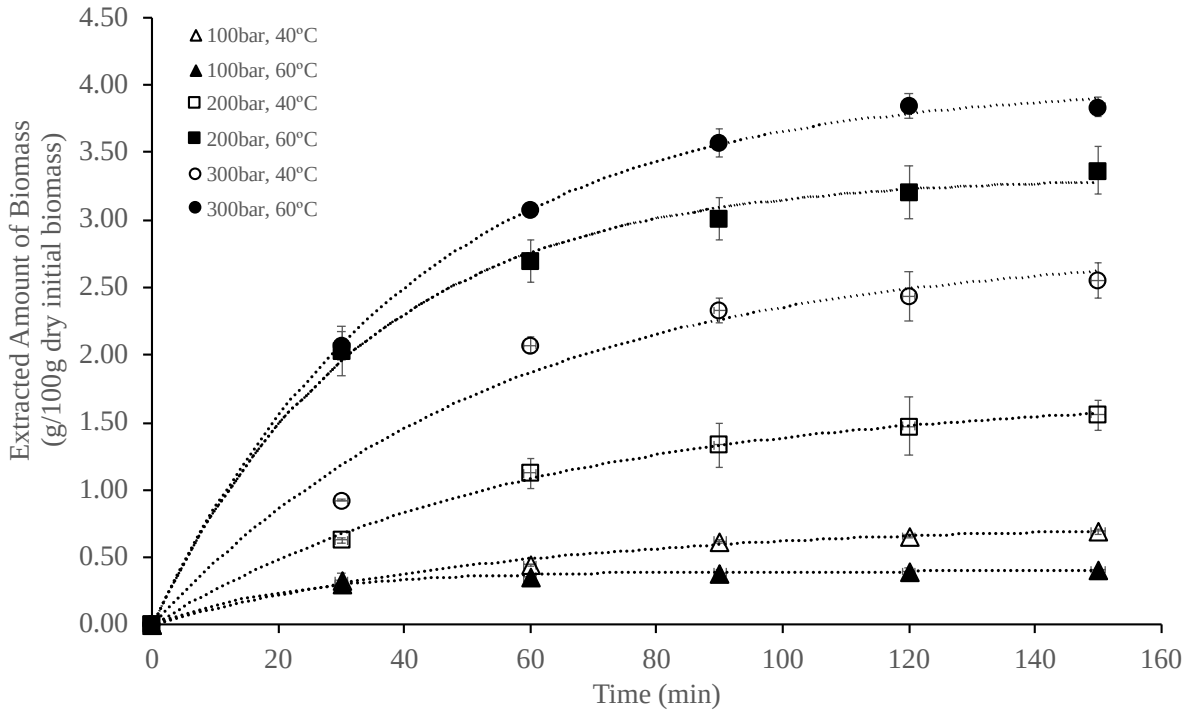


**Figure 2.** Total extracted amount of biomass (100% lipids) with respect to the initial dry amount of microalgae-based biomass (g/100g) by supercritical carbon dioxide extraction. The results are expressed as means ± standard deviations of 2 replicate experiments analysed in duplicate (4 experimental results). The vertical interval lines represent standard deviation of the means. Discontinuous lines represent the fitted model by Modified Brunner's Equation using the parameters in Table 1.

The influence of pressure on the SC-$CO_2$ extraction had divergences at the two studied temperatures. For 40ºC, the increment from 100 to 200 bar doubled the lipid extraction yield while an increase up to 300 bars led to a 39% improvement which corresponded with a lipid extraction yield of 50% at 150 minutes. This increase on the SC-$CO_2$ lipid extraction yield with pressure was expected because of the increase in solvent density and solvation power

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with pressure [43]. Nevertheless, these results suggest a scarce enhancement of lipid extraction yields with pressure for those higher than 300 bar. The study by Bahadar et al. [18] did not show a significant increase on the extraction yield at 40ºC when the pressure was increased from 275 to 655 bar during the SC-$CO_2$ extraction on *Chlorella vulgaris*.

