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Vítor Hugo Dos Louros Rodrigues

Produção de extratos bioativos a partir de resíduos da CMC-Biomassa SA por extração supercrítica com solventes verdes

Bioactive extracts production from CMC Biomassa SA residues using supercritical extraction and green solvents

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Engenharia Química, realizada sob a orientação científica do Doutor Carlos Manuel Santos da Silva, professor auxiliar do Departamento de Química da Universidade de Aveiro e coorientação da Doutora Maria Inês Purcell de Portugal Branco professora auxiliar do Departamento de Química da Universidade de Aveiro.

Dedico este trabalho aos meus pais pelo constante apoio e confiança.

O júri

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Palavras-chave

Ácidos triterpénicos, cossolvente, *Eucalyptus globulus*, extração supercrítica, folhas, rendimento de extração.

Resumo

Esta dissertação teve como objetivo estudar a extração e caraterização de compostos bioativos com valor acrescentado, nomeadamente os ácidos triterpénicos (TTAs) (ácidos betulínico, betulónico, oleanólico, ursólico e derivados acetilados dos dois últimos) da folhagem de *Eucalyptus globulus*. Efectuaram-se extracções com metanol, etanol e diclorometano, pelo método Soxhlet, e com dióxido de carbono na extração supercrítica (SFE), modificado ou não com etanol (cossolvente). Analisaram-se os extractos por FTIR-ATR, GC-MS e SEM, em conjunto com métodos hierárquicos de análise de agrupamentos (clusters) com vista à comparação dos diferentes extractos entre si, com a biomassa original e com os TTAs puros.

Verificou-se que as folhas de eucalipto contêm uma quantidade elevada de extractáveis, com rendimentos totais de 30.34 % (m/m) para extrações Soxhlet com metanol, e 7.32 % com diclorometano. Por SFE os rendimentos totais variaram entre 1.52 e 3.16 % para extracções realizadas com CO_2 a 200 bar com 0 e 5.0 % (m/m) de etanol, respectivamente. Após optimização alcançaram-se rendimentos totais de 3.95 % e de TTAs de 0.67 % (m/m), para extracções a 250 bar com CO_2 contendo 5.0 wt.% etanol, a 40 ^{o}C e caudal de 12 gCO_2 min^{-1}.

A remoção específica de ceras das folhas foi estudada com êxito obtendo-se posteriormente extratos particularmente ricos em TTAs. Realizou-se um estudo cinético para as condições ótimas de SFE, usando biomassa tal e qual (estilhas sem pré-tratamento) ou pré-tratada para redução de granulometria (moagem) ou remoção de ceras. Destes resultados destaca-se o elevado rendimento total obtido com a amostra moída (5.90 %, ao fim de 6 h) e o bom desempenho da biomassa sem tratamento em termos de extração de TTAs (rendimento 0.64 % e concentração de 16.5 %). A biomassa com pré-remoção das ceras gerou extratos com concentrações de TTAs similares aos da biomassa não tratada.

No geral, o presente trabalho fornece informação útil para o estudo da extração de compostos bioativos da folhagem de *E. globulus*, assim como detalhes de composição e rendimentos que podem constituir o ponto de partida para estudos ainda mais detalhados a realizar futuramente.

Keywords

Cosolvent, *Eucalyptus globulus*, extraction yield, leaves, supercritical fluid extraction, triterpenic acids

Abstract

This work focuses the extraction and characterization of high added value bioactive compounds obtained from *Eucalyptus globulus* leaves, namely triterpenic acids (TTAs) such as betulonic, betulinic, oleanolic and ursolic acids, and the acetylated derivates of the last two. The extractions were done using methanol, ethanol, and dichloromethane for the Soxhlet method, and carbon dioxide for the supercritical fluid extraction (SFE), modified or not with ethanol (cosolvent). The extracts were characterized by FTIR-ATR, GC-MS and SEM, together with the hierarchical cluster analysis method for comparison of the different extracts between themselves and with the original biomass and with the pure TTAs.

The results revealed *Eucalyptus* leaves have high content of extractives, namely 30.34 wt.% for Soxhlet extraction with methanol and 7.32 wt.% with dichloromethane. With SFE the total extraction yield varied between 1.52 and 3.16 wt.%, for extractions at 200 bar with CO_2 containing 0 wt.% and 5.0 wt.% of ethanol, respectively. After optimization, total extraction yields of 3.95 wt.% and 0.67 wt.% for TTAs were attained, at 250 bar with CO_2 containing 5.0 wt.% ethanol, temperature of 40 ^{o}C and flow rate of 12 gCO_2 min^{-1}

The removal of waxes from the leaves was successfully studied enabling the production of extracts rich in TTAs. Kinetic studies were performed at the SFE optimized conditions to compare the effect of crushing or dewaxing the leaves in relation to untreated leaves. Highest yields were obtained with the crushed leaves (5.90 wt.%, after 6 h) while in terms of TTAs extraction the best results were obtained with untreated biomass (yield 0.64 wt.%, and concentration 16.5 wt.%). The extracts obtained with untreated and dewaxed biomass samples presented similar concentrations of TTAs.

The present work presents useful information for the extraction of bioactive compounds from *E. globulu*s leaves as well as attainable yields and composition that may guide future comprehensive studies.

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I. Motivation and Structure of the Thesis

Over the last years, increasing environmental awareness and tighter regulations have motivated industrial companies to search for new products or process improvements, aiming the maximization of their resources output. Under this scope, residues or by-products from renewable resources are not considered useless anymore, since they can be transformed into high added value products as preconized by the biorefinery concept [1,2]. The agro-forestry industrial sector is specially challenged by this new vision since their resources are predominantly of vegetable nature. For instance, the raw materials utilized in the logging/timber industry generate 30 – 35% of residues (bark, leaves and fruits), which are left in the forests or burned for energy production [2]. In Portugal, forestry occupies around 35 % of the country's area with the dominant species being *Eucalyptus globulus* [3]. This fast-growing tree is the main source of wood for the pulp and paper industry, which is important to Portugal's economy.

Over the last years, researchers at the Chemistry Department of University of Aveiro have been focusing on extraction processes to add value to biomass residues or by-products, such as *E. globulus* bark [4–6]. In particular, triterpenic acids (TTAs) due to unique biological activities that can be used in pharmaceutical, nutraceutical, cosmetic or food products. Within this context, supercritical fluid extraction (SFE) based on high-pressure carbon dioxide as extraction solvent [7,8] has been particularly investigated for the production of TTAs rich extracts. This "green" technology is preferred due to the easy and complete removal of the solvent after the extraction process, but also because of the (intact) natural character of the extracts. Moreover, the Confederation of European Paper Industries (CEPI) recently elected supercritical CO_2 as a breakthrough technology for the 2050 world [9] and is certainly a driving force for industrial and fundamental research on SFE processes.

The present work aims the production and characterization of extracts from *E. globulus* leaves supplied by CMC Biomassa SA. This small and medium enterprise (PME), located in Leiria – Portugal, is dedicated to the collection, storage and transport of biomass from timber and recycling industries and to the production of pellets, with special interests in new applications of biorefinery products. Within this thesis, different Soxhlet and SFE

extraction methods were used to evaluate the richness of the biomass (*E. globulus* leaves) and the effects of the leaves superficial wax layer on the extraction yield and extractives composition. The extracts were characterized by FTIR-ATR, GC-MS and SEM, together with the hierarchical cluster analysis method. Whenever possible relations were established with previous studies of extracts obtained from other *Eucalyptus* species and/or morphological parts, to enrich the meaning of the conclusions presented. On a subsequent stage, SFE conditions were optimized in terms of pressure and cosolvent content using a statistical model to map the dependence of the extraction yields on the said operating conditions. Finally, kinetic curves were measured under optimum SFE conditions, looking for specific insights related to the pros and cons of undergoing pretreatments of the biomass.

The described work was accomplished with two scientific publications, as follows:

Publication 1 [10]: "Supercritical fluid extraction and characterization of *Eucalyptus globulus* leaves." This article covers Soxhlet extraction of the leaves using different solvents and SFE with CO_2 at different conditions. The results were analysed with techniques such as FTIR-ATR, GC-MS and SEM. A wax removal treatment was performed and the influence of the cuticular wax layer is debated. A cluster analysis method was performed using FTIR-ATR and GC-MS data to obtain comparative insights between the extracts compositions and their similarity to biomass and the pure TTAs.

Publication 2 [11]: "Optimization of the supercritical fluid extraction of *Eucalyptus globulus* leaves. Influence of operating conditions and biomass pretreatment". This article reports the Design of Experiments and Response Surface Methodology (DoE-RSM) implemented to optimize the SFE operating conditions (namely pressure and ethanol content as cosolvent) in terms of total and TTAs extraction yields. Finally, kinetic curves were measured under optimum SFE conditions to further understand the effect of biomass pretreatments (grinding and dewaxing).

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II. Publication 1

Supercritical extraction and characterization of *Eucalyptus globulus* leaves

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Keywords

Cluster Analysis, *Eucalyptus globulus,* Soxhlet, Supercritical CO_2 extraction, Triterpenic acids

1. Introduction

The genus *Eucalyptus* belongs to the Myrtaceae family and includes about 600 species and subspecies [1]. It is an evergreen tall tree original from Oceania presently found worldwide, occupying an estimated global area between 16 and 19 million hectares [1]. Its commercial exploitation occurs mainly in countries such as Brazil, China, India, Tasmania, South Africa and Portugal. *Eucalyptus globulus* is the dominant species in Portugal with the planted area (ca. 812 kha) representing 26 % of the forested areas in a country where 35 % of the landmass is occupied by forests [2]. An important part of *E. globulus* timber is consumed in the pulp and paper industry, with the leaves, branches and bark being considered by-products burned for energy recovery or soil amendment. However, within the biorefinery scope these biomass residues are a source of potentially valuable bioactive extracts.

In recent years, some studies have been performed focusing on *E. globulus* bark as a source of triterpenic acids (TTAs), a family which includes valuable compounds such as betulinic, betulonic, oleanolic and ursolic acids and the acetylated forms of the last two [3–7]. Although recognized for their antioxidant, anti-inflammatory, anti-cancer, anti-microbial and anti-ulcerogenic activities [8–12] their potential has not yet been completely exploited by the pharmaceutical industry [8]. On the other hand, *E. globulus* leaves have not been thoroughly assessed as a source of bioactive compounds despite the important market of essential oil extracted from *Eucalyptus* leaves [13,14].

Eucalyptus leaves can be processed by several extraction techniques each one with its pros and cons. For instance, hydro-distillation and steam extraction operate at high temperatures which may cause degradation of thermolabile compounds and loss of the more volatile ones [15,16]. Organic solvent extraction is not particularly selective for the desired compounds (TTAs) and the final step of solvent removal from the extract is frequently incomplete causing contamination of the final product [4,15,16]. Supercritical fluid extraction (SFE) is an increasingly important alternative, particularly when environmental friendly carbon dioxide (CO_2) is used as solvent [17,18]. Since the properties of supercritical fluids are easily manipulated (fine-tuned) the extraction process is efficient and extract recovery at the final stage is complete. Moreover, the supercritical CO_2 extracts retain their natural character

which increases their potential for pharmaceutical, cosmetic, nutraceutical and food applications [18].

The use of supercritical carbon dioxide (SC-CO_2) for the extraction of *Eucalyptus* leaves has been reported and compared to different extraction methods. For instance, Singh et al [16] investigated hydro-distillation, solvent extraction, ultrasonic assisted extraction and SC-CO_2 extraction of *E. globulus* leaves; Zhao et al [15] compared hydro-distillation, Soxhlet extraction and SC-CO_2 extraction of *E. loxophleba* leaves; El-Ghorab et al [19] studied solvent extraction and SC-CO_2 extraction of *E. camaldulensis* leaves; and Francisco et al [20] examined hydro-distillation and SC-CO_2 extraction of *E. camaldulensis* leaves. In the present work *E. globulus* leaves extracts were obtained by Soxhlet extraction with various solvents and SC-CO_2 extraction with and without ethanol (added as cosolvent).

The extracts were analysed by different techniques, namely Fourier Transform Infra-Red spectroscopy with Attenuated Total Reflectance (FTIR-ATR) and Gas Chromatography-Mass Spectroscopy (GC-MS) combined with chemometric methods for data analysis. The chemical complexity of natural extracts often leads to spectra and chromatograms with overlapping peaks that can be tackled with chemometric resolution techniques. Over the last years, chemometric methods have been used with FTIR and GC-MS to distinguish and identify species based in their habitat or origin [21–24], and also to evaluate the quality of food products [25]. Different methods and metrics can be used but the general goal is to group or distinguish a group of samples in a preliminary stage, depending on the analytical data [21,26]. The hierarchical clustering method used in this work allows the grouping of variables in homogeneous groups in terms of one or more characteristics. Hence, each object of a cluster must be similar to all the others within the cluster and different from the objects in the others clusters [27,28]. Clusters are formed according to dissimilarities (Euclidean distances) between objects being computed, for instance, by the furthest neighbour method (i.e. the distance between two clusters is the maximum distance between the two objects that are furthest apart) [29].

2. Materials and Methods

In this section, we describe the chemicals and biomass used, the analytical methods and the experimental procedures to perform Soxhlet extraction and SFE.

2.1. Chemicals

Carbon dioxide (CO_2, purity 99 %) was supplied by Air Liquid (Algés, Portugal). Ethanol (purity 99.5 %), dichloromethane (purity 99.98 %) and *n*-hexane (purity 99 %) were supplied by Fisher Scientific (Leicestershire, United Kingdom). Methanol (purity 99.9 %), pyridine (purity 99.5 %), N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, purity 98 %) and chlorotrimethylsilane (TMSCl, purity 99 %) were supplied by Sigma Aldrich (Steinhein, Germany). Acetone (purity 99.5 %) and petroleum ether (purity 99 %) were supplied by VWR International (Fontenay-sous-Bois, France) and Chem-Lab NV (Zedelgem, Belgium), respectively. The betulinic, oleanolic and ursolic acids (purity 98 %) were supplied by Aktin Chemicals, Inc. (Chengdu, China).

2.2. Biomass

The biomass used in the present work consisted in leaves of *Eucalyptus globulus* leaves supplied by CMC Biomassa S.A. (Leiria, Portugal). The leaves represent 80 wt.% of the leaves and have a moisture content of 6.74 wt.% (evaluated by drying at 60 °C for 72 hours). The leaves were cut in small pieces with roughly 1 cm wide, as illustrated in Figure 1, and used without any further treatment.

