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Enhancement of the supercritical fluid extraction of grape seed oil by using enzymatically pre-treated seed

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ABSTRACT

The supercritical fluid extraction of grape seed (*Vitis vinifera* L.) oil using carbon dioxide has been carried out at constant temperature (313.15 K) and solvent flow rate ($1.7 \times 10^{-4} \text{ kg s}^{-1}$), at 160, 180 and 200 bar, using both untreated and enzymatically pre-treated seeds. The pre-treatment of triturated seeds has been performed with a cell wall degrading enzyme cocktail containing cellulase, protease, xylanase, and pectinase, in order to enlarge the broken/intact cells ratio, thus increasing oil availability. The maximum extraction yield obtained was 16.5%, which is 44% higher than the 11.5% yield obtained with untreated seeds.

The cumulative extraction curves measured show two characteristic periods: a first linear part where the majority of the oil is obtained, and a second asymptotic branch which contributes with only 3–8% to the total oil removed.

As pressure rises, the mass of CO₂ needed to reach a definite extraction yield decreases and the linear part of the extraction curves of treated and untreated seed approach themselves.

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1. Introduction

Grape seed (*Vitis vinifera* L.) is a well known oilseed crop containing typically 8–15% (w/w) of oil with recognised quality due to its high level of unsaturated fatty acids, namely oleic and linoleic [1] and antioxidant-rich compounds. In terms of applications, it is becoming increasingly popular for culinary, pharmaceutical, cosmetics, and medical purposes [2]. It is also an appealing product due to its large availability as a major by-product of wine industry [3,4]. Grapes give origin to approximately 25% (w/w) of dry pomace, of which one third is seed [5]. Hence, despite winemaking residues have been considered traditionally an economic and environmental problem, they are now becoming increasingly recognised as valuable commodities for the production of added value products.

Industrially, the process commonly adopted to obtain edible oil from vegetable seeds foresees several stages: clean up of the biomass, drying, crushing, and pressing. During pressing most of the oil is extracted from the seeds, but a considerable amount remains in the final cake. This is then extracted with *n*-hexane which is evaporated afterwards. Finally, if necessary, the oil has to be refined to fulfil the requirements for human consumption [6].

One obvious drawback of this process, that may inclusively dictate its future viability, is the utilization of *n*-hexane in last stages. In fact, even seeds coming from biological agriculture loose their biological status and, consequently, the extracted oil too. Supercritical (SC) carbon dioxide emerges as a suitable solvent to produce these oils.

The use of supercritical fluids (SCF) has been attracting widespread interest owing to their unique properties (e.g., liquid-like solvent power, negligible surface tension, and gaslike transport properties), versatile applications, and changes in environmental regulations which foster the utilization of green solvents. In this field, carbon dioxide has been especially adopted since it is essentially non-toxic, non-flammable, inexpensive, can be recycled, is totally dissipated from extracts at atmospheric pressure, and has easily accessible critical conditions [7,8].

The supercritical fluid extraction (SFE) of grape seed oil has proven to reach extraction yields equivalent to those achieved by conventional Soxhlet with *n*-hexane [3].

The semi-continuous SFE may be characterised by extraction curves, a plot of the accumulated amount of extract against time or, equivalently, against the amount of solvent passed through the extractor [4]. They usually comprise two extraction periods, a first one characterised by a rapid oil extraction followed by a period of slow to very slow additional oil yield. This represents a two-mechanism extraction process where a rapid extraction of sur-

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Nomenclature

D_{12}	tracer diffusion coefficient
FPR	forward pressure regulator
k_c	convective mass transfer coefficient
m	mass
\dot{m}	mass flow
MFM	mass flow meter
NV	needle valve
P	pressure
Re	Reynolds number
Sc	Schmidt number
Sh	Sherwood number
T	temperature
Y	oil solubility

Greek letters

η	extraction yield (% w/w)
μ	viscosity
ρ	density

face and shallow subsurface oil is followed by diffusion-controlled extraction of the more deeply embedded oil [9]. With respect to modelling, the sudden reduction of the extraction rate cited above was taken into account introducing the concept of broken and intact cells [10]. The first ones prevail close to the surface, where cell walls have been damaged by pre-treatment processes, while the particle core contains intact cells. Mass transfer resistance through cell walls is high, which justifies the large difference found between the transfer rates of both regions [4].