In contrast, the effect of the pressure was highly pronounced when increased from 100 to 200 bars at 60ºC. The SC-$CO_2$ lipid extraction yield at 60ºC and 150 minutes increased from 8% at 100 bar to 66% at 200 bar. Afterwards, the increase on pressure to 300 bar resulted in a slight enhancement of the lipid extraction (75% at 150 minutes).

Contrary to this behaviour, Crampon et al. [19] observed a proportional improvement on the lipid extraction yield at 60ºC when the pressure increased during the SC-$CO_2$ extraction of *Spirulina platensis* (14% of initial lipids content). They obtained SC-$CO_2$ lipid extraction yields of 28, 57, and 92% at 100, 200, and 300 bars for 180 min, respectively.

To determine the kinetic of lipid extraction from microalgae-based biomass, the effect of time was evaluated from 0 to 150 minutes for all the temperature and pressure conditions. Generally, a prominent increase was observed at 30 min while only experiments at 200 and 300 bar showed a further increase. After 120 min, all the values remained almost constant. Zinnai et al. [44] also detected this significant increase (50%) in the first minutes on the lipid extraction from *Schizohytrium sp*. at 250 bar and 40ºC; but in this case the lipid extraction continued to increase up to 150 min. Tang et al. [45] also reported a significant lipid extraction yield of 25% at 30 min, 40ºC, and 350 bar using the microalgae powder *Schizohytrium*, but without remarkable improvement at higher times, reaching lipid extraction yields of 30, 32, and 33.5% at 60, 90, and 120 min.

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All of the total SC-$CO_2$ extraction yields obtained in this study were fitted to a kinetic model according to Modified Brunner's equation (Eq. 2) with $R^2 > 0.995$ (Figure 2). The fitting parameters are shown in Table 1.

Table 1: Kinetic model parameters of Modified Brunner's Equation for experiments of SC-$CO_2$ extraction from microalgae biomass grown in pig manure

| Extraction pressure and temperature | SC-$CO_2$ $Y_0$ (%) | k ($min^{-1}$) |
|---|---|---|
| 100 bar, 40ºC | 0.751 | 0.018 |
| 100 bar, 60ºC | 0.400 | 0.046 |
| 200 bar, 40ºC | 1.706 | 0.018 |
| 200 bar, 60ºC | 3.322 | 0.030 |
| 300 bar, 40ºC | 2.801 | 0.018 |
| 300 bar, 60ºC | 4.000 | 0.024 |

From these results, the maximum possible SC-$CO_2$ lipid extraction yield at the range of pressure and temperature of this study is 78% (300 bar, 60ºC). The total SC-$CO_2$ extraction yields obtained at 60ºC and 150 minutes were very close to the SC-$CO_2$ $Y_0$ values calculated from Equation 2. However, the total SC-$CO_2$ extraction yield achieved only 90% of the total potential SC-$CO_2$ extraction yields in experiments at 40ºC.

Identical values of the rate coefficients (0.018 $min^{-1}$) were obtained by fitting all the experiments at 40ºC independently of the applied pressure. However, the SC-$CO_2$ $Y_0$ values rose from 0.75 to 2.8 when the pressure increased from 100 to 300 bars. Therefore, the increase of pressure improved the extraction yield without effecting the kinetic of the extraction. Regarding the kinetic results at 60ºC, the rate coefficient decreased with the pressure, from 0.046 $min^{-1}$ at 100 bar to 0.024$min^{-1}$ at 300 bar. The increase on the total potential SC-$CO_2$ extraction yield with pressure at 60ºC was even higher than at 40ºC, from values as low as 0.4 at 100 bar, to 4.0 at 300 bar, both at 60ºC. Zinnai et al. [44] applied the Modified Brunner's equation to fit the results of SC-$CO_2$ extraction from the marine

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microalgae *Schizochytrium* sp, working in a wide range of pressures (250 to 700 bar) and temperatures (40 and 55ºC) for 4 hours of extraction time. In these experiments, the pressure had a clear and positive influence on the rate coefficients (the coefficients increased 5 times at 40ºC and 12 times at 55ºC). This is most likely related to the high accumulation of long chain lipids which is characteristic of this microalgae species.