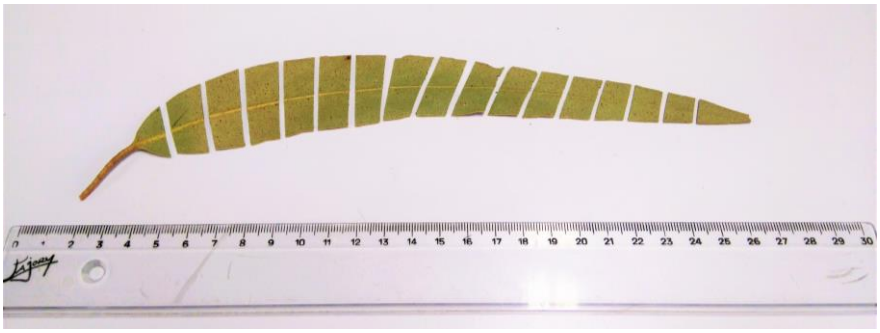

Figure 1- Photo of the typical cut applied to the *E. globulus* leaves before extraction.

Removal of the superficial waxes from the leaves followed the procedure described by Domingues et al [30]. Very briefly, the cut leaves (ca. 60 g) were immersed during 30 s in a

glass flask containing a mixture of solvents (600 mL) at 50 °C. This step was repeated 6 times and afterwards the solvent was filtrated and evaporated using a rotary evaporator. The solvent was a 1:1 (v/v) mixture of petroleum ether/acetone or n-hexane/acetone, being identified as Wax S2 and Wax S3, respectively (see Table 1).

2.3. Scanning Electron Microscopy (SEM) analysis

Scanning electron microscopy (SEM) images of the surface of *E. globulus* leaves were obtained using a SEM Hitachi S4100 microscope. Prior to SEM analysis a gold/palladium (Au/Pd) alloy was deposited on the leaf samples, namely: an original one (without treatments or extractions), one after wax removal pretreatment, and another after dichloromethane Soxhlet extraction.

2.4. Fourier Transform Infrared-Attenuated Total Reflectance (FTIR-ATR) spectroscopy analysis

FTIR spectra of Soxhlet and SFE extracts were collected in a Bruker Tensor 27 spectrometer fitted with an attenuated total reflectance (ATR) accessory. The spectra were obtained by co-adding 256 scans with a resolution of 4 cm^{-1} and afterwards a baseline correction was performed. Pure betulinic, oleanolic and ursolic acids, and the original (ground) biomass were also analysed.

2.5. Gas Chromatography Mass Spectroscopy (GC-MS) analysis

Soxhlet and SFE extracts were analysed by GC-MS after trimethylsilylation, according to a procedure from the literature [4,6]. For each extract, two aliquots of about 20 mg were analysed in duplicate (the results reported are the average) using a Trace Gas Chromatograph 2000 series equipped with a DB-1 J&W capillary column (30 mm x 0.32 mm i.d., 0.25 µm film thickness) and coupled with a Finnigan Trace MS mass spectrometer. Helium was the carrier gas (35 cm s^{-1}) and the chromatographic conditions were as follows: furnace initial temperature 80 °C for 5 min, heating ramp at 4 °C min^{-1}, and final temperature 285 °C for 10 min; injector temperature 250 °C; transfer-line temperature 290 °C; split ratio 1:50. The MS was operated in the electron impact mode with electron impact energy of 70 eV and data collected at a rate of 1 scans s^{-1} over a range of m/z of 33-750. The ion source was maintained at 250 °C.

For quantification of TTAs in the extracts internal (tetracosane) and external (TTAs) standards were used.

2.6. Cluster Analysis

For complex systems or large data sets, it is difficult to analyse all the chemically relevant information systematically without chemometric tools, such as the hierarchical clustering method applied to FTIR-ATR and GC-MS data in this work. Clusters were computed by the furthest neighbour method (i.e. the distance between two clusters is the maximum distance between the two objects that are furthest apart) [29], using IBM SPSS Statistics 23 software. The analysis was performed as follows: (i) for FTIR-ATR clustering: Soxhlet, SFE and dewaxing extracts, pure TTAs (betulinic, oleanolic and ursolic acids) and natural biomass (ground) were all analysed; (ii) for GC-MS clustering: Soxhlet, SFE and dewaxing extracts were analysed, and also a mix comprising three pure TTAs.

2.7. Soxhlet extraction

Soxhlet extractions were performed with dichloromethane, ethanol or methanol to evaluate the effect of solvent polarity on the extraction yield, using biomass (cut leaves) with and without the aforesaid wax removal pretreatment (runs S1 to S5, in Table 1). The extractions lasted 6 h since it has been verified that higher extraction times do not increase the extraction yield [15]. Afterwards, the extract samples were evaporated, weighed, and analysed by FTIR-ATR and GC-MS.

Total extraction yield ($\eta_{\mathrm{Total}}, \%$) was calculated according to Equation 1. The triterpenic acids yield ($\eta_{\mathrm{TTA}}, \%$) and their concentration in the extract ($c_{\mathrm{TTA}}, \%$) were calculated according to Equations 2 and 3, respectively.

$$\eta_{\mathrm{Total}} = \frac{m_{\mathrm{extract}}}{m_{\mathrm{biomass}}} \cdot 100 \quad (1)$$

$$\eta_{\mathrm{TTA}} = \frac{m_{\mathrm{TTA}}}{m_{\mathrm{biomass}}} \cdot 100 \quad (2)$$

$$c_{\mathrm{TTA}} = \frac{m_{\mathrm{TTA}}}{m_{\mathrm{extract}}} \cdot 100 \quad (3)$$

Here m_{extract} corresponds to the mass of the extract weighed after solvent evaporation, m_{biomass} is the mass of dried leaves (6.74 wt.% moisture) used in the extraction, and m_{TTA} is

the mass of triterpenic acids quantified by GC-MS using internal and external standards. The experimental conditions for all Soxhlet extractions are listed in Table 1.

2.8. Supercritical Fluid Extraction (SFE)

The extractions were performed in a lab scale Speed SFE unit, a model of Helix SFE System-Applied Separations, Inc. (USA) schematically presented in Figure 2 and operating as follows: liquid carbon dioxide is pressurized in a cooled liquid pump and then heated in a vessel before the extractor, where CO_2 reaches the desired supercritical conditions (P,T). The supercritical fluid enters the extractor (loaded with 50 g of leaves) and flows upwards at constant flow rate (Q_{CO_2}). At the end of each cycle the effluent stream is depressurized in a heated back-pressure regulator and the extractables are collected in a cooled chamber containing two vessels, where the solutes precipitate or get solubilized in ethanol. Then the ethanolic solutions of the extracts are evaporated, weighed and analysed. The SFE experimental conditions are presented in Table 1 (runs SFE1 to SFE3). Note that in run SFE2 ethanol was used as cosolvent to modify the polarity of SC-CO_2.

Table 1 - Experimental conditions of the extraction and dewaxing assays carried out.

Run	Type of Extraction	Solvent	P (bar)	T (°C)	Q_{CO_2} (g min^{-1})	$m_{biomass}$ (g)	Wax removal	t (h)
S1	Soxhlet	CH_2Cl_2	-	-	-	5.289	-	6
S2	Soxhlet	CH_2Cl_2	-	-	-	5.041	Wax S2	6
S3	Soxhlet	CH_2Cl_2	-	-	-	5.277	Wax S3	6
S4	Soxhlet	EtOH	-	-	-	5.973	-	6
S5	Soxhlet	MeOH	-	-	-	5.107	-	6
SFE1	SFE	CO_2	200	40	12	50.080	-	6
SFE2	SFE	CO2:EtOH (95:5 wt.%)	200	40	12	50.054	-	6
SFE3	SFE	CO_2	300	40	12	50.010	-	6
Wax S2	SLE	petroleum ether:acetone (1:1, V/V)	-	50	-	61.302	-	0.05
Wax S3	SLE	*n*-hexane:acetone (1:1, V/V)	-	50	-	10.012	-	0.05

EtOH - ethanol; MeOH - methanol; SFE - Supercritical fluid extraction; SLE - Solid liquid extraction

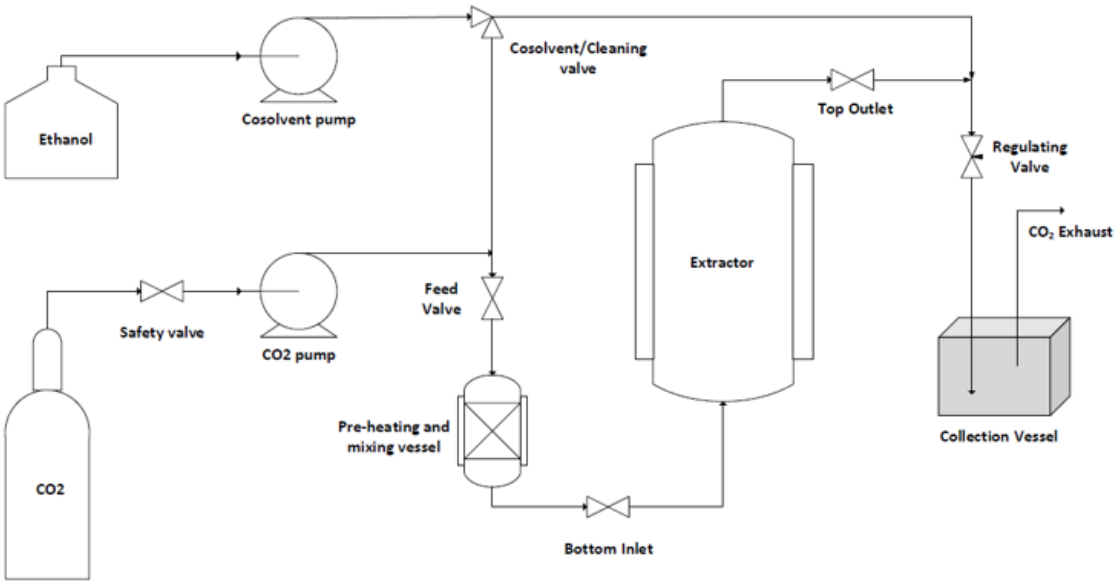

Figure 2 - Simplified scheme of the SFE installation.

3. Results and Discussion

This section is dedicated to the presentation and analysis of results. It is divided in three parts: Section 3.1 focuses the full extracts; the volatile compounds are analysed in Section 3.2; and Section 3.3 discusses the Cluster Analysis of FTIR-ATR and GC-MS data.

3.1 Characterization of full extracts

3.1.1 Total extraction yield

The analysis of the results started by the calculation of the total extraction yields (η_{Total}), which is a quantitative but undifferentiating indicator of the amount of extract produced in each experiment. Table 2 presents the values of η_{Total} for the Soxhlet and SFE experimental assays using *Eucalyptus globulus* leaves as biomass.

At first glance, the results (Table 2) evidence a significant variability, marked by the very high yields obtained for the extraction with the polar solvents, ethanol and methanol, in contrast to dichloromethane and SC-CO_2. While the maximum yield was attained for methanol, with η_{Total} = 30.34 wt.%, the minimum amount (only 1.52 wt.%) was attained in run SFE1, which comprises SC-CO_2 without cosolvent at 200 bar and 40 ^{o}C.

Table 2 – Extraction yields and TTAs content of the Soxhlet and supercritical extractions.

Run	Type of Extraction	Solvent	$\boldsymbol{P}$ (bar)	$\boldsymbol{T}$(ºC)	Wax removal pretreatment	$\boldsymbol{\eta_{Total}}$ (wt.%)
S1	Soxhlet	CH_2Cl_2	-	-	-	7.32
S2	Soxhlet	CH_2Cl_2	-	-	Wax S2	2.38
S3	Soxhlet	CH_2Cl_2	-	-	Wax S3	2.89
S4	Soxhlet	EtOH	-	-	-	25.90
S5	Soxhlet	MeOH	-	-	-	30.34
SFE1	SFE	CO_2	200	40	-	1.52
SFE2	SFE	CO_2:EtOH (95:5 wt.)	200	40	-	3.16
SFE3	SFE	CO_2	300	40	-	2.02

EtOH = ethanol; MeOH = methanol.

Soxhlet assays - Considering only the Soxhlet results, two main factors seem to have dictated the differences between the η_{Total} values: the polarity of the solvent, and the decision to remove waxes as pretreatment. Regarding the former, ethanol gave rise to a slightly lower yield than that of methanol, attaining 25.90 wt.%. In turn, dichloromethane was only able to reach 7.32 g per 100 g eucalypt leaves if no pretreatment was employed (Run S1) and 2.38-2.89 wt.% if employed.

In order to track the coherence of the attained values for the different extraction systems, it is worthwhile to compare our results with available data for leaves of *Eucalyptus* species. Accordingly, Figure 3 presents a comparison of our η_{Total} values with those attained by Singh et al [16], who studied leaves of *E. globulus* using batch solid-liquid extractions and SFE; El Ghorab et al [19], who studied mature leaf extracts from *E. camaldulensis var. brevirostris* both by Soxhlet and SFE; and Francisco et al [20], who also studied leaves of *E. camaldulensis by* SFE. Within the conditions presented, the assays can vary in the pretreatment (grinding, lyophilization or none), the extraction time for both Soxhlet extraction and SFE only varies between 6 and 7 h with the exceptions of the SFE runs from Francisco et al and Singh et al which took 2 h. The SC-CO_2 flow ranged from 6 to 17 g min^{-1} with the exception of Francisco et al whose runs were performed at 200 g min^{-1}.

The results from Singh et al [16] showed that when solid-liquid extraction (SLE) is employed using a non-polar solvent like *n*-hexane, the η_{Total} substantially decrease from levels of Soxhlet (i.e. 7.32 wt.% as in run S1 with dichloromethane) and attain 2.0 wt.%, which is much comparable to SFE values (the same authors report η_{Total} = 3.2 wt.% for SFE at 350

bar, 80 ^{o}C and 0 wt.% EtOH). Hence, this result suggests that the great abundance of fresh solvent at boiling point is what justifies the higher yields attainable by Soxhlet using weakly polar solvents, and not the intrinsic solvent power of this solvent when in contact with the eucalypt leaves. On the other hand, the results obtained by El. Ghorab et al [19] for ethanolic extracts using Soxhlet suggest that *E. globulus* leaves might be richer in polar extractives than *E*. *camaldulensis*, since it yielded 20.0 wt.% against 25.90 wt.% for *E. globulus*.