In this work, the extraction of grape seed oil has been carried out with supercritical CO₂. Moreover, the effect of an enzymatic pre-treatment of the seed upon the process yield and the cumulative curves has been also investigated. The pre-treatment has been accomplished using a cell wall degrading enzyme cocktail with pectinase, xylanase, protease, and cellulase activities. The choice upon the type of enzymes used was based on the knowledge that the oil removal can be favoured upon partial hydrolysis of the plant cell walls by means of appropriate enzymes [11], using the best operating conditions previously assessed [12].

2. Experimental

2.1. Materials and reagents

Seeds were collected from grapes (*Vitis vinifera* L.) of the red variety 'Touriga Nacional' harvested for red wine manufacture in Bairrada Appellation (Anadia, Portugal) during September 2007. Cellulase produced from *Aspergillus niger* (commercial code no. 22178), hemicellulase produced from *Thermomyces lanuginosus* (commercial code no. X2753), pectinase produced from *Aspergillus aculeatus* (commercial code no. P2611), and protease produced from porcine pancreas (commercial code no. 93614) have been purchased from Fluka Sigma–Aldrich Co. (St. Louis, MO). Other reagents were of analytical grade or higher available purity.

2.2. Seed preparation and enzymatic pre-treatment

2.2.1. Seed preparation, size reduction and screening

Seeds were collected during transfer of the musts in wine fermentation, and separated from pulp and skins by decantation and sieving. A first wash removed immature grains floating at water surface. Subsequently, the seeds were submitted to several washes

with water (200 g/L) under gentle stirring with a magnetic bar at 4 °C during a minimum of 3 days, with two water exchanges per day, until a minimum constant turbidity was observed. The purified seeds were finally washed with ethanol, air dried at room temperature, and stored at 4 °C until use. Finally, milling was carried out on a domestic coffee mill, and the particles were classified in a standard sifter with several mesh sizes. For the experiments performed, only particles with an average size of 0.75 were selected.

2.2.2. Enzymatic pre-treatment

Prior to the SFE of grape seed oil, an enzymatic pre-treatment was performed based on a previous research [12]. The grape seeds have been treated with a cocktail of cellulase = 29, protease = 1191, xylanase = 21, and pectinase = 569 U/g seed sample, which have been added to flasks containing 40 mL of distilled water + milled seed. The enzymatic suspension to seed ratio has been kept equal to 4 L/kg through all experiments. The pH 4 has been fixed with a buffer solution of citric acid and sodium hydrogenphosphate. The reaction proceeded isothermally at 40 °C under continuous stirring at 200 rpm during 24 h, and was stopped by freezing the suspension with liquid nitrogen. Then, the water was removed by freeze-drying the content of the flasks.

The above-mentioned operating conditions were fixed after analysing the effect of each parameter upon the enzymatic activity, in an attempt to increase grape seed oil availability [12]. Carrying out Soxhlet measurements it was shown that the extraction yield increases with both increasing enzymes concentration and treatment time, whereas pH and temperature give rise to opposite behaviours. GC-FID analysis were performed to evaluate oil content, and allowed the authors to confirm that the global increment observed was due to triacylglycerides only.

2.3. Supercritical fluid extraction

2.3.1. Equipment

The SFE experiments were carried out with carbon dioxide under semi-continuous operation in an apparatus built/assembled at the University of Aveiro. A simplified scheme of the equipment is given in Fig. 1. The extraction chamber is cylindrical, stainless steel, with $1.6 \times 10^{-4} \text{ m}^3$ (length $H=0.13 \text{ m}$, internal diameter i.d.=0.04 m). The CO₂ withdrawn from a container is firstly liquefied in a refrigerated bath, to approximately 265 K, and then pressurised by an air driven liquid pump to a high-pressure vessel. The pressure inside the extractor is regulated via the forward pressure regulator FPR1. The mass flow meter (MFM) measures the instantaneous flow rate and total quantity of solvent delivered. The solvent is brought to the extraction temperature by means of a long tubing coil placed inside the oven. After percolating the seed bed, the extract stream passes through the forward pressure regulator FPR2 and a micrometering valve (NV), reaching atmospheric pressure; NV is used to control solvent flow rate. The FPR2 and the adjoining line are heated to prevent blocking up due to oil and CO₂ freezing. The recovery vessel at the exit has an internal volume of $3.0 \times 10^{-4} \text{ m}^3$.