Finally, the SEM analysis showed the possible cell wall breakthrough of the investigated microalgae material after the supercritical carbon dioxide extraction. Comparing the photos before and after 150 minutes of SC-$CO_2$ extraction, for all the conditions, there was no noticeable differences between them. These results seem indicate that the extraction method applied does not appreciably break the cell wall when using these conditions and this type of very robust biomass grown in wastewater treatment plants. In the case of the Bahadar et al. [18] study, the SEM analysis showed ruptured oil pouches of *Chlorella vulgaris* algae after supercritical carbon dioxide extraction applied in much more severe conditions (620 bar and 80ºC, 180 minutes).

### *3.2. Subcritical water extraction results*

To achieve a sequential recovery of the other macromolecular components (carbohydrates and proteins) of microalgae-based biomass, subcritical water extraction was applied to the exhausted solid fraction remaining from the selected SC-$CO_2$ experiment (300 bar, 60ºC, 150min). 96.16 g of SC-$CO_2$ exhausted solid fraction with a composition of 32.5% carbohydrates, 45.7% proteins, and 1.3% lipids were obtained from 100 g of dry raw biomass. Moreover, SWE was also carried out with the initial raw material biomass in order to determine the feasibility of the previous application of supercritical carbon dioxide extraction (Figure 1).

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Table 2 shows the mass of the SWE raffinate solid fraction after SWE experiments, on the basis of 100 g of the initial dry raw microalgae-based biomass for both substrates (the initial raw material and the SC-$CO_2$ exhausted solid fractions). As expected, the total solubilisation increased with the extraction temperature. Despite no clear disruption of the cell wall found in the SEM analysis of the SC-$CO_2$ exhausted solid fraction extraction, the results show a remarkable effect that the previous SC-$CO_2$ extraction step had on the solubilisation of the biomass by subcritical water extraction.

Figure 3 shows the retained carbohydrate, protein, and lipid yields in the SWE raffinate solid fractions. The solubilised amounts of carbohydrates and proteins were remarkably higher for SWE of the SC-$CO_2$ exhausted solid fractions than for SWE of the raw biomass for all the studied operation conditions. Subcritical water extraction solubilised 41-57 % of the lipids from the raw biomass, but no solubilisation of lipids was detected by SWE of the exhausted solid fractions after SC-$CO_2$ extraction (Table 2).