SFE results – For the supercritical fluid extractions, the η_{Total} values ranged from 1.52 wt.% for SFE1 (200 bar, 40 ^{o}C and 0.0 wt.% of EtOH) to 3.16 wt.% for SFE2 (200 bar, 40 ^{o}C and 5.0 wt.% of EtOH). It is worth to note that when pressure is increased to 300 bar (SFE3), the total extraction yield only reaches 2.02 wt.%, thus remaining considerably far from the value attained with cosolvent (SFE2). Hence, the SFE results suggest the importance of a polar cosolvent in order to attain higher yields, particularly if it is taken into account that the SC-CO_2 pressure of runs SFE1 and SFE3 fall within the typical range of values reported in the literature (i.e. 200-400 bar) [17]. In turn, ethanol is the most employed cosolvent (it has been preferred 53 % of the times) to modify the polarity of the SC-CO_2 in extractions of vegetal biomass [17], and its importance to increment η_{Total} has been demonstrated in several experimental optimization works [31–34], including the SFE of *E. globulus* bark [3,35–38].

Comparing our SFE results with available data from the literature (plotted in Figure 3), it is worth noting that the assay SFE2 yielded almost as much as what Singh et al [16] attained using ground *E*. *globulus* leaves at the harsher SC-CO_2 $P-T$ conditions of 350 bar and 80 ^{o}C, i.e. η_{Total} = 3.2 wt.% vs. 3.16 wt.% (run SFE2). Nevertheless, it should be noted that these authors used a much lower extraction time (t =2 h). On the other hand, the available results for *E. camaldulensis* show what may be expected if the working conditions are substantially incremented: P to 400 bar, T to 70 ^{o}C, and ethanol concentration to 9.3 wt.% (10 %, v/v) and 13.3 wt.% (15 %, v/v): yields jump to 12.0 and 16.6 wt.%, respectively. Such results are somehow expectable, at least if the Soxhlet with ethanol assay remains in mind (η_{Total}= 20.0 wt.%): the greater the enrichment of cosolvent the higher the tendency of the SFE results to approach the plateau yield of that cosolvent as defined by the Soxhlet extraction with it.

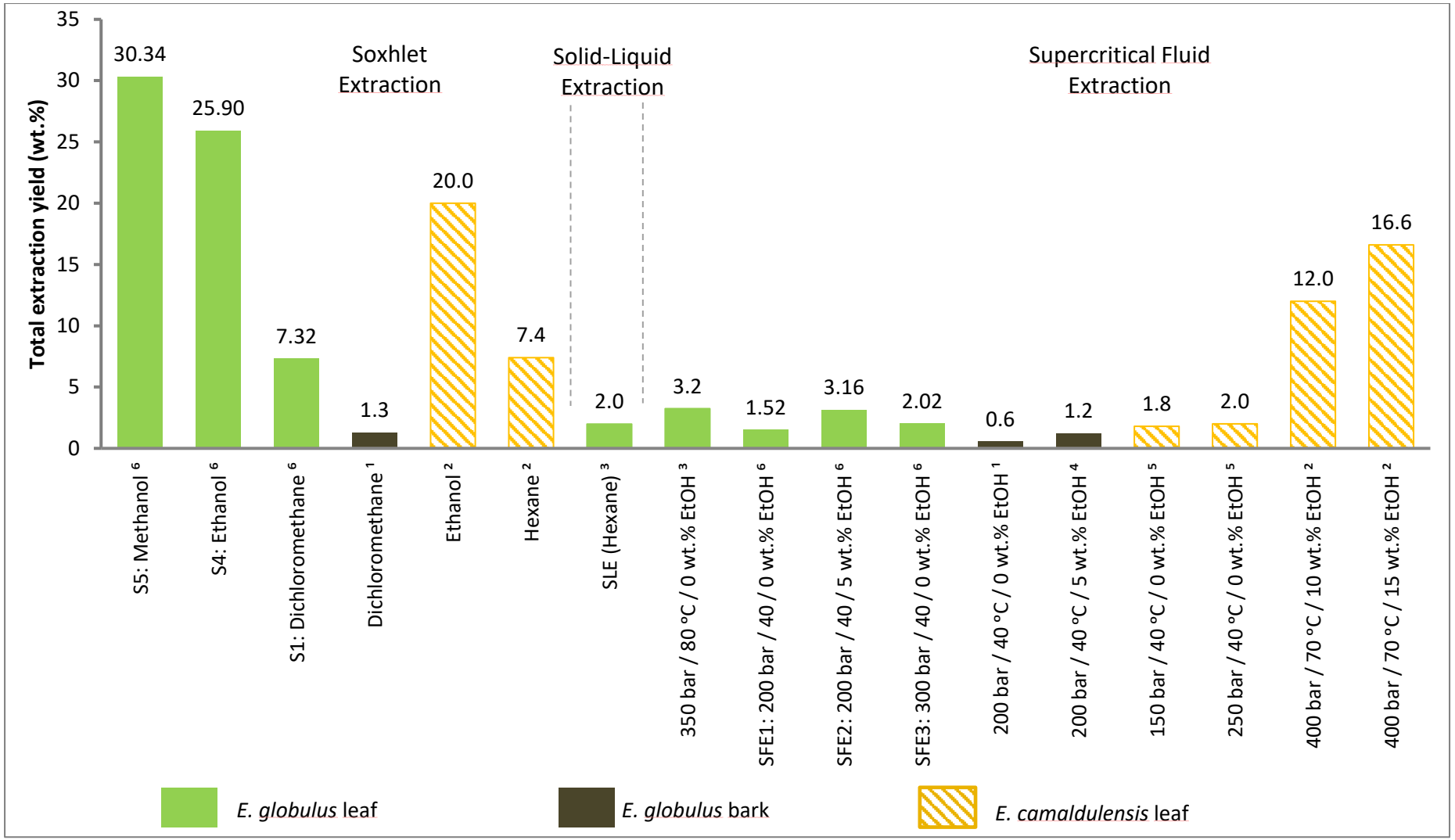

Figure 3- Illustrative comparison of the total extraction yields obtained with different extraction methods and conditions for different eucalypt species and morphological parts. The superscripts in the abscissa refer to the source, being: 1 from Melo et al [4], 2 from El-Ghorab et al [19], 3 from Singh et al [16], 4 from Domingues et al [5], 5 from Francisco et al [20], and 6 from this work.

Moreover, it is also interesting to compare our results with those attained for other morphological parts of *E. globulus*, especially deciduous bark, which has been strongly investigated in recent years due its richness in triterpenic acids [5,37]. The data for such comparison is plotted in Figure 3, encompassing a Soxhlet extraction with dichloromethane and two SFE assays at the same conditions of runs SFE1 and SFE3. When contrasted with leaves, *E. globulus* bark evidences much lower content of lipophilic compounds, yielding only 1.3 wt.% against 7.32 wt.% in the case of run S1. Nevertheless, the SFE assays performed at the conditions of SFE1 and SFE3 were able to remove, respectively, 46 % and 92 % of the amount the dichloromethane Soxhlet yielded (7.32 wt.%). These values highly contrast with our results for leaves, where SFE1 and SFE3 assays reached only 19 % and 26 %, respectively, of the amount of Soxhlet with dichloromethane. In fact, the best performance was for SFE2, which was able to reach 41 % of the Soxhlet yield by means of the use of 5 wt.% ethanol as cosolvent, suggesting a greater resistance of the leaves to the supercritical extraction.

3.1.2 Waxes removal

The leaves of *E. globulus* contain significant amounts of cuticular wax that might hinder extraction due to an additional resistance to mass transfer of the solutes. The existence of specific procedures to remove wax from leaves opens a way to fractionate the extracts produced from *E. globulus* leaves, namely through a first extract richer in waxes, followed by a second containing essentially non-superficial solutes. The experiments Wax S2 and Wax S3 targeted this possibility, differing only in terms of the less polar solvent used in the mixture, namely petroleum ether/acetone (run Wax S2) or n-hexane/acetone (run Wax S3) (see Tables 1 and 2).

The obtained results are listed in Table 3, with the waxes extraction yield (η_{waxes}) and the cumulative yield ($\eta_{\mathrm{Total}} + \eta_{\mathrm{waxes}}$) being reported separately. Accordingly, the η_{waxes} were similar (Wax S3 9.7 % higher) between runs Wax S2 and S3, thus suggesting that the use of *n*-hexane or petroleum ether mixed with acetone are indistinct, at least from the perspective of the total amount recovered. The η_{waxes} values reached 4.17 and 4.10 wt.%, respectively, and when summed to the subsequent extraction yield by Soxhlet with dichloromethane gave rise to $\eta_{\mathrm{Total}} + \eta_{\mathrm{waxes}}$ equal to 8.04 and 8.05 wt.%. These values are 9.8 and 10.0 % higher than those attained by Soxhlet without pretreatment (whose single

extract represented a yield of 7.32 wt.%). Although it only takes a total of three minutes, this increase might be caused by the polar solvent higher removal during the dewaxing procedure.

Table 3 – Waxes extraction yield of the Soxhlet extractions.

Run	Waxes removal pretreatment	$\eta_{\mathbf{waxes}}$ (wt.%)	$\eta_{\mathbf{Total}} + \eta_{\mathbf{waxes}}$ (wt.%)
S1	-	-	7.32
S2	Wax S2	-	8.04
S3	Wax S3	-	8.05
Wax S2	petroleum ether:acetone	5.66	-
Wax S3	*n*-hexane:acetone	5.16	-

In order to understand the influence of waxes on the extracts production, a comprehensive study devoted to cuticle layers of *Eucalyptus* species [39] should be cited, where one of the main conclusions is that the leaf cuticle corresponds to a modified cell wall that contains additional lipids. This is pertinent in light of the fact that some researchers assume the cuticle waxes as non-interacting entities in relation to the target extractives (e.g. oil or TTAs), whose extractions might take place in parallel [40]. Contrarily, Guzman et al [39] suggest the waxes reinforce the cell walls thus acting as an additional resistance to mass transfer of intracellular solutes. In practice, this might imply harder conditions for solvents to penetrate into the vegetable cells where target compounds can be dissolved, and also greater difficulties for these solutes to migrate to the bulk solution. In the whole, for uncrushed biomass particles (i.e. having a small fraction of broken (accessible) cells), the removal of intracellular compounds may be considered a phenomenon occurring in series to the removal of waxes, i.e. interdependent. Under these insights the comparable values of $\eta_{\mathrm{Total}} + \eta_{\mathrm{waxes}}$ for runs S2 and S3 in relation to S1 for similar extraction times (pretreatment is negligible), might mean that a significant amount (5.66 wt.%) of extractives is found in a more accessible layer and that the remaining (at least 1.72 wt.%) might be obtained from an inner position (intracellular), under lower diffusion/extraction rate.

In order to further investigate this topic, SEM images (Figure 4) were acquired for the original leaf samples (containing waxes), for the same samples after being Soxhlet extracted, and for the leaves right after the waxes removal procedure (and before any subsequent

extraction). The wax cuticle of the original leaf samples can be visualized in Figures 4.A and 4.B, and its partial (or total) absence after the wax removal procedure in Figures 4.E and 4.F. The said procedure seems to have changed the surface layer, which evidences a flatter aspect and a softened roughness. Once again it should be referred that the biomass was not ground, which ensures that the observed differences are only due to the waxes removal pretreatment. Finally, Figure 4.C and 4.D show the biomass samples without waxes removal pretreatment but after Soxhlet extraction with dichloromethane. Here one can see that the leaves resemble the original ones but with a cleaner/washed aspect. Such resemblance seems to confirm that dichloromethane is not able per se to remove the cuticle waxes.

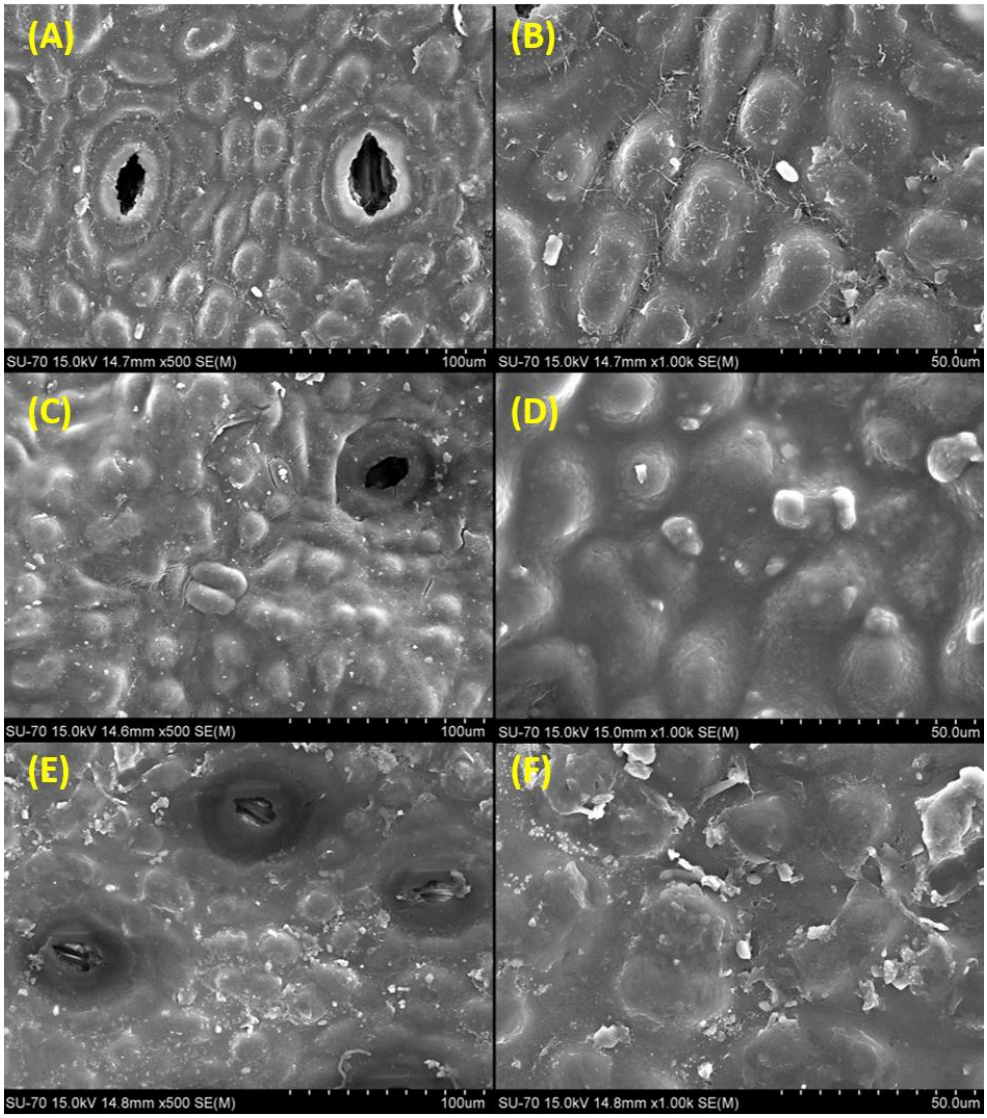

Figure 4- SEM images for the leaves surface with a magnification of 500x (micrographs on the left column) and 1000x (micrographs on the right column): (A) and (B) original leaf samples; (C) and (D) original leaf samples after being Soxhlet extracted with dichloromethane; (E) and (F) leaf samples after the waxes removal pretreatment.