2.3.2. SFE experimental conditions and procedure

Approximately 0.07 kg of grape seeds previously prepared were charged into the extractor, and a small amount of steel shreds was packed at the top to prevent seed powder to escape. To analyse both the effect of the pressure (i.e., solvent density, ρ) and the enzymatic treatment upon the SFE, experiments were accomplished at $P=160, 180, \text{ and } 200 \text{ bar}$, at constant temperature ($T=313.15 \text{ K}$) and carbon dioxide flow rate ($\dot{m}_{\text{CO}_2} = 1.7 \times 10^{-4} \text{ kg s}^{-1}$), for both untreated and pre-treated samples. The extraction curves were obtained by representing the yield or the quantity of extracted oil against consumed

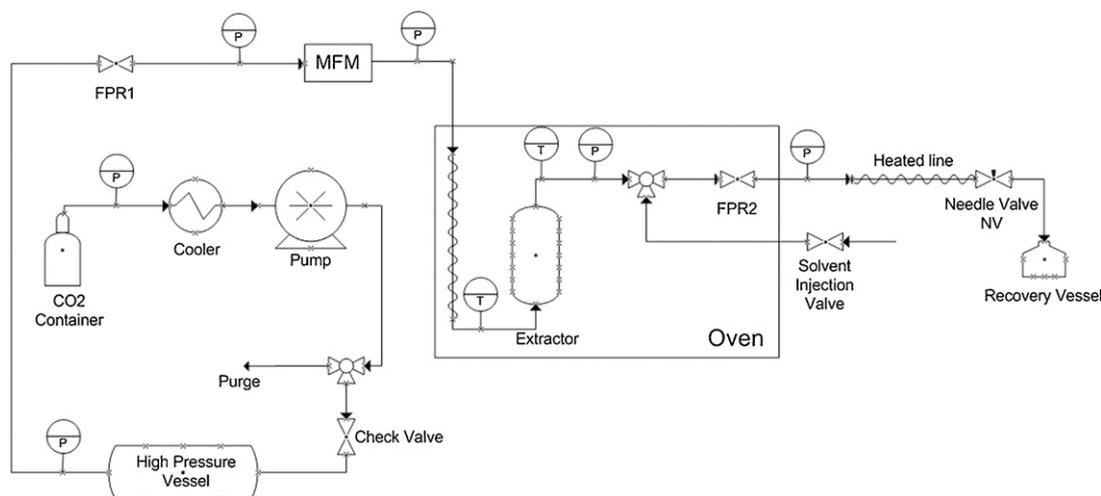


Fig. 1. Simplified scheme of the supercritical fluid extraction installation.

CO₂. The yield (η) of the process is defined as the mass of extracted oil divided by the mass of dried seed loaded in the extractor. Whenever an experiment was interrupted to assess a new data point of its extraction curve, the oil precipitated along the pressure drop section (which includes the section between FPR2 and NV) was recovered by washing with *n*-hexane, and then weighted.

Conventional extraction was carried out for comparison using 150 mL of *n*-hexane in a Soxhlet apparatus (250 mL round bottom flask; Soxhlet chamber of 50 mL capacity; 23 mm × 100 mm cartridge) during 4 h.

2.4. Oil recovery

The mass of extracted oil was determined gravimetrically after solvent evaporation. Furthermore, to ensure complete removal of the water, prior to solvent evaporation, the extracted samples were passed through sodium sulphate anhydrous powder on a G1 sintered glass filter, and evaporated in a rotary evaporator also under vacuum at 303 K. The oil was then transferred to speed-vacuum tubes and dried by centrifugal evaporation.

3. Results and discussion

3.1. Extraction curves for untreated seed

When analysing the effect of solvent pressure on the SFE, the average particle diameter of crushed seed has been fixed because it is one of the most influent parameters in the process (e.g., [12,13]). Hence, particles with an average size of 0.75 have been always used. The bed density and porosity were experimentally determined by gravimetry as 688 kg m⁻³ and 0.37, respectively.