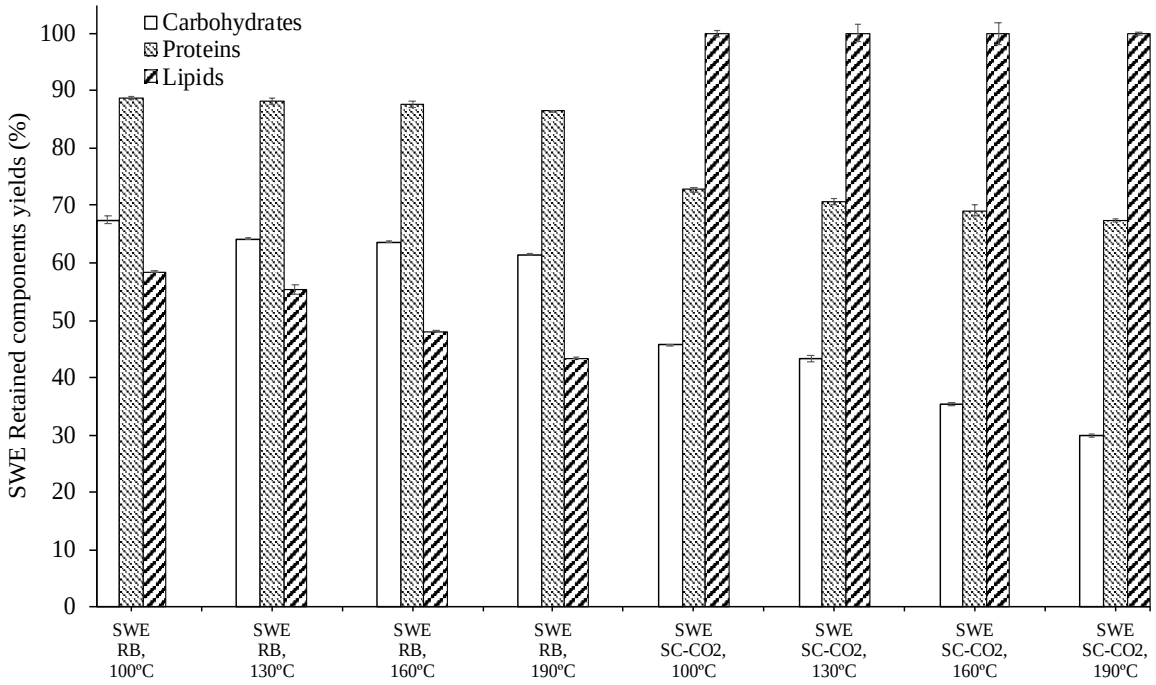

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**Figure 3.** Retained component yields (%) in the raffinate solid fractions after the subcritical water extraction at different temperatures with respect to the components in the raw biomass (RB) and in the SC-$CO_2$ exhausted solid fraction (300 bar, 60ºC, 150 minutes). The results are expressed as means ± standard deviations of 2 replicate experiments analysed in duplicate (4 experimental results). The vertical interval lines represent standard deviation of the means.

As shown in Table 2, the carbohydrate solubilisation yield was remarkably affected by the temperature of subcritical water extraction for the SC-$CO_2$ exhausted solid samples, increasing from 54% at 100ºC to 70% at 190ºC. However, the SWE temperature effect resulted in very low carbohydrate solubilisation yields from the initial raw material (from 32% at 100ºC to 38% at 190ºC). Fu et al. [46] also observed the limited impact of the temperature on carbohydrate solubilisation by SWE working at 120 and 200ºC with raw *Chlorella pyrenoidosa*.

Table 2: Masses (g) of dry SWE raffinate solid fraction; carbohydrates, proteins and lipids solubilised by SWE and monosaccharides and proteins in the SWE liquid fraction by 100 g of dry initial microalgae-based biomass (96.16 g of SC-$CO_2$ exhausted solid fraction).

| SWE Substrate[b] | RB | RB | RB | RB | SC-$CO_2$ | SC-$CO_2$ | SC-$CO_2$ | SC-$CO_2$ |
|---|---|---|---|---|---|---|---|---|
| SWE Temperature | 100ºC | 130ºC | 160ºC | 190ºC | 100ºC | 130ºC | 160ºC | 190ºC |
| SWE raffinate solid fraction | 87.89± 0.01 | 84.96 ± 0.04 | 82.37 ± 0.00 | 81.43 ± 0.00 | 71.30 ± 0.01 | 69.50 ± 0.09 | 66.08 ± 0.12 | 63.57± 0.03 |
| Carbohydrates solubilised by SWE | 10.12 ± 0.68 | 11.14 ± 0.21 | 11.33 ± 0.16 | 12.00 ± 0.09 | 16.84 ± 0.01 | 17.63 ± 0.57 | 20.13 ± 0.34 | 21.80 ± 0.29 |
| Proteins solubilised by SWE | 4.93 ± 0.35 | 5.19 ± 0.45 | 5.47 ± 0.61 | 5.95 ± 0.08 | 12.04 ± 0.37 | 12.96 ± 0.52 | 13.64 ± 0.88 | 14.36 ± 0.27 |
| Lipids solubilised by SWE | 2.13 ± 0.11 | 2.29 ± 0.79 | 2.66 ± 0.02 | 2.90 ± 0.24 | n.d.[c] | n.d [c] | n.d [c] | n.d.[c] |
| Monosaccharides in SWE liquid fraction | 8.16 ± 0.34 | 9.08 ± 0.19 | 9.19 ± 0.00 | 9.73 ± 0.15 | 15.23 ± 0.15 | 15.67 ± 0.59 | 17.51 ± 0.23 | 18.73± 0.02 |
| Proteins in SWE liquid fraction | 2.32 ± 0.04 | 2.34 ± 0.02 | 2.51 ± 0.12 | 2.67 ± 0.09 | 5.47 ± 0.14 | 5.71 ± 0.27 | 5.87 ± 0.42 | 5.99 ± 0.17 |

[a]: The results are expressed as means ± standard deviations of 4 experimental results (replicated experiments analysed in duplicate).
[b]: RB: raw microalgae biomass grown in pig manure; SC-$CO_2$: exhausted solid fraction after supercritical $CO_2$ extraction at 300 bar, 60ºC, 150 minutes of microalgae biomass grown in pig manure
[c] non detectable.