3.1.3 FTIR-ATR results

Taking into account that η_{Total} results are generic and do not consider distinctions between the extracts at chemical level, FTIR-ATR spectroscopy was applied to the Soxhlet extracts (S1, S5) and to the extracts produced by SFE (SFE1 to SFE3). In addition, the analysis also included the original *E. globulus* leaf (in a powdered form), and the individual TTAs, namely betulinic, oleanolic and ursolic acids. The attained results can be observed in Figure 5. The main bands identified in all samples (see top of Figure 5) were discriminated using data from the literature and systematized in Table 4.

For an easier understanding of Figure 5, the diversity of compounds is expected to decrease from the top to the bottom, since the spectra refer to progressively simpler samples in terms of the number of compounds: one starts with natural biomass and ends in the three single compounds (i.e., ursolic, oleanolic and betulinic acids). Hence, the FTIR-ATR spectra have a resemblance with the η_{Total} values, in the sense that biomass represents the theoretical case of η_{Total} = 100 wt.% and the final three spectra represent the theoretical case where $\eta_{\mathrm{Total}} \approx \eta_{\mathrm{TTA}}$.

In general, the main bands found in the spectra correspond to the hydrogen bond O-H , then the C-H stretching in methylene and methyl groups (CH_2 and CH_3), carbonyl stretching band C=O, stretching of C-O and deformation of C-H (C-H_{def}), which correspond to the characteristic peaks found in eucalypt leaf extracts [41]. As we go down in Figure 5, it can be observed that the bands associated to some polar groups present in the biomass and extracts of polar Soxhlets (ethanol and methanol), namely O-H, C=O and C-O_{def}, gradually lose importance in the spectra, until reaching the supercritical extracts where they do not appear at all. This emphasizes the great appetence of ethanol and methanol to remove polar extractives such as phenolics, when compared to dichloromethane and SC-CO_2, even if CO_2 is slightly modified with ethanol. Although ethanol, even in small contents, is expected to tune the polarity of SC-CO_2 [17], from the FTIR-ATR data obtained it looks like the variety of compounds might not have increased (the polar bands are not as present as in the polar Soxhlet extractions). This contrasts with the fact that the overall quantity extracted (η_{Total}) increased, which might be due to an easier penetration through the cuticular waxes of the eucalypt leaf surface, as well as an easier extraction of the solutes.

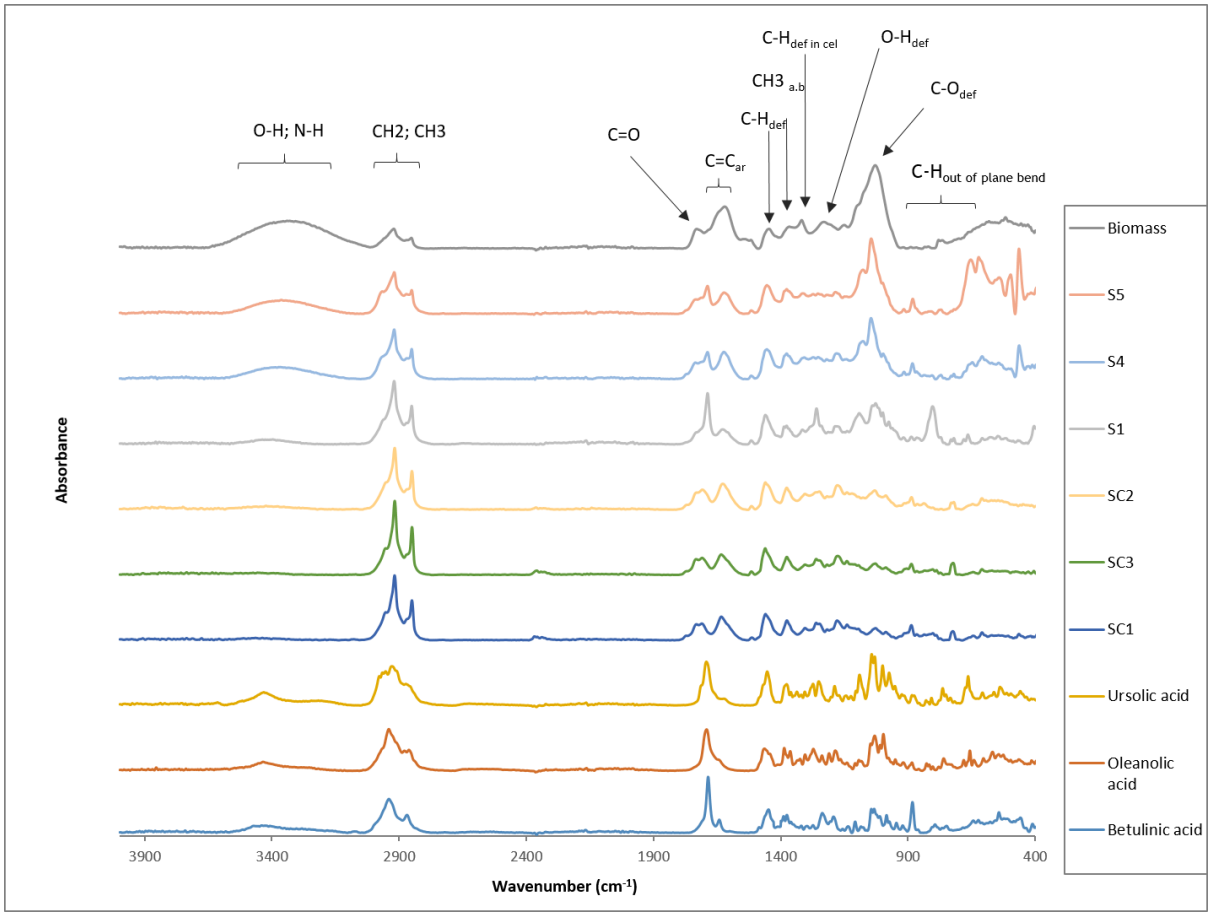

Figure 5 - FTIR-ATR spectra of the Soxhlet and supercritical extracts, of three triterpenic acids (ursolic, oleanolic and betulinic acids) and of the original biomass sample.

Table 4 –Bands assignments in FTIR-ATR spectra of Soxhlet and SFE extracts of *E. globulus* leaves and of triterpenic acids with the respective wavelength and reference.

Wavelength (cm^{-1})	Band assignment	Reference
3200-3600	Hydrogen bond (O-H) N-H stretching in primary amines (N-H)	[42–46]
2920	C-H stretching of methylene groups (CH_2)	[42,44,46,47]
2850	C-H stretching of methyl groups (CH_3)	[42,47–49]
1730	C=O stretching modes (C=O) attributed to oxygenated functionalities (carbonyl/carboxyl groups)	[47–50]
1600-1640	Aromatic skeletal vibrations (C=C)	[47,48,50,51]
1450	C-H deformations ($C\text{-}H_{def}$)	[42,48,52,53]
1389	Characteristic peaks of ursane triterpenoids	[46,54]
1370	CH_3 asymmetrical bending ($CH_{3;a.b.}$)	[41,43,44,48]
1367	Characteristic peaks of ursane triterpenoids	[46,54]
1356	Characteristic peaks of ursane triterpenoids	[46,54]
1317	Characteristic peaks of ursane triterpenoids	[46,54]
1315	C-H deformation in cellulose ($C\text{-}H_{cel}$)	[42]
1290	Characteristic peaks of ursane triterpenoids	[46,54]
1254	Characteristic peaks of ursane triterpenoids	[46,54]
1222	O-H deformation ($O\text{-}H_{def}$)	[48]
1160	Antisymmetrical bridge oxygen stretching (C-O-C)	[42]
1100-1000	C-O stretching; C-N stretching for a liphatic amines	[42,43,48,55]
900-700	C-H aromatic out of plane deformation ($C\text{-}H_{o.f.p.\ bend}$)	[42,50]

3.2. Volatile extractives

3.2.1 GC-MS results

In order to complement the structural information acquired by FTIR-ATR, GC-MS analyses were performed to better characterize and/or quantify the volatile fraction present in the extracts of the runs shown in Table 2.

In Table 5 the peaks of volatile compounds detected in the Soxhlet and supercritical extracts are listed, assigned by family of compounds and also their total number. In terms of families of chemical compounds, four main categories were considered: monoterpenes (MTp) and sesquiterpenes (STp), fatty acids (FA) and long chain aliphatic alcohols (LCAA), sterols (ST), and triterpenoids (TT). For the assignment of peaks, retention time (Rt) ranges were defined for each family based on published chromatographic results for *E. globulus* [4,16], as follows:

MTp/ STp comprise Rt < 28 min; those of FA/LCAA comprise 28 min < Rt < 53 min; ST are located at 53 min <Rt < 59 min ; and TT encompass peaks with Rt > 59 min.

Table 5 - Number of peaks detected for lipophilic compounds in the Soxhlet and supercritical extracts.

Run	Chemical families				Total Peaks detected
	MTp/STp	FA/LCAA	ST	TT	
S1	3	4	2	18	27
S2	4	4	5	13	26
S3	5	7	3	18	33
S4	9	18	1	10	38
S5	8	18	3	15	44
SFE 1	16	15	4	20	55
SFE 2	15	15	10	26	66
SFE 3	12	14	12	26	64
Wax S2	1	2	0	13	16
Wax S3	1	1	1	12	15

MTp = monoterpenes; STp = sesquiterpenes; FA = fatty acids; LCAA = long chain aliphatic alcohols; ST = sterols; TT = triterpenoids.

As a starting point, 27 peaks were detected in the dichloromethane Soxhlet extract (S1), which is lower than those obtained for the dichlorometahe Soxhlet after wax pretreatment with *n*-hexane:acetone (S3), where 33 were registered. The difference is mainly noticed in the MTp/STp and FA/LCAA families, which increased the number of peaks by 2 and 3 compounds, respectively. This means that the wax pretreatment, even just for a small extraction time (6 x 30 s), reduced the resistance of the leaves to extraction. Furthermore, the fact that the dewaxing already extracts a significant part of the solutes gives more importance to the 33 detected peaks. As far as the wax rich extracts are concerned, the number of peaks amounted 16 for the petroleum ether:acetone assay (Wax S2) and 15 for the *n*-hexane:acetone run (Wax S3). These extracts show almost no presence of compounds in the MTp/STp, FA/LCAA and ST categories, and a concentration of peaks in the TT region.

The polar Soxhlet extractions presented a higher number of detected peaks – 38 in the case of ethanol and 44 in the case of methanol - which is in accordance with the global yields in Table 2. The pronounce difference between the volatiles present in weakly polar and

polar Soxhlets, S1 and S4 respectively, can be observed in Figure 6 (A and B), where richness of the ethanol extract (S4) is very clear between retention time of 20 and 40 min, which correspond to compounds of the MTp/STp and FA/LCAA families. In turn, the number of TT peaks is much lower, scoring only 10 (against 18 of run S1).

Furthermore, the higher number of chromatographic peaks from all runs was obtained with the supercritical fluid extractions where the SFE1 got 55, then SF3 (higher pressure) has 64 and the SFE2 (with ethanol) got 66. The enhancement was attained mostly at expenses of all family compounds, with a special emphasis to the TT family, in which 20 to 26 peaks were detected, to MTp/STp where 12 to 16 peaks were detected, and also to FA/LCAA which totalized 14 to 15 compounds. These results confirm the high affinity of the SC-CO_2 to lipophilic solutes and shows that under harsher conditions the extracted compounds can increase.

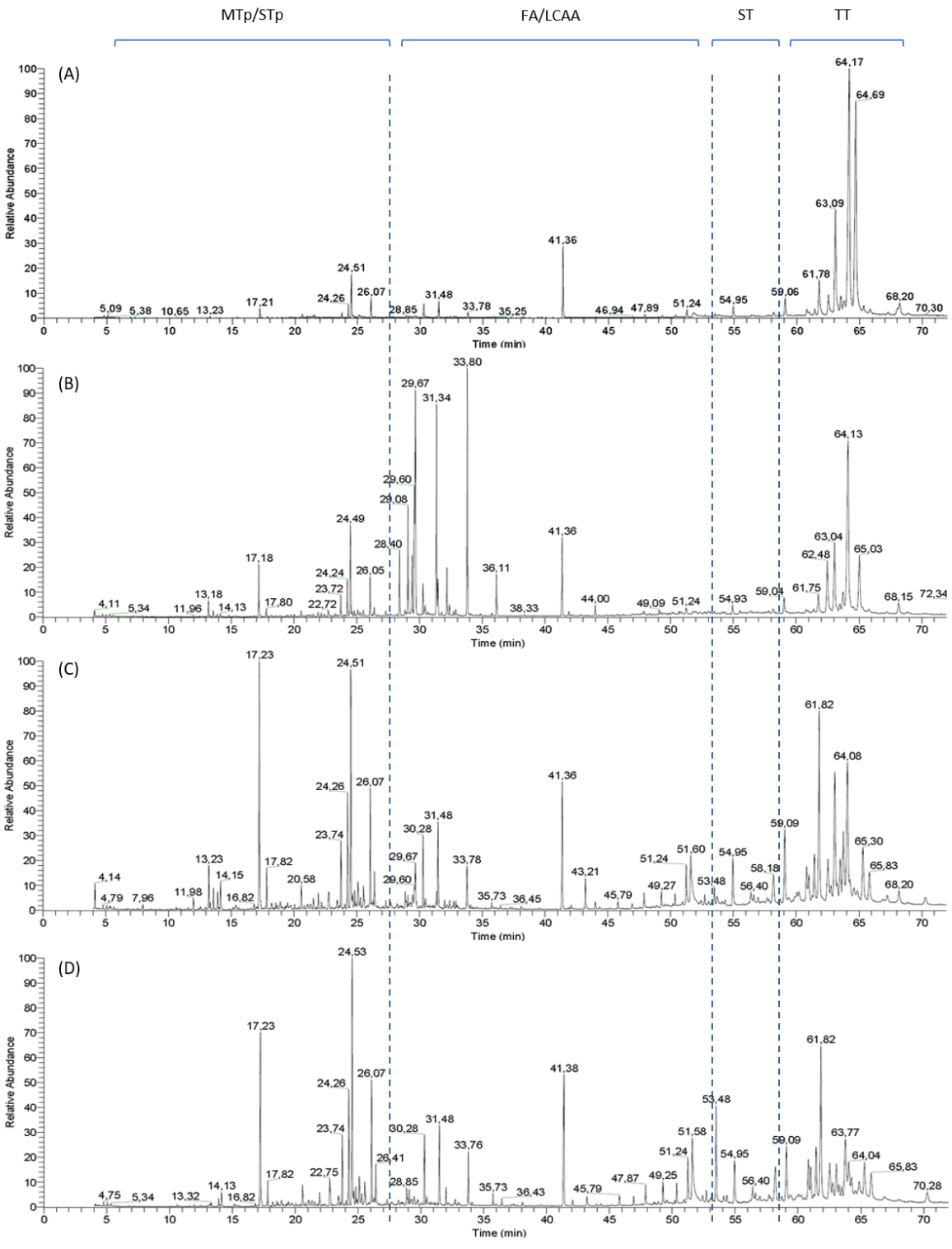

Figure 6 – GC-MS chromatograms of the Soxhlet extracts using dichloromethane (A) and ethanol (B), and SFE with (C) and without (D) cosolvent. Nomenclature: MTp = monoterpenes; STp = sesquiterpenes; FA = fatty acids; LCAA = long chain aliphatic alcohols; ST = sterols; TT = triterpenoids.