The cumulative curves measured in this work with untreated seed at $T = 313.15$ K, $\dot{m}_{\text{CO}_2} = 1.7 \times 10^{-4}$ kg s⁻¹, and $P = 160, 180,$ and 200 bar are shown in Fig. 2. The yield (% w/w) is plotted against solvent consumption to seed charge ratio ($m_{\text{CO}_2}/m_{\text{seed}}$). All of the curves exhibit the two characteristic extraction periods. The first one, where the yield increases approximately linearly, extends until almost $\eta = 11.5\%$, which is the maximum value common to the three curves. The last part has a smooth asymptotic shape: it is nearly invariable, representing only 3–8% of the total oil extracted. Such trends corroborates the hypothesis of the broken + intact cells model proposed by Sovová [4,10] which assumes the extraction kinetics conforms a two-mechanism process, comprehending a rapid extraction from surface (i.e., oil convection from broken cells)

followed by a diffusion-controlled period from inner intact cells.

Additionally, Fig. 2 points out the chief influence of the pressure on the results. Under isothermal conditions, higher pressures give rise to higher densities which increases the oil solubility in the SC CO₂, and so the driving force to mass transfer. It is possible to demonstrate that its effect upon the convective mass transfer coefficient, k_c , is comparatively small. In fact, taking into account an empirical correlation of the type $Sh = \alpha Re^\beta Sc^\gamma$ to estimate k_c , and an hydrodynamic equation similar to the Wilke–Chang model to calculate tracer diffusivities [14], $D_{12} \propto T/\mu$, where μ is solvent viscosity, one shall observe the following relation at two different pressures, while fixing temperature and velocity:

$$\frac{k_c(P_2)}{k_c(P_1)} = \left(\frac{\mu_1}{\mu_2}\right)^{1+\beta-2\gamma} \left(\frac{\rho_2}{\rho_1}\right)^{\beta-\gamma}$$

Substituting $\gamma = 0.25$ and $\beta = 0.35$ [15], one finds: $k_c(180 \text{ bar})/k_c(160 \text{ bar}) = 0.95$ and $k_c(200 \text{ bar})/k_c(180 \text{ bar}) = 0.96$; for $\gamma = 1/3$ and $\beta = 0.83$ [16], the same ratios result. On the contrary, the density dependence of the oil solubility given by the model of del Valle and Aguilera [17] is $Y \propto \rho^{10.724}$; therefore, $Y(180 \text{ bar})/Y(160 \text{ bar}) = 1.39$ and $Y(200 \text{ bar})/Y(180 \text{ bar}) = 1.29$. These calculations demonstrate that, under the experimental conditions of this essay, the predominant effect of the pressure is upon the oil solubility and not on the mass transfer coefficient. It is worth noting that Fig. 2 supports these calculations, because η increments prevail when passing from 160 to 180 bar, although the vertical spacing between the curves for 180 and 200 bar is still significant, especially between $m_{\text{CO}_2}/m_{\text{seed}} = 2$ and 3. An operational conse-

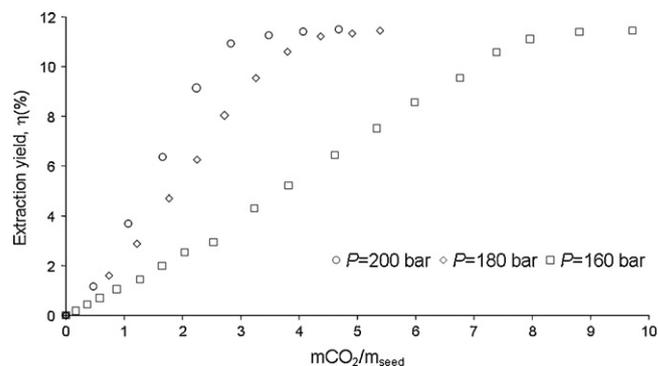


Fig. 2. Extraction curves for the SFE of untreated grape seed oil at 313.15 K.

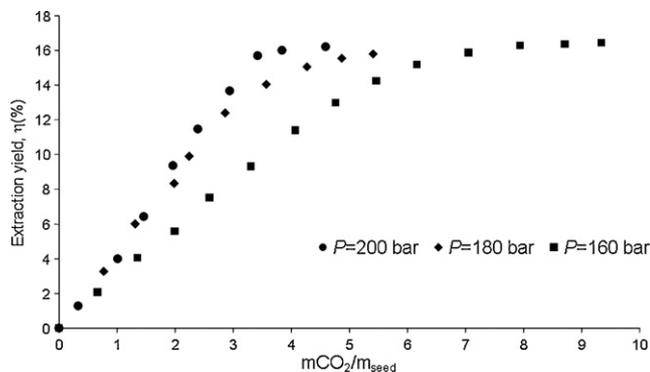


Fig. 3. Extraction curves for the SFE of pre-treated grape seed oil at 313.15 K.