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Subcritical water extraction solubilised lower amounts of proteins than carbohydrates in all the experiments (Table 2). The SWE retained protein yields was slightly dependent on the temperature of the subcritical extraction for the raw biomass, with values of SWE retained protein yields higher than 87% for all the experiments using the raw biomass. However, values of solubilisation of proteins ranging from 27% at 100ºC to 33% at 190ºC were obtained by SWE from the SC-$CO_2$ exhausted solid fractions. Phusunti et al. [25] also reported no influence of the temperature of SWE on the protein solubilisation from *Chlorella vulgaris* working at 150, 180, and 200ºC. However, they obtained higher protein solubilisation than those obtained in this work (around 66% for extraction at 90 min and 42% at 180 min). A similar effect of temperature on protein solubilisation by SWE was reported by Fu et al. [46]. The low protein solubilisations in this work could be related to the high resistance of the cell wall in the microalgae species grown in pig manure treatment plants (mainly *Scenedesmus*, in this case).

The SWE lipid solubilisation yield increased with the extraction temperature for the raw biomass. Other authors such as Reddy et al. [47] obtained lower values of lipid solubilisation by SWE (from 20 to 38%) using the raw biomass of *Nannochloropsis Salina* at 160 and 190ºC, respectively.

Monosaccharides, proteins, and possible by-products were analysed in the SWE liquid fractions to quantify the degradation and the monosaccharide recovery yields during the subcritical water extraction. The amount of recovered monosaccharides and proteins in the SWE liquid fractions calculated on the basis of 100 g of the initial dry raw biomass are shown in Table 2. SWE carbohydrate degradation remained independent of temperature for the raw

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biomass, with values close to 7%. As result, the SWE monosaccharide recovery yields were around 29% for the raw biomass, increasing slightly with the temperature.

The previous SC-$CO_2$ extraction improved the SWE monosaccharide recovery yields, with values increasing with temperature from 49% at 100ºC to 60% at 190ºC. Again, SWE carbohydrate degradation was scarcely dependent on temperature with values ranging from 5% at 100ºC to 10% at 190ºC. Awaluddin et al. [24] reported a negative effect of SWE temperature on the monosaccharide recovery yields, but in their work, they achieved a SWE monosaccharide recovery yield of 30% when working with *Chlorella vulgaris* at temperatures as high as 277ºC for 5 min. The percentage of SWE protein degradation was around 7% for SWE of the raw biomass and ranged from 15% at 100ºC to 19% at 190ºC for SWE experiments with the SC-$CO_2$ exhausted solid fraction.

Table 3 shows the concentration of the analysed by-products in the SWE liquid fractions. The concentration of most of the by-products increased with temperature, but succinic and lactic acids remained almost constant. The main by-products at 100ºC were formic acid (24%), oxalic acid (22%), acetic acid (21%), citric acid (14%), and malic acid (10%).

Table 3: Total and individual concentrations of by-products (g/L) in the liquid fractions-extracts obtained after subcritical water extraction (SWE)[a].