3.2.2 - Triterpenic acids (TTAs) extraction yield

Since the general motivation of this work is set on the triterpenic acids existing in the leaves of *E. globulus*, the quantification of these compounds in the extracts was also accomplished. The results are given in Table 6 in terms of TTAs extraction yield (η_{TTA}) and concentration of TTAs in the extracts (c_{TTA}).

As can be noticed, the yield results ranged from 0.15 wt.% (run SFE1) to 7.79 wt.% (run S5). In fact, the polar Soxhlet extractions (runs S4 and S5) led to the highest TTAs yields, with ethanol rendering 5.39 wt.%, followed by the dewaxing extracts (Wax S2 and Wax S3), which rendered η_{TTA} values of 2.79 and 1.77 wt.%, respectively. For the weakly polar organic solvent Soxhlets, the TTAs yields ranged from 0.63 wt.% (run S2) to 1.34 wt.% (Run S1).

Regarding the SFE assays, the highest values obtained of TTAs yield corresponded to SFE2 (run with 5.0 % of ethanol), i.e. 0.53 wt.%, a value that is more than the double those of the best SFE assay without cosolvent, namely SFE3, in which η_{TTA}= 0.24 wt.%. Nevertheless, all supercritical assays led to yields much below 1 wt.%, which clearly contrast with the values attained by Soxhlet extraction, particularly if extracted with no wax related pretreatments.

In what concerns the TTAs content of the extracts, the higher c_{TTA} values were obtained for wax enriched extracts, namely runs Wax S2 and Wax S3, which rendered c_{TTA} = 66.8 wt.% and 43.1 wt.%, respectively. Remarkably, the Soxhlet extracts produced after these pretreatments led also the extracts more enriched in TTAs than if no wax removal is accomplished: 26.7-39.4 wt.% (runs S2 and S3) vs. 18.2 wt.% (run S1).

With reference to the TTAs content of the polar solvents and supercritical assays, the Soxhlet with methanol (run S5) provided a c_{TTA} = 25.7 wt.%, value that decreases to 20.8 wt.% if ethanol is used instead, but still higher than if dichloromethane is employed (S1). The lowest value of c_{TTA} was once again obtained in extract SFE1, the one with milder conditions, where c_{TTA} = 9.8 wt.%. In turn, the best SFE result was for the assay having ethanol as cosolvent (SFE2), whose extracts reached a TTAs content of 16.7 wt.%.

Table 6 – TTAs extraction yield and TTAs content of the Soxhlet and supercritical extractions.

Run	Type of Extraction	Solvent	P (bar)	T (ºC)	Wax removal	η_{TTA} (wt.%)	c_{TTA} (wt.%)
S1	Soxhlet	CH_2Cl_2	-	-	-	1.34	18.2
S2	Soxhlet	CH_2Cl_2	-	-	Wax S2	0.63	26.7
S3	Soxhlet	CH_2Cl_2	-	-	Wax S3	1.14	39.4
S4	Soxhlet	EtOH	-	-	-	5.39	20.8
S5	Soxhlet	MeOH	-	-	-	7.79	25.7
SFE1	SFE	CO_2	200	40	-	0.15	9.8
SFE2	SFE	CO_2:EtOH (95:5 wt.)	200	40	-	0.53	16.7
SFE3	SFE	CO_2	300	40	-	0.24	11.6
Wax S2	-	petroleum ether:acetone (1:1, v/v)	-	-	-	2.79	66.8
Wax S3	-	*n*-hexane:acetone (1:1, v/v)	-	-	-	1.77	43.1

EtOH = ethanol; MeOH = methanol.

The poor performance of SFE assays for *E. globulus* leaves clearly contrast with the results previously obtained for *E. globulus* deciduous bark [4,36]. For the same experimental conditions of SFE1 and SFE2, c_{TTA} values of *E. globulus* bark were 32.2 and 40.2 wt.%, respectively, which represents extracts two to three times richer in TTAs. Despite this advantage on extracts concentration, under the said SFE conditions, bark yielded 0.18 and 0.49 wt.%, which is comparable to the η_{TTA} values obtained in this work for leaves. As a result, between bark and leaves, the potential to remove TTAs seems the same, but the coextraction of non-target compounds is stronger for leaves.

Cluster Analysis was applied to the data acquired by FTIR-ATR and by GC-MS for all the extracts for a comprehensive chemical analysis of the full extracts produced and of their volatile fraction, respectively.

3.3 Cluster Analysis

3.3.1 Full extracts

Cluster analysis was employed to data acquired by FTIR-ATR of Soxhlet and SFE extracts, original biomass (*E. globulus* leaves) and the pure TTAs, using the complete linkage or Furthest Neighbor method previously described (Section 2.6). First, the complete data series were normalized to prevent the statistical analysis results from being affected by the absolute intensities of FTIR-ATR spectra, inspired by the approach reported for FTIR comparisons involving *Cortex eucommiae* bark [22]. All bands were normalized in relation to the C-H (methylene group) stretching band, located at 2920 cm^{-1}. This band was chosen since it is clearly present in all spectra and is not particularly informative about less abundant chemical compounds, i.e. it cannot be used as a differentiation factor.

The dendrogram obtained with the FTIR-ATR data is illustrated in Figure 7. For vertical cut at a distance of 5 units, the diagram is partitioned into four clusters: one group (orange lines) composed of runs SFE1, SFE2, SFE3, Wax S3 and S1; a second one (grey lines) for the pure TTAs; a third group (green lines) joining the polar Soxhlet extracts, S4 (ethanol) and S5 (methanol); and, a fourth group (black lines) comprising only the natural biomass. These results are in accordance with ones' expectation since the pure compounds and biomass are very distinct from each other at the chemical level, but also in relation to the extracts. Moreover, the further sorting of the extracts according to the polar and weakly polar character of the solvent is also in agreement with what was obtained for the total extraction yields (higher yields for the alcoholic extractions).

Even though the cluster analysis results could be further refined by cutting the dendrogram at even lower distance units, a minimum threshold of 4 units was accepted. This seems to be needed to differentiate the three pure TTAs in a distinct cluster (see Figure 7). Accordingly, the shaded region in the dendrogram delimits an exclusion zone where no further insights about the extracts were sought. If in turn the dendrogram is cut looser (for instance, at a distance between 6 and 9 distance units) the clustering is reduced to three groups, with the main difference being the combination of dichloromethane soxhlet, SFE, and wax rich extracts, plus the three pure TTAs in a single group. These reflect the greater resemblance of the triterpenic acids (slightly polar) with the chemical bonds found in the

lipophilic extracts, rather than with those found in the alcoholic extracts, which may encompass compounds such as glycosides or phenolics. Moreover, if the dendrogram is cut only at a distance between 10 and 25 units (the broadest division), two very asymmetric clusters are produced: one comprising only the vegetal raw material (biomass leaves) and another one containing all the remaining samples (extracts and pure TTAs). This is easy to explain, since none of the extracts grouped in that cluster represent more than 30 wt.% of the biomass weight.

In the whole, cluster analysis of FTIR-ATR data suggests greater differences between the produced extracts and the original biomass, than between the former and the target compounds (TTAs). This is particularly true for the lipophilic extracts, namely those involving dichloromethane or SC-CO_2, but also for the wax rich extracts arising from the cleaning pretreatment of the biomass.

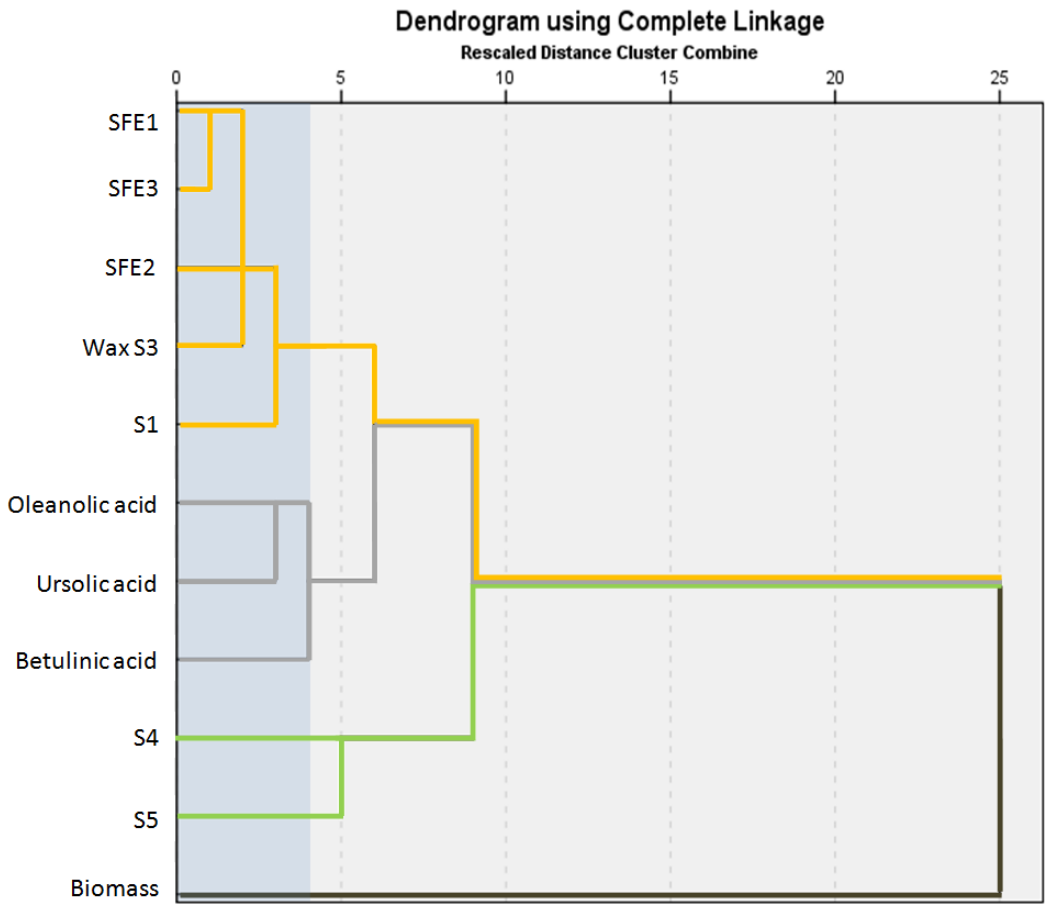

Figure 7 - Dendrogram obtained using FTIR-ATR spectral data for the various Soxhlet (S1 to S5) and SFE extracts (SFE1 to SFE3), the original (ground) biomass, and the oleanolic, betulinic and ursolic acids. The dark gray shaded area refers to a region where differences between the extracts should not be considered. The four clusters are identified by coloured dendrogram lines (orange, grey, green and black).

3.3.2 Volatile extractives

In principle, cluster analysis applied to GC-MS results enables extract assessment based on the individual volatile compounds, thus a providing a more specific comparison between extracts and the pure compounds than the one accomplished using FTIR-ATR data. This is so not only because volatiles are a partial fraction of the full extracts produced, but also because the GC-MS results refer to individual compounds and not to chemical bonds found in a group of molecules (as in the case of FTIR spectra).

The dendrogram obtained with GC-MS data is depicted in Figure 8. When the vertical cut is located between 3 and 10 distance units, six clusters are obtained. This is a high number of clusters considering that the volatiles analysis was based on 10 samples. Nevertheless, the grouping is rational with the mixture of pure triterpenic acids being classified as an independent cluster (grey lines), and the alcoholic extracts (green lines) being deemed different from those of weakly polar solvents. Moreover, the analysis differentiates the SFE extracts (yellow lines) from dichloromethane Soxhlet extracts (orange lines), and the latter from the volatiles found in the wax rich extract (blue lines). Curiously, the wax rich extracts (Wax S2 and Wax S3) were not combined in the same group claiming individual clusters.

If the dendrogram cut distance is broaden (e.g. increase to 10 or 15 units), a first cluster merge is noticed comprising the combination of alcoholic extracts and those of SFE assays. Although this closeness between SFE and polar Soxhlet extracts might look odd (recall that these extracts were closer the FTIR-ATR cluster – see Figure 7), it can be understood by comparing the data obtained from GC-MS analysis (Table 5 and Figure 6). In fact, the general appearance of the chromatograms and the number of peaks are both more similar between SFE and polar Soxhlet extracts than with weakly polar (dichloromethane) extracts. Alternatively, if the cut limit is set at a larger distance of 19 units, the dichloromethane Soxhlet extracts are further merged with the alcoholic ones and SFE assays, leaving only the wax rich extracts as independent clusters, apart from the pure TTAs mixture. Jumping the two dewaxing extracts, if a distance cut is set between 23 and 25 units, there will be only two groups, namely: the TTAs mixture group and another group comprising all the extracts (i.e. a general group that resembles the same type of result obtained for FTIR-ATR under the broadest cutting criterion). Grouping of TTAs in a single cluster is in agreement with ones

expectations since this group only exhibited four peaks (the three TTAs plus the internal standard used for normalization) out of the ninety peaks found globally in the GC-MS chromatograms of all samples.

In the whole, the first conclusion of this cluster analysis is that the volatile fractions of the extracts produced are much closer to one another than in relation to the pure target compounds (TTAs). This reflects the unmanageable selective extraction of an exclusive family of compounds or individual molecules, and thus hinders the correct appraisal of the extract value for commercial trade [17]. The second conclusion is that the progressive appearance of new clusters as the cut criterion is progressively refined somehow follows/correlates with the trend of TTAs concentration in the extracts: TTAs = 100 wt.%, wax rich extracts = 43.1-66.8 wt.%, dichloromethane extracts = 18.2-39.4 wt.%, alcoholic extracts = 20.8-25.4 wt.%, and SFE extracts = 9.8-16.7 wt.%. The third conclusion is that the clustering results can be correlated with the number of peaks detected in the GC-MS chromatograms. Here, the samples with the highest number of peaks were the ones that needed lower distances to be distinguished in different clusters, thus demanding more refined limits to be distinguished between each other.