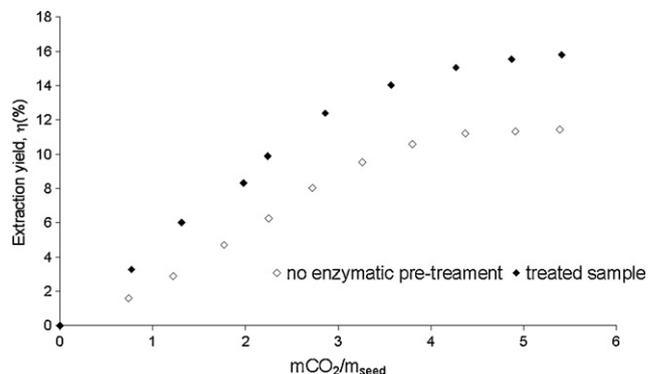


Fig. 5. Comparison between the measured extraction curves for untreated and pre-treated grape seed at 313.15 K and 180 bar.

quence of this relation is that the time (or, equivalently, the mass of CO_2) needed to reach a definite extraction yield decreases at higher pressures. According to Fig. 2, one spends approximately 4, 5, and 9 $\text{kg}_{\text{CO}_2}/\text{kg}_{\text{seed}}$ to reach maximum yield ($\eta = 11.5\%$) at 200, 180 and 160 bar, respectively. Similarly, by increasing the pressure from 160 to 180 bar, the CO_2 necessary to extract 50% of the oil (i.e., $\eta = 5.75\%$) decreases about 46%; the extra increment of 20 bar (i.e., passing from 180 to 200 bar) reduces the CO_2 consumption in 28% only. These data corroborate the results previously described by Fiori [6] who studied the extraction of grape seed oil in the range of 280–550 bar, and showed it raises 60% in the interval of 280–350 bar and only 8% between 350 and 550 bar.

3.2. Extraction curves for pre-treated seed

The cumulative curves of the SFE of grape seed oil measured for enzymatically pre-treated seeds are shown in Fig. 3. Experiments were carried out at the same experimental conditions to allow comparisons with previous results: $T = 313.15 \text{ K}$, $\dot{m}_{\text{CO}_2} = 1.7 \times 10^{-4} \text{ kg s}^{-1}$, and $P = 160, 180, \text{ and } 200 \text{ bar}$. Once more, all these curves exhibit both characteristic extraction periods (i.e., linear plus asymptotic) related with the two-mechanism process described above. Nonetheless, the maximum extraction yield is $\eta = 16.5\%$, independently of the operating pressure conditions used, which represents an increment of 43% over the η for untreated seeds. Such enhancement is due to the enzymatic action, which destroys the cellular and sub-cellular organisation of plant tissues, as their walls and cytoplasm become disrupted [12,18], augmenting seed oil availability.

In Figs. 4–6 a comparison between the results obtained with treated and non-treated grape seed at 160, 180 and 200 bar,

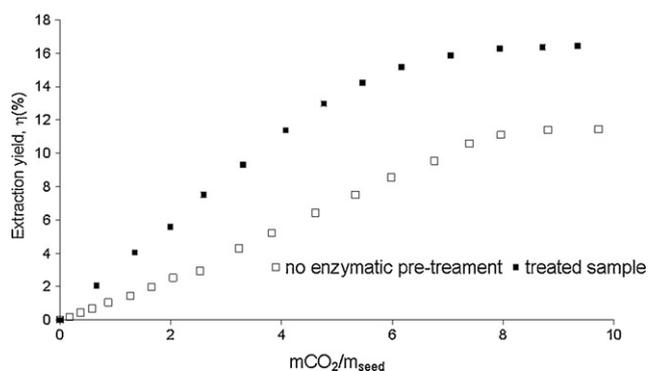


Fig. 4. Comparison between the measured extraction curves for untreated and pre-treated grape seed at 313.15 K and 160 bar.

respectively, is presented. It is possible to verify that each pair of correspondent curves reaches their maximum extraction yields for nearly the same CO_2 consumption level (9, 5, and 4 $\text{kg}_{\text{CO}_2}/\text{kg}_{\text{seed}}$, respectively).