| SWE Substrate[b] | RB | RB | RB | RB | SC-$CO_2$ | SC-$CO_2$ | SC-$CO_2$ | SC-$CO_2$ |
|---|---|---|---|---|---|---|---|---|
| SWE Temperature | 100ºC | 130ºC | 160ºC | 190ºC | 100ºC | 130ºC | 160ºC | 190ºC |
| Oxalic acid | 0.09 ± 0.00 | 0.06 ± 0.00 | 0.10 ± 0.00 | 0.09 ± 0.00 | 0.05 ± 0.00 | 0.02 ± 0.00 | 0.03 ± 0.00 | 0.01 ± 0.00 |
| Citric acid | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.01 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Malic acid | 0.15 ± 0.01 | 0.12 ± 0.01 | 0.22 ± 0.01 | 0.23 ± 0.01 | 0.07 ± 0.00 | 0.05 ± 0.00 | 0.11 ± 0.00 | 0.10 ± 0.01 |
| Succinic acid | 0.16 ± 0.01 | 0.42 ± 0.00 | 0.19 ± 0.01 | 0.18 ± 0.01 | 0.12 ± 0.00 | 0.12 ± 0.00 | 0.16 ± 0.00 | 0.14 ± 0.00 |
| Lactic acid | 0.14 ± 0.01 | 0.24 ± 0.01 | 0.14 ± 0.00 | 0.15 ± 0.00 | 0.14 ± 0.00 | 0.10 ± 0.00 | 0.10 ± 0.00 | 0.14 ± 0.02 |
| Formic acid | 0.16 ± 0.01 | 0.07 ± 0.00 | 0.15 ± 0.01 | 0.16 ± 0.01 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.17 ± 0.00 | 0.17 ± 0.01 |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Acetic acid | 0.31 ± 0.03 | 0.33 ± 0.02 | 0.34 ± 0.01 | 0.38 ± 0.01 | 0.51 ± 0.02 | 1.38 ± 0.03 | 0.48 ± 0.01 | 0.21 ± 0.00 |
| Levulinic acid | 0.18 ± 0.02 | 0.15 ± 0.00 | 0.32 ± 0.01 | 0.34 ± 0.02 | 0.07 ± 0.00 | 0.03 ± 0.00 | 0.56 ± 0.01 | 0.58 ± 0.00 |
| Butyric acid | 0.24 ± 0.01 | 0.15 ± 0.01 | 0.14 ± 0.01 | 0.14 ± 0.01 | 0.03 ± 0.00 | 0.00 ± 0.00 | 0.25 ± 0.01 | 0.72 ± 0.02 |
| HMF | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.04 ± 0.00 | 0.01 ± 0.00 | 0.03 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 |
| Total | 1.46 ± 0.04 | 1.57 ± 0.01 | 1.64 ± 0.06 | 1.74 ± 0.05 | 1.02 ± 0.03 | 1.64 ± 0.03 | 1.97 ± 0.03 | 2.08± 0.04 |

[a]: The results are expressed as means ± standard deviations of 4 experimental results (replicated experiments analysed in duplicate).
[b]: RB: raw microalgae biomass grown in pig manure; SC-$CO_2$: exhausted solid fraction after supercritical $CO_2$ extraction at 300 bar, 60ºC, 150 minutes of microalgae biomass grown in pig manure.

Finally, the SEM analysis clearly showed the breakthrough of microalgae cell walls after the subcritical water extraction but was more aggressive in the samples from the SC-$CO_2$ exhausted solid fractions where large cuts of cells were observed. This shows that the most efficient cell wall destruction was achieved with sequential exposure to supercritical and subcritical extractions, rather than by subcritical alone. A similar effect was observed by Fu et al. [46] when applying a thermal pretreatment before subcritical water extraction. Awaluddin et al. [24] also analysed the rupture of the cell of *Chlorella vulgaris* after SWE by SEM analysis. They found that the thermal pretreatment agglomerated the cells, and a further subcritical water extraction step had a segregation effect on these agglomerated cells.

### *3.3. Fertiliser analysis*

The possible application of the SWE raffinate solid fractions as fertiliser was evaluated due to their high protein concentration. Table 4 shows the composition of C, N, P, and the mineral compounds of interest in agriculture. As expected, from the subcritical extraction results, the nitrogen content decreased slightly with respect to the raw material by protein solubilisation, resulting in an NPK ratio in the range of 7.3 to 7.7%. The NPK values of all the SWE raffinate solid fractions exceeded the minimum EU legal threshold value of 7% (w/w), which would be acceptable for its use as an organic fertiliser. Similarly, the C/N ratio of the SWE raffinate solid fractions was barely lower than that of the raw material because of the

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solubilisation of the components; but it ranged from 6.7 to 7.6, values far below the maximum allowed ratio of 15. The As content of all the analysed samples was lower than 14 mg/kg, far below the maximum permitted of 50 mg/kg. Supplementation of Cu, Fe, and Mn would be required to use these materials as fertilisers for extensive and grazing cultivation, while no addition would be necessary for fertigation or horticultural use, as well as for foliar application [48].