In terms of a definition of an extraction strategy/plan, dichloromethane extracts obtained with or without wax removal remained in the same cluster, which suggests a similarity between the volatiles of these runs despite the wax removal treatment. Secondly, the fractionated wax rich extracts exhibited a volatiles profile distinct from any other extracts, even between each other, thus implying that their volatiles profile should be considered unique in the sense that they do not resemble the extracts profile (including those relying on single organic solvents). Finally, SFE assays seem to produce also a distinct category of extracts. However, as discussed before, the attained results are still far from the potential of this technology therefore demanding an optimization of the working conditions.

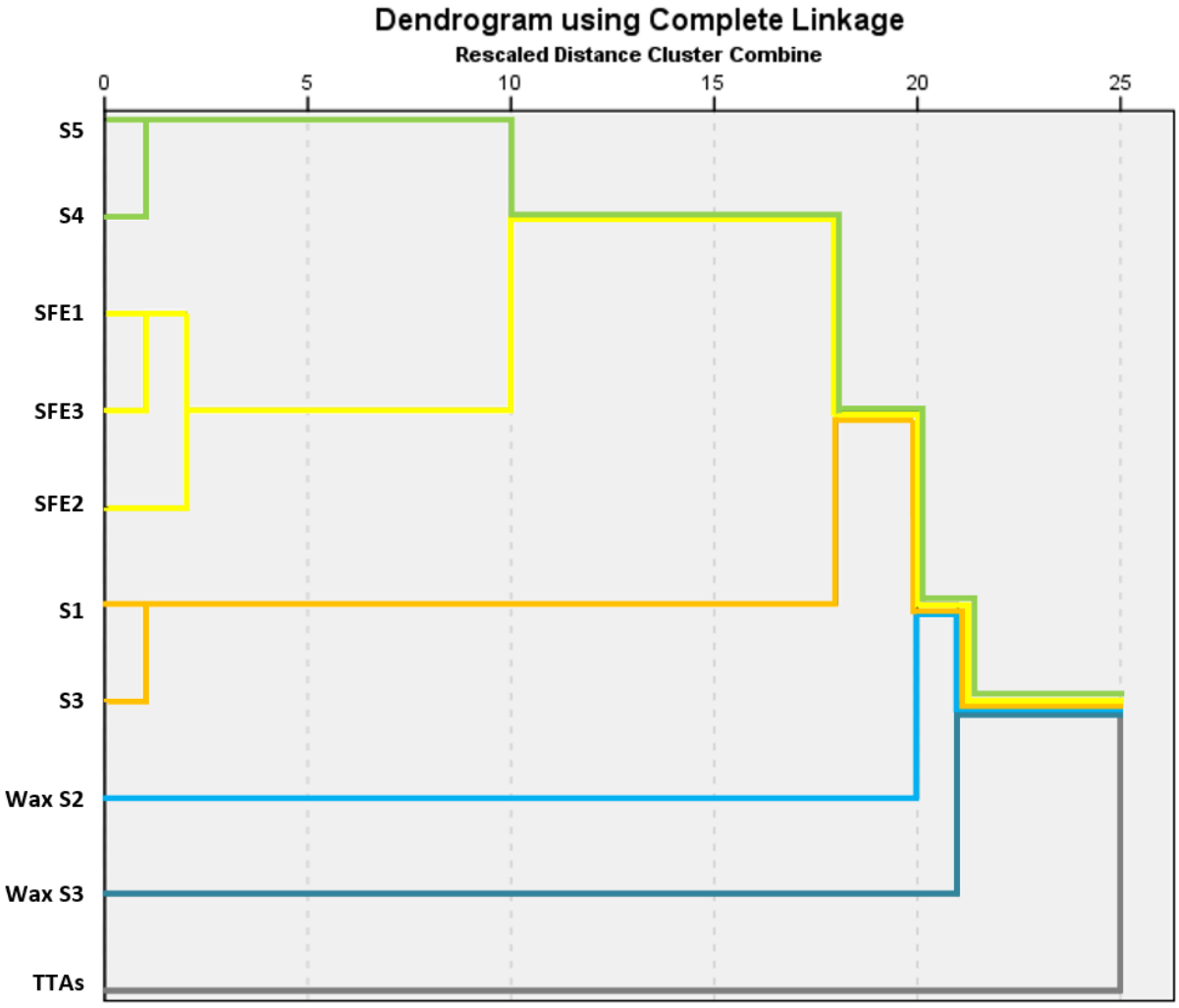

Figure 8 - Dendrogram of the extracts obtained by different methods and conditions (see Table 1) and TTAs, analysed by GC-MS.

4. Conclusions

In this work, *E. globulus* leaves (biomass) were extracted using ethanol, methanol, and dichloromethane for Soxhlet extraction, and carbon dioxide for supercritical fluid extraction (SFE). The total extraction yields (η_{Total}) ranged from 1.52 wt.%, attained for SFE at 200 bar without cosolvent, and 30.34 wt.% for Soxhlet extraction with methanol. In terms of triterpenic acids (TTAs), the extraction yields ranged from 0.24 wt.% to 7.79 wt.% (for the same conditions/methods mentioned for η_{Total}).

The cuticular wax layer covering the surface of *E. globulus* leaves causes an additional resistance to extraction and thus a specific characterization study was envisaged to address

wax removal. With the solid liquid extraction process implemented the wax extraction yields scored 5.66 wt% and the TTAs content of this fraction reached 66.8 %. Moreover, the cumulative amount of extract obtained by performing a wax removal treatment followed by Soxhlet extraction with dichloromethane, is similar to the η_{Total} attained directly by Soxhlet extraction of the original biomass (i.e. wax-rich leaves) with the same solvent.

The extracts were characterized by FTIR-ATR and GC-MS analysis and the data were thoroughly discussed using a cluster analysis technique. The FTIR-ATR spectra of the extracts are quite different from the original biomass spectrum. The main bands correspond to the hydrogen bond (O-H), methylene and methyl groups (CH_2 and CH_3), carbonyl stretching (C=O), stretching of C-O and deformation of C-H (C-H_{def}). Pronounced differences were observed in the O-H bond bands for extracts obtained with polar or weakly polar solvents. Cluster analyses of FTIR-ATR data allowed sample grouping in three mains clusters, namely: (i) biomass, (ii) alcoholic extracts, and (iii) pure TTAs, leaving the dichloromethane, SC-CO_2 and wax extracts closed to the target compounds. The same chemometric method applied to the GC-MS chromatograms of the samples extracted with weakly polar solvents showed that: these extracts are different from pure TTAs mixtures; dichloromethane extracts are similar with or without wax removal pretreatment; SFE volatiles are chemically closer to polar Soxhlet volatiles than to weakly polar ones; and, finally, wax fractionation does not originate extracts significantly different from those obtained from leaves rich in waxes.

In the whole, this study provides arguments for an informed selection of different extraction methods and solvents to produced extracts enriched in TTAs from the leaves of *E. globulus.*

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III. Publication 2

Optimization of the supercritical fluid extraction of *Eucalyptus globulus* leaves. Influence of operating conditions and biomass pretreatment

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Keywords

DoE-RSM*, Eucalyptus globulus*, Supercritical CO_2 extraction, Triterpenic acids

1. Introduction

Eucalypt is one the most exploited trees in the world, with an occupied global area estimated at 16 to 19 million hectares [1]. In Portugal, *Eucalyptus globulus* is the dominant species, occupying 26 % of the national forest area [2]. The harvested wood is fed to the pulp and paper industries from which significant amounts of residues and/or by-products are obtained, namely bark, leaves, fruits and knots. These, although rich in bioactive compounds, are ultimately burned for energy production [3]. However, with the raising interest on biobased materials and bioactive compounds, pulp and paper mills are being challenged to biorefine these vegetal matrices in an integrated system, in order to capitalize from the high added value compounds that can be extracted without affecting their main industrial outputs, i.e. pulp and paper products.

In previous studies [3], the extractives of *E. globulus* bark and leaves have been assessed using several extraction methods and solvents, namely their content of triterpenic acids (TTAs), which exhibit pertinent bioactive properties [4]. For the deciduous bark, the state of the art [5,6] shows that a green extraction with supercritical carbon dioxide (SC-CO2) is a promising approach to recover TTAs, sustained by the optimization of operating conditions [7], kinetics modelling [8] and scale-up tests [9]. In turn, *E. globulus* leaves have already been object of preliminary extractions with SC-CO_2 and alternative methods [10,11]. For instance, leaves can render a total extraction yield (η_{Total}) of 30.34 wt.% when Soxhlet extracted with methanol (polar solvent), 7.32 wt.% with dichloromethane (weakly polar solvent), and 3.16 wt.% with SC-CO_2 modified with 5.0 wt.% ethanol at 250 bar/40 °C.

In the case of *E. globulus* leaves, the success of their supercritical fluid extraction (SFE) may be limited by the cuticular wax layer on the external surface of the leaves [12], which may represent an additional resistance to the recovery of target solutes. While this challenge is not exclusive of *E. globulus* [13–15], the existence of specific methods to accomplish the dewaxing of leaves offer the possibility to pretreat this raw material in order to overcome eventual constraints to mass transfer, which implies a fractionation of the final extracts in two different products: a wax rich and a wax poor extract. Alternatively, an intensive particle size reduction could also overcome this problem, since the wax layer is damaged and the contact

area between leaf particles and solvent would increase. However, both procedures increase the costs of industrial process, and this is very pertinent in light of one of the key reasons behind the rising potential of SFE technology: the lower number of operations needed adds interest to the industrial scale operation of this processes [16].

In order to further improve the SFE results obtained with non-treated *E. globulus* leaves, an experimental optimization of operating conditions is addressed in this study. Accordingly, a Design of Experiments (DoE) was built to identify the significant effects, and the Response Surface Methodology (RSM) was applied to obtain response functions. In the last years, this approach has been widely applied in SFE studies, with more than 70 different vegetable matrices being studied with these methods [17], standing as an effective approach to improve the performance of SFE processes.

The article is structured as follows: after this introduction the Materials and Methods section is presented, where the chemicals and biomass utilized, the analytical method, the extraction process and the statistical model used are described. Section 3 is the Results and Discussion and comprises: Experimental Optimization Results (Section 3.1), which is divided into the analysis of total extraction yield and TTAs extraction yield (Sections 3.1.1 and 3.1.2); Following, the optimized results are compared with literature data for other morphological parts of *E. globulus* (Section 3.2); The study of biomass pretreatments upon SFE performance is assessed in Section 3.3. Finally, the main conclusions are compiled in Section 4.

2. Materials and Methods

2.1. Chemicals and Biomass

The CO2 (purity 99 %) was supplied by Air Liquid (Algés, Portugal), the ethanol (purity 99.5 %) was supplied by Fisher Scientific (Leicestershire, United Kingdom). The pyridine (purity 99.5 %), N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, purity 98 %) and chlorotrimethylsilane (TMSCl, purity 99 %) were supplied by Sigma Aldrich (Steinhein, Germany). The betulinic, oleanolic and ursolic acids (purity 98 %) were supplied by Aktin Chemicals, Inc. (Chengdu, China).

The biomass was supplied by CMC Biomassa S.A. (Leiria, Portugal) and consists in industrial grade leaves of *Eucalyptus globulus*. The leaves correspond to 80 wt.% of the biomass weight and present a moisture content of 6.7 wt.% (after drying at 60 °C for 72 hours). The biomass was cut in similar gross pieces with roughly one-centimetre wide.

2.2. GC-MS analysis

The extracts were analysed by GC-MS using about 20 mg of each dried extract once they were converted into trimethylsilyl (TMS) derivatives according to the literature [6,18]. Two aliquots of each extract were analysed in duplicate, being the reported results the average of the measurements. The GC-MS analyses were performed in a Trace Gas Chromatograph 2000 series coupled with a Finnigan Trace MS mass spectrometer, using helium as carrier gas (35 cm s^{-1}), equipped with a DB-1 J&W capillary column (30 mm x 0.32 mm i.d., 0.25 μm film thickness). The detailed description method can be consulted elsewhere [6]. For the quantification of TTAs, calibration with reference compounds, namely, betulinic, oleanolic and ursolic acids, was performed. Each sample was injected in duplicate.

2.3. Supercritical Fluid Extraction

The supercritical extractions were performed in a 0.5 dm^3 lab unit, model Speed-SFE from Applied Separations, Inc. The installation can be visualized in Figure 1, and includes the possibility to work with a cosolvent, in this case ethanol. The extraction starts pressurizing the liquid carbon dioxide in a cooled liquid pump, and eventually mixing with cosolvent under specified proportions. The liquid stream is then heated in a vessel before the extractor to reach the supercritical state. After, the supercritical solvent (pure or modified SC-CO_2) flows the extractor upwards and percolates the vegetal bed (ca. 50 g of leaves per run). The extract stream is then depressurized through the heated back-pressure regulator in order to collect the solutes in a cooled chamber containing two vessels. These are partially filled ethanol so that the gaseous solvent is bubbled and the solutes trapped in the solution, while at the same time CO_2 is vented out of the vessel. Finally, the extracted compounds (usually named extract) are obtained after ethanol evaporation and analysed.

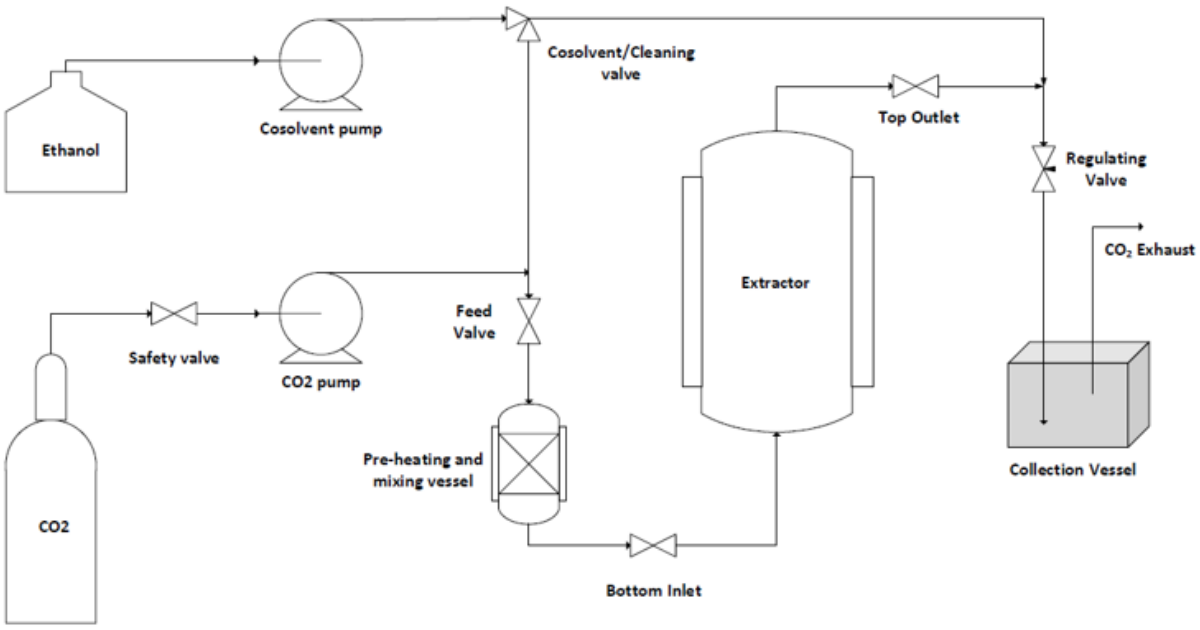

Figure 1 - Simplified scheme of the SFE unit of this work [10].