It is worth noting that the slopes of both cumulative curves at $P = 160 \text{ bar}$ (see Fig. 4) are clearly different from each other, which means the output extract is not saturated along time. If it was the case, the first linear period of extraction should overlap. Therefore, under the experimental conditions run, the extraction is not exclusively controlled by equilibrium. The difference is the enzymatic pre-treatment, which enlarges the broken/intact cells ratio, so increasing the oil availability in contact with the solvent. Although less remarkable, Fig. 5 ($P = 180 \text{ bar}$) illustrates an analogous finding. On the contrary, the first branch of the cumulative curves assessed at 200 bar are approximately coincident (see Fig. 6), which indicates the extract stream may be saturated at exit. As has been mentioned above, mass transfer is highest at 200 bar, and so the extraction rate; thus the saturation of CO_2 appears to be attained during the space time of the system.

The operating pressure is unquestionably one of the most relevant process parameters that can be used to tune the selectivity of a SCF. The general rule is: the higher is the pressure, the larger is the solvent power though the smaller is the extraction selectivity. Several studies present results for extremely high pressures [6,13,19,20] in order to achieve good solvent capacity of CO_2 , for instance. In this work one shows it is possible to improve oil production with mild operating conditions if the SFE is combined with a suitable enzymatic pre-treatment. In this manner, selectivity is not engaged being possible to manipulate it with advantage. For a future industrial application, the enzymes used in the seed pre-

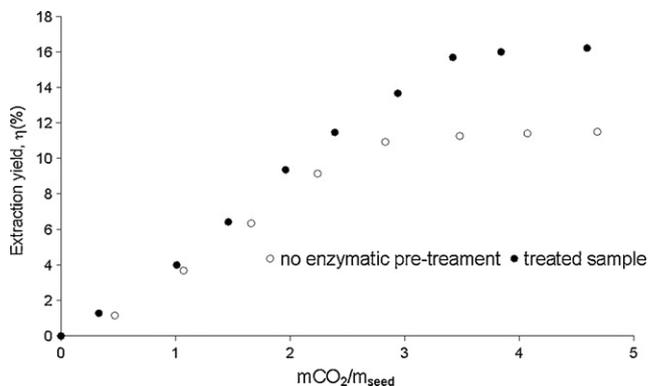


Fig. 6. Comparison between the measured extraction curves for untreated and pre-treated grape seed at 313.15 K and 200 bar.

treatment need to be recycled. As they are soluble in water, a simple decantation of the seeds, a filtration, or a centrifugation will allow to obtain an aqueous solution ready to be reused. For this, studies of the loss of enzyme activity need to be determined. Another unsurpassable point is the cost associated to high-pressure processes: their operation is more expensive, owing to equipment design and construction, as well as the additional safety features that are indispensable. Further, the capital cost of a high-pressure process is not linear with pressure because the pressure ratings of certain vital equipment are available in discrete steps (e.g., 60 and 100 bar) [7]. In addition, the number of companies with experience in high-pressure process design drops dramatically as the operation pressure rises above 200 bar [7]. Clearly, these caveats strongly recommend operating at pressures as low as possible.

4. Conclusions

In this essay, the SFE of grape seed oil using CO₂ has been accomplished isothermally at 313.15 K, and at 160, 180, and 200 bar, using both untreated and enzymatically pre-treated seed. The pre-treatment has been carried out with cell wall degrading enzyme cocktail of cellulase, protease, xylanase and pectinase. For untreated seed the maximum extraction yield obtained was 11.5%, whereas the enzymatic pre-treatment increased this value by 43.5%, attaining $\eta = 16.5\%$. Such results evidence the validity of the hypothesis of the broken + intact cells model, once that pre-treatment enlarges the broken/intact cells ratio, increasing the oil availability in the milled seed. The cumulative extraction curves measured in this work, with both treated and untreated seed, exhibited the common first linear part and a second asymptotic branch; the last part contributes with only 3–8% to the total oil removed. Under the experimental conditions adopted, we found that the process during the first extraction period was not controlled exclusively by equilibrium. With respect to pressure, results confirm that this variable has a large impact on the SFE: (i) the mass of CO₂ needed to reach a definite extraction yield decreases at higher pressures, and (ii) as pressure rises, the first part of the cumulative extraction curves of the treated and untreated biomass become closer, tending to overlap.

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