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Table 4: Main components for the characterisation as a fertiliser of the raw biomass and the raffinate solid fractions after the subcritical water extraction

| Samples[a] | Al[b] | As[c] | C[b] | Ca[b] | Cr[c] | Cu[b] | Fe[b] | Hg[b] | K[b] | Mg[b] | Mn[b] | N[b] | P[b] | Pb[c] | S[b] | Zn[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RB | 0.11 | <14 | 45.12 | 4.38 | <14 | 0.01 | 0.06 | <0.00 | 0.98 | 0.51 | 0.01 | 6.38 | 0.38 | <14 | 0.66 | 0.05 |
| SWE_RB_100ºC | 0.06 | <14 | 42.9 | 2.31 | <14 | 0 | 0.05 | <0.00 | 0.94 | 0.42 | 0.01 | 5.67 | 0.27 | <14 | 0.57 | 0.04 |
| SWE_RB_130ºC | 0.05 | <14 | 42.93 | 2.3 | <14 | 0 | 0.05 | <0.00 | 0.94 | 0.41 | 0.01 | 5.84 | 0.28 | <14 | 0.58 | 0.04 |
| SWE_RB_160ºC | 0.05 | <14 | 42.29 | 2.21 | <14 | 0 | 0.05 | <0.00 | 0.93 | 0.41 | 0.01 | 5.82 | 0.27 | <14 | 0.57 | 0.04 |
| SWE_RB_190ºC | 0.05 | <14 | 42.15 | 2.17 | <14 | 0 | 0.05 | <0.00 | 0.92 | 0.40 | 0.01 | 5.56 | 0.27 | <14 | 0.56 | 0.04 |
| SWE_SC-$CO_2$_100ºC | 0.05 | <14 | 41.71 | 2.27 | <14 | 0 | 0.05 | <0.00 | 0.93 | 0.40 | 0.01 | 5.84 | 0.27 | <14 | 0.57 | 0.04 |
| SWE_SC-$CO_2$_130ºC | 0.05 | <14 | 41.64 | 2.05 | <14 | 0 | 0.05 | <0.00 | 0.91 | 0.39 | 0.01 | 5.85 | 0.27 | <14 | 0.55 | 0.04 |
| SWE_SC-$CO_2$_160ºC | 0.05 | <14 | 40.42 | 2.06 | <14 | 0.01 | 0.05 | <0.00 | 0.92 | 0.41 | 0.01 | 5.56 | 0.37 | <14 | 0.52 | 0.04 |
| SWE_SC-$CO_2$_190ºC | 0.06 | <14 | 39.78 | 2.24 | <14 | 0 | 0.05 | <0.00 | 0.92 | 0.42 | 0.01 | 5.90 | 0.28 | <14 | 0.55 | 0.04 |

[a]RB: raw material. SWE_RB: raffinate solid fraction after subcritical water extraction of initial raw biomass; SWE_SC-$CO_2$: raffinate solid fraction after subcritical water extraction of the exhausted solid fraction from SC-$CO_2$ extraction at 300 bar, 60ºC, 150 minutes.
[b]Percentage in dry weight (g*100/ g dried)
[c]mg/kg dry weight

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In order to determine if these samples can be used as fertilisers, the amino acid content must also be determined and quantified (Figure 4). The main amino acids in all the samples were glutamic acid, aspartic acid, alanine, as well as the essential amino acids (leucine, valine, and arginine). Their respective composition in the raw biomass material was 14, 11, 9, 9, 7, and 7%. Moreover, serine, threonine, and arginine were the most solubilised amino acids by SWE which resulted in a lower amount of these amino acids in the SWE raffinate solid fractions. Thus, the raffinate solid fractions of microalgae biomass grown in pig manure after SWE have the potential to be used as fertilisers.