Three quantitative responses were investigated: total extraction yield (η_{Total}), triterpenic acids extraction yield (η_{TTA}), and TTAs concentration in extracts (c_{TTA}). The corresponding definitions are:

$$\eta_{\mathrm{Total}} = \frac{m_{\mathrm{extract}}}{m_{\mathrm{biomass}}} \times 100 \quad (1)$$

$$\eta_{\mathrm{TTA}} = \frac{m_{\mathrm{TTA}}}{m_{\mathrm{biomass}}} \times 100 \quad (2)$$

$$c_{\mathrm{TTA}} = \frac{m_{\mathrm{TTA}}}{m_{\mathrm{extract}}} \times 100 \quad (3)$$

where m_{extract} is the mass of extract after solvent evaporation (i.e. dry), m_{biomass} is the mass of leaves in the extractor bed, and m_{TTA} corresponds to the mass of triterpenic acids quantified by GC-MS.

2.5. Design of Experiments (DoE) and Response Surface Methodology (RSM).

Response surface methodology consists of a group of mathematical and statistical techniques based on fitting empirical models to experimental data for subsequent analysis. The data are typically produced under the formalism of design of experiments (DoE), which

aims at minimizing the experimental effort while maximizing the information obtained [19]. In this respect, the relative importance of independent variables (factors) influencing a desired dependent variable (response) can be statistically assessed, including their crossed interactions and non-linear effects.

In this essay, a full factorial DoE comprising 2 factors and 3 levels was chosen, which results in a total of 3^2 = 9 SFE experiments. The studied factors are the extraction pressure (P) and ethanol content in the supercritical fluid mixture (EtOH wt.%), and the three levels are 200/250/300 bar and 0.0/2.5/5.0 wt.%, respectively. The full list of experiments is presented in Table 1 along with other parameters that have been fixed along this study, such as extraction temperature (set at 40 ºC), time (set at 6 h) and CO_2 flow rate (set at 12 g min^{-1}). In order to minimize the influence of unknown and uncontrolled effects upon results (nuisance factor), randomization of experiments was accomplished.

Table 1 - Experimental conditions of the extraction assays carried out in this work.

Run	P (bar)	EtOH content (wt.%)	T (ºC)	Q_{CO_2} (g min^{-1})	t (h)
SFE 1[a]	200	0.0	40	12	6
SFE 2	200	2.5	40	12	6
SFE 3[a]	200	5.0	40	12	6
SFE 4	250	0.0	40	12	6
SFE 5	250	2.5	40	12	6
SFE 6	250	5.0	40	12	6
SFE 7[a]	300	0.0	40	12	6
SFE 8	300	2.5	40	12	6
SFE 9	300	5.0	40	12	6

[a] retrieved from [10]

The factors were codified (into -1, 0 or +1, see Table 2) according to Eq. (4) so that the impact of jumps between levels can have a common comparison basis:

$$X_k = \frac{x_k - x_0}{\Delta x_k} \quad (4)$$

where X_k refers to the codified value of the independent variable x_k, x_0 is the variable value in its center point and Δx_k is the step change between levels for the k variable.

The experimental results that undergo RSM analysis are usually well described by a second order polynomial function formally written as:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \beta_{ij} X_i X_j \quad (5)$$

where Y is the studied response (η_{Total}, η_{TTA} or C_{TTA}), β_0 is a constant, β_i, β_{ij} and β_{ij} refers to model coefficients associated with linear effects, quadratic effects, and interaction effects, respectively.

Finally, STATISTICA software (version 5.1, StatSoft Inc., Tulsa, USA) was applied to treat the results. Analysis of variance (ANOVA) was employed to assess the significant factors and interactions using Fisher's test and its associated probability $p(\mathrm{F})$, while t-tests were performed to evaluate the significance of the fitted coefficients of each model. The determination coefficients, R^2, and their adjusted values, R^2_{adj}, were used to evaluate the goodness of the fit.

Table 2 - Correspondence of three different levels for the two factors considered in codified and non-codified form.

Variable	Level correspondence		
	Low (-1)	Medium (0)	High (+1)
Pressure (X_{P}, bar)	200	250	300
Ethanol content (X_{EtOH}, wt. %)	0.0	2.5	5.0

3. Results and Discussion

3.1 Experimental optimization results

The optimization performed in this work comprises the joint goal of producing high amount of *E. globulus* bulk extract by SFE, given by the total extraction yield, η_{Total}, with special emphasis on the TTA recovery from the vegetal matrix, measured by TTAs extraction yield, η_{TTA}, and TTAs concentration in the extracts, c_{TTA}. Since the latter can be calculated from the other two ($c_{\mathrm{TTA}} = \eta_{\mathrm{TTA}}/\eta_{\mathrm{Total}}$), the combined DoE-RSM approach of this work will only focus the yield responses.

The experimental results for the 9 assays performed are listed in Table 3. For the DoE considered, the total extraction yield varied between 1.52 wt.% for run SFE1 (200 bar, 0.0 wt.% EtOH) to 3.95 wt.% for run SFE6 (250 bar, 5.0 wt.% EtOH). In comparison to the best result reported recently by Rodrigues et al. [10], this new maximum (SFE6) represents an enhancement of 25.0 % in the value of η_{Total}. Regarding TTAs extraction yield, the results ranged from 0.13 wt.% for run SFE4 (250 bar, 0.0 wt.% EtOH) to 0.67 wt.% for run SFE6 (250 bar, 5.0 wt.% EtOH). Once again, SFE6 represented an enhancement in relation to the previous best result in the literature [10], in this case of 26.4 %. Finally, in terms of c_{TTA}, the results ranged from 7.2 wt.% for run SFE4 (250 bar, 0.0 wt.% EtOH) to 16.7 wt.% for run SFE3 (200 bar, 5.0 wt.% EtOH). Contrarily to the other responses, the best TTAs concentration reported by Rodrigues et al. [10] was not surpassed here, which suggests that the increments of both η_{Total} and η_{TTA} counterbalanced each other with negative impact on c_{TTA}.

Table 3 - Results of the DoE experiments carried out in this work.

Run	X_{P}	X_{EtOH}	η_{Total} (wt.%)	η_{TTA} (wt.%)	c_{TTA} (wt.%)
SFE1	-1	-1	1.52	0.15	9.8
SFE 2	-1	0	2.98	0.23	11.6
SFE3	-1	1	3.16	0.53	16.7
SFE 4	0	-1	1.76	0.13	7.2
SFE 5	0	0	2.68	0.23	7.7
SFE 6	0	1	3.95	0.67	12.2
SFE7	1	-1	2.02	0.32	11.9
SFE 8	1	0	2.77	0.32	11.6
SFE 9	1	1	3.69	0.60	16.2

Concerning η_{Total} and η_{TTA} modelling, Table 4 presents the fitting coefficients of the full model (FM), where the bold values correspond to significant coefficients at 95 % confidence level. The results show that only two fitting parameters were considered statistically significant, namely β_0 and β_2 for both responses, with the latter representing the linear effect of ethanol content, and the former being the typical constant of the polynomial model (not related to any factor). While for η_{Total} all non-significant parameters (and thus effects) score well above the exclusion limit (β_1 is the closest with p=0.406, which is notoriously far from 0.05), for the η_{TTA} response β_{22} is the closest and scores much nearer

to that limit, p=0.096. This suggests that a quadratic effect for ethanol content is closer to being significant, which reinforces the importance of this factor, possibly due to TTAs solubility variations in supercritical mixtures of increasing ethanol percentage.

Despite the model seemed to fit reliably well the data ($R^2 = 0.935$), the value of $R^2_{\text{adj}} = 0.741$ is significantly lower than R^2, which implies that the number of parameters is dictating the quality of the adjust, and not the robustness of the proposed function. The same can be observed for η_{TTA}, although the gap between the two coefficients is inferior, $R^2 = 0.968$ *versus* $R^2_{\text{adj}} = 0.871$. As in the previous case, the number of parameters is supporting the results obtained for TTAs yield.

Based on the information of Table 4, the non-significant coefficients (p-value > 0.05) were purged from the full models, giving rise to the reduced models (RM) containing only the significant ones. Each RM was fitted to experimental data and then uncoded, i.e. converted into the real factor variables and respective level values by back substitution of Eq. (4). The final uncoded models are presented in Table 5.

Table 4 - Regressed coefficients and individual significance at 95% confidence level of the RSM polynomial given by Eq. (2), along with the calculated determination coefficients for the full model (FM).

	Total extraction yield, η_{Total}		TTAs extraction yield, η_{TTA}	
	FM	*p*	FM	*p*
β_0	2.88111	**0.002** (*)	0.31	**0.005** (*)
β_1	0.13667	0.406	0.04	0.178
β_2	0.91667	**0.008** (*)	0.215	**0.003** (*)
β_{11}	-0.10667	0.693	-0.03	0.503
β_{22}	-0.12667	0.642	0.095	0.096
β_{12}	0.0075	0.968	-0.0025	0.934
R^2	0.935		0.968	
R^2_{adj}	0.741		0.871	

(*) Values in bold represent significant coefficients.

Table 5- Reduced experimental models (RM) fitted to the responses listed in Table 3.

Response	Reduced model	R^2	R^2_{adj}	Eq.
η_{Total} (wt.%)	$Y_1 = 1.80889 + 0.36667 \times \text{EtOH}$	0.905	0.873	(6)
η_{TTA} (wt.%)	$Y_2 = 0.13833 + 0.086 \times \text{EtOH}$	0.879	0.838	(7)

3.1.1 Total extraction yield

As discussed above for Table 4, ethanol content was the only factor with important impact upon η_{Total}. It exhibits a positive linear contribution to this response as it jumps from 0.0 to 5.0 wt.%, which is clear from Figure 2 where the plotted 3-D surface corresponds to a plane, i.e. horizontal for pressure variations (non-significant factor) and sloped according to the ethanol content. From the calculated determination coefficients, $R^2 = 0.905$ and $R^2_{\mathrm{adj}} = 0.873$ (Table 5), one may consider the reduced model is adequate to fit our experimental data, although their values became lower after eliminating the non-significant parameters of Table 4 from the full model.

The relevance of the ethanol concentration is understandable when comparing the edges of the surface plotted in Figure 2. Here, from the corner of 200 bar and 0.0 wt.% EtOH (run SFE1) to that at 200 bar and 5.0 wt.% EtOH (run SFE3), η_{Total} jumps from 1.52 to 3.16 wt.%, i.e. more than doubles the extraction yield. In turn, an increment of 100 bar passing from the same starting point (run SFE1: 200 bar, 0 wt.% EtOH) to 300 bar and 0.0 wt.% EtOH (run SFE7) enhances η_{Total} by only 33 %. The dominance of ethanol concentration in relation to pressure may be analysed from two perspectives. (i) In one hand, the range of extraction pressures considered in this study falls within the usual working region found in the literature (*ca*. 200-400 bar) [17], which suggests this factor is already in a typically good operating region for SFE applications. Moreover, the role played by pressure above 300 or 400 bar would be only slightly productive taking into account the supercritical solvent density is already high. Similar results were found for spent coffee grounds [20] and apricot pomace [21]. (ii) On the other hand, the intrinsic importance of cosolvent content under the context of SFE of *E. globulus* leaves should be considered recalling the large gap between the yields attained when this biomass is Soxhlet extracted using ethanol and when Soxhlet extracted with dichloromethane (taken as a reference weakly polar solvent for SC-CO_2). According to available data [10], η_{Total} for ethanolic Soxhlet extracts reaches 25.90 wt.%, while the same method produces only 7.32 wt.% with dichloromethane. Even though such results refer to distinct temperatures (i.e., boiling points), the key variable is unquestionably the solvent polarity. Hence the said gap may justify itself the importance of increasing the content of

ethanol in the supercritical mixture, as it may expand the attainable yields just like when a weakly polar solvent (dichloromethane) is replaced by a polar one (ethanol).

In the whole, our results show that the optimized operating conditions for the production of a bulk extract from *E. globulus* leaves rely on the highest ethanol content studied (5.0 wt.%), being independent of pressure as long as it remains within 200 and 300 bar. This maximum can be observed in the red region of Figure 2 as the line at the top of the plane.