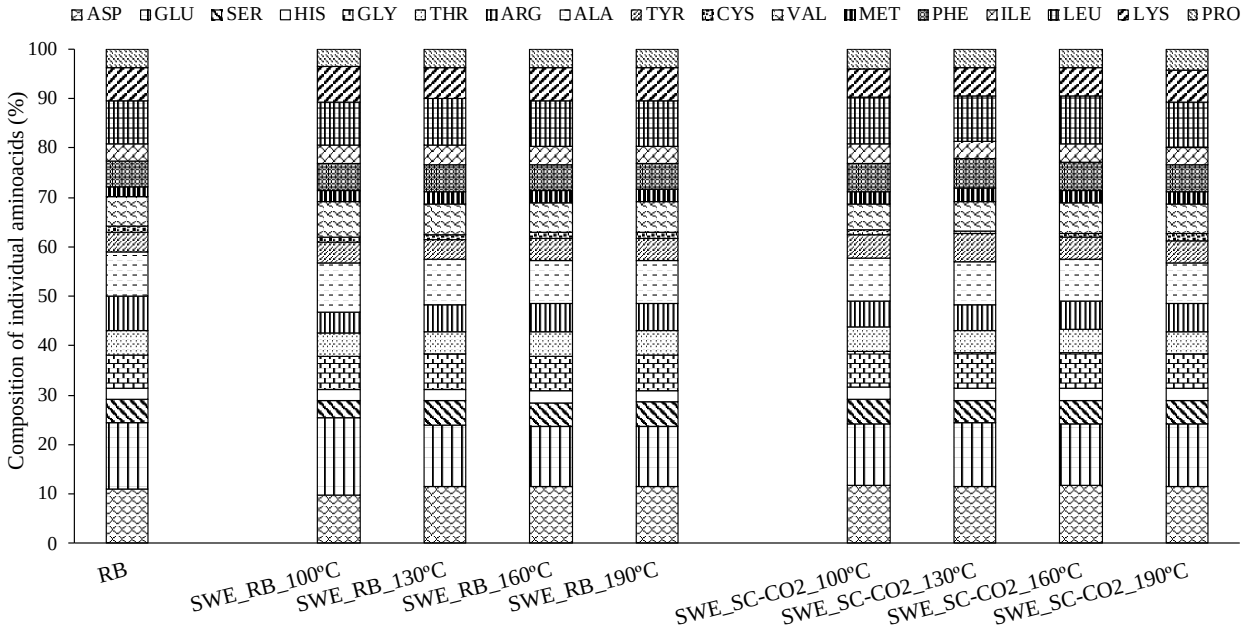


**Figure 4.** Composition of the proteins in the initial raw material, and in the raffinate solid fractions after the subcritical water extraction in terms of individual amino acids (%). RB: raw material. SWE_RB: raffinate solid fraction after subcritical water extraction of initial raw biomass; SWE_SC-$CO_2$: raffinate solid fraction after subcritical water extraction of the exhausted solid fraction from SC-$CO_2$ extraction.

## 4. Conclusions

Sequential extraction of the fraction accumulated by microalgae grown in pig manure was evaluated at laboratory scale with successful results. Initially, SC-$CO_2$ extraction accounted for 3.84 g of the extracted lipid per 100 g of the initial biomass (300 bars, 60ºC).

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Subcritical water extraction disrupted the cell wall, solubilising 70% of the carbohydrates and 33% of the proteins from the exhausted solid fractions after SC-$CO_2$ extraction and 38, 13, and 57% of carbohydrates, proteins, and lipids from the raw material at 190ºC for 10 min. The maximum monosaccharide recovery yield was achieved by SWE of the exhausted solid fraction after SC-$CO_2$ extraction with a value of 60% while only 32% of the SWE monosaccharide recovery yield was achieved from the raw biomass. In order to include quality aspects in the comparison of alternative processes, further research will address the characterisation of possible high value products in the obtained fractions. Finally, the composition of the raffinate solid fraction after subcritical water extraction – with NPK higher than 7% (w/w) and C/N lower than 15 – allows for its use as fertiliser.

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## Declaration of authors contributions

- Judit Martín Juárez: Collection and assembly of the data. Analysis and interpretation of the data. Drafting of the article.
- Jelena Vladic: Technical and logistic support. Acquisition of data.

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- Silvia Bolado Rodriguez: Conception and design. Critical revision and final approval of the article.
- Senka Vidovic: Conception and design. Analysis and interpretation of the data. Critical revision and final approval of the article.

## Declaration of competing interest

No conflicts, informed consent, or human or animal rights are applicable to this study.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version of the paper.

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