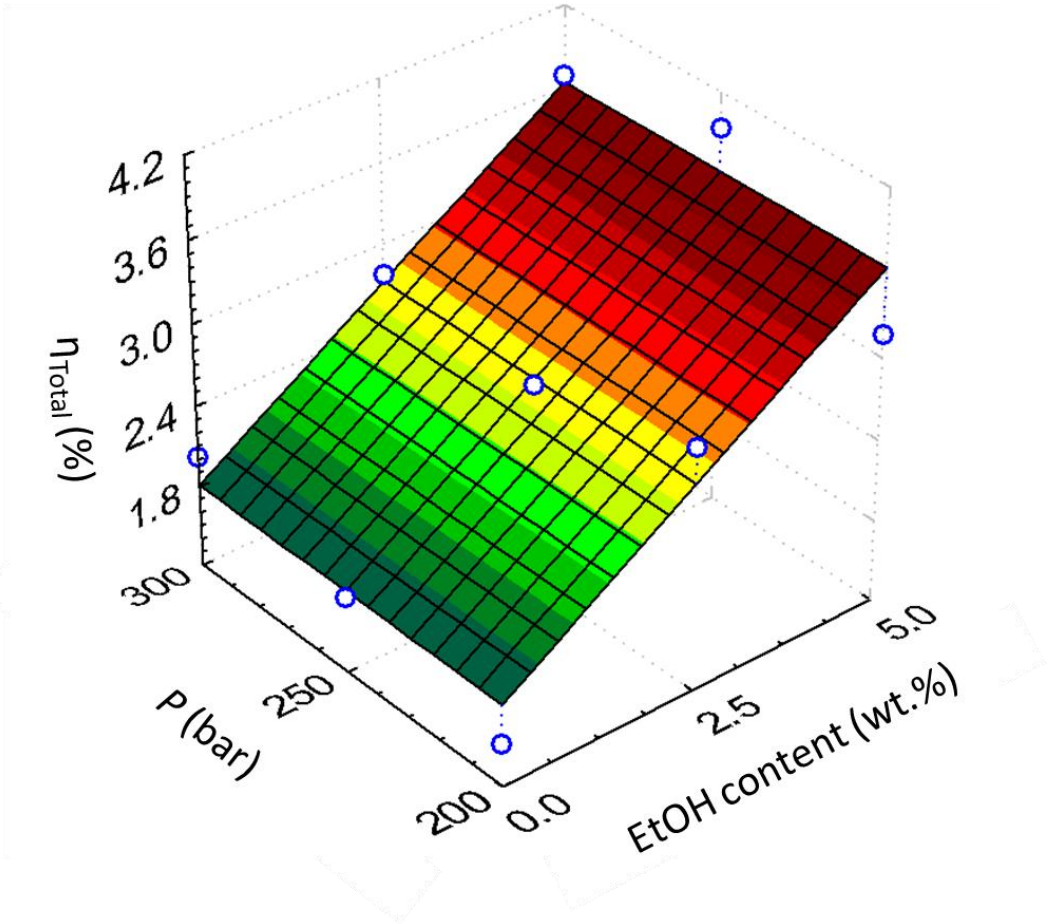

Figure 2 - Response surface plotting the influence of pressure and ethanol content on total extraction yield (reduced model). Dots are experimental data, and surface is given by Eq. (6) (Table 5).

3.1.2. TTAs extraction yield

The fitted η_{TTA} surface (reduced model) is plotted in Figure 3 together with experimental results, and its visual aspect is identical to the above discussed η_{Total} model. The influence of pressure was once again negligible in comparison with ethanol content. The

reduced model can be considered adequate to represent TTAs yield, as the respective determination coefficients obtained are very similar: $R^2 = 0.879$ and $R^2_{\text{adj}} = 0.838$.

The positive impact of ethanol concentration can be emphasised by comparing vertex conditions of η_{TTA} surface: between 200 bar/0.0 wt.% EtOH (run SFE1) and 200 bar/ 5.0 wt.% EtOH (run SFE3), η_{TTA} goes from 0.15 to 0.53 wt.%, respectively, i.e. more than triples the yield value. In turn, the weak effect of pressure can be assessed by picking the same starting vertex (run SFE1: 200 bar, 0.0 wt.% EtOH) and comparing it with the one at 300 bar/0.0 wt.% EtOH (run SFE7): in this case an increment of 113 % from 0.15 to 0.32 wt.% was observed. Nonetheless, if the same pressure step is considered under fixed 5.0 wt.% ethanol (SFE3 *vs*. SFE9), the TTAs yield increase is only 13 %.

As a result, and similarly to the previously discussed for η_{Total} (Section 3.1.1), the optimized conditions for η_{TTA} are located along the surface top edge (depicted in red color), which corresponds to the maximum EtOH content studied.

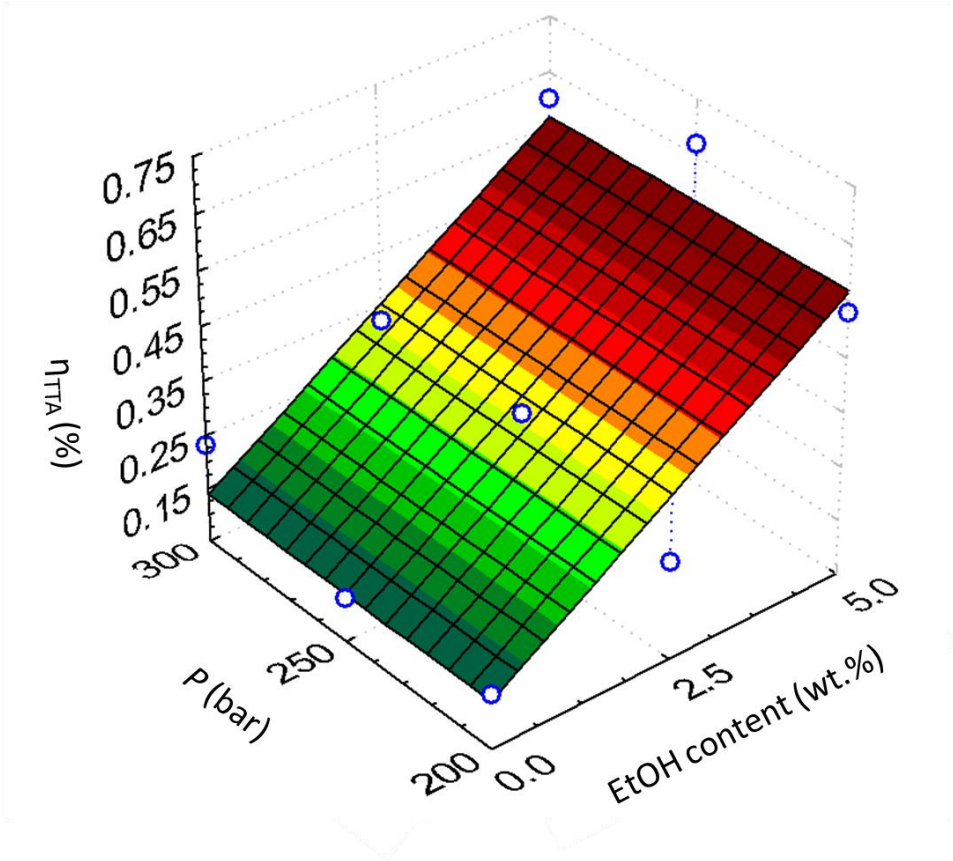

Figure 3 - Response surface plotting the influence of pressure and ethanol content on triterpenic acids extraction yield (reduced model). Dots are experimental data, and surface is given by Eq. (7).

3.2 Comparative analysis of *E. globulus* leaves and bark

This section presents a comparison between the results of this work and those taken from literature for *Eucalyptus globulus* leaves and bark. In all cases the extraction yields of Soxhlet with dichloromethane are taken as reference values, and the SFE results correspond to the best experimental conditions reported. Accordingly, Table 6 compiles the following η_{Total}, η_{TTA} and c_{TTA} values: (i) experimental results of run SFE6 (this work; *E. globulus* leaves); (ii) dichloromethane Soxhlet results for *E. globulus* leaves published by Rodrigues et al [10]; (iii) SFE of *E. globulus* bark at 200 bar/40 °C/5.0 wt.% EtOH, retrieved from Domingues et al [7]; and (iv) dichloromethane Soxhlet assay for *E. globulus* bark from de Melo et al [6].

Instructive insights can be taken when the optimised results of Table 6 are compared. For instance, the SFE of leaves exhibits a TTAs yield advantage in relation to bark, ($\eta_{\mathrm{TTA}} = 0.67$ wt.% against 0.50 wt.%), but a significant disadvantage in terms of TTAs concentration of extracts ($c_{\mathrm{TTA}} = 17.0$ wt.% versus 40.2 wt.%). Consequently, bark is a poor source of TTAs when supercritical solvents are used, but generates extracts much richer in TTAs than leaves.

Table 6 - Values of η_{Total}, η_{TTA} and c_{TTA} for dichloromethane Soxhlet extraction and SFE (under optimized conditions) of *E. globulus* bark and leaves. The ratio $\eta(\mathrm{SFE})/\eta(\mathrm{Soxhlet})$ estimates the recovery of bulk extract or of TTAs by SFE.

Response	*E. globulus* bark			*E. globulus* leaves		
	Soxhlet (CH_2Cl_2)	SFE (200 bar/5.0 wt.% EtOH)	$\frac{\eta(\mathrm{SFE})}{\eta(\mathrm{Soxhlet})}$	Soxhlet (CH_2Cl_2)	SFE 6 (250 bar/5.0 wt.% EtOH)	$\frac{\eta(\mathrm{SFE})}{\eta(\mathrm{Soxhlet})}$
η_{Total} (wt.%)	1.31[a]	1.23[b]	0.9	7.32	3.95	0.5
η_{TTA} (wt.%)	0.67[a]	0.50[b]	0.8	1.34	0.67	0.5
c_{TTA} (wt.%)	50.1[a]	40.2[b]	-	18.3	17.0	-

[a] data taken from Melo et al (2012) [6]; [b] data taken from Domingues et al (2013) [22].

From the perspective of total yield, while Soxhlet extraction of leaves rendered η_{Total} about 5.6 times higher than bark (7.32 *vs*. 1.31 wt.%), the SFE of leaves triples the result for bark (3.95 *vs.* 1.23 wt.%). This shows that the potential of the two morphological parts is

considerably different, where leaves offer a much higher amount of extractives than bark. Nevertheless, when dealing with *E. globulus* bark, an optimized SFE removes 90 % of the reference Soxhlet value, which contrasts with 50 % for *E. globulus* leaves. Such dissimilar performances might be due to the distinct granulometries of leaves (gross pieces) and bark (ground), and also to existence of a cuticular wax layer at leaves surface, which has been object of specific studies recently [10,12].

3.3 Supercritical extraction curves of pretreated/untreated biomass

The SFE performance of *E. globulus* leaves has still margin for improvement, namely in terms of pretreating the biomass before extraction. Therefore, three kinetic curves were measured during 6 h under the optimised conditions of section 3.1 (run SFE6: 250 bar/5.0 wt.% EtOH) during 6 h, using the following biomass:

(i) original or untreated eucalypt leaves. Assay hereafter called “SFE Natural”;

(ii) ground leaves with average particle size d_p > 20 mesh (d_p < 1 mm). Assay hereafter labelled “SFE Ground”;

(iii) dewaxed gross size leaves, based on the removal procedure of Domingues et al. [3]. Assay henceforth called “SFE Dewaxed”.

The kinetic curves, $\eta_{\mathrm{Total}}(t)$, obtained for the biomasses listed above are graphed in Figure 4. At $t = 6$ h, the total extraction yield of “SFE Natural” is extremely coherent with the previously measured run SFE6, as $\eta_{\mathrm{Total}}(t = 6\ \mathrm{h};\ \text{"SFE Natural"}) = 3.86$ wt.% and $\eta_{\mathrm{Total}}(\mathrm{SFE6}) = 3.95$ (Table 6).

When ground leaves are used, the final yield ($t = 6$ h) reaches 5.90 wt.%, which represents an enhancement of 52.8 % over the bulk extract obtained in “SFE Natural”. It thus confirms that decreasing particle size in this range strongly influences mass transfer since interfacial area increases and intraparticle resistance decreases. Equivalent improvements were obtained by Fiori et al. with SFE of grape seed oil [23], and de Melo et al. with SFE of cork samples [24]. Furthermore, the advantage of grinding the *E. globulus* leaves is particularly noticed during the first period of extraction, where the mass of extract collected

in the first hour was 2.38 times higher than for untreated biomass. Consequently, a kinetic gain owing to the biomass grinding is notorious for the studied system.

With reference to the dewaxed biomass, the SFE curve exhibits a much slower pace than the untreated biomass, attaining only $\eta_{\mathrm{Total}}(t = 6\ \mathrm{h}) = 1.66$ wt.%. This cannot be confounded with a kinetic disadvantage imparted by the dewaxing procedure, because one should bear in mind that this pretreatment removes a significant amount of non-wax extractives [12], giving rise to a preliminary fractionation of the raw material. Accordingly, the biomass that was extracted after the dewaxing protocol was in fact poorer in extractives by about 4.17 wt.% [10] (the yield rendered by the dewaxing procedure per se). Hence, if the dewaxing removes part of the solutes, an inferior η_{Total} curve could be measured in the case of run "SFE Dewaxed". If the two extracts in series are summed (the dewaxing one and the SFE one for t = 6 h), a global yield of 5.83 wt.% is attained, which is curiously similar to the yield reached by "SFE Ground" after 6 h. In conclusion, from the point of view of bulk extract, both approaches (grinding or dewaxing) are equivalently more productive than if the SFE relies on the straightforward processing of untreated *E. globulus* leaves.

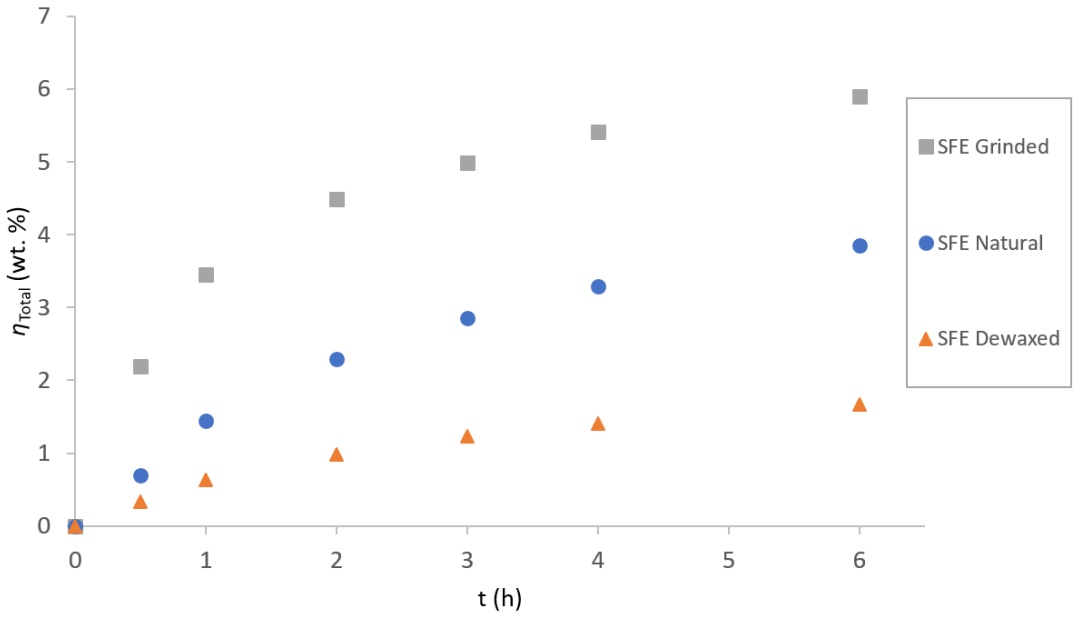

Figure 4 - Cumulative curves of total extraction yield measured under the optimised conditions of run SFE6 (200 bar/5.0 wt.% EtOH) in the case of: pretreated ("SFE Dewaxed" and "SFE Ground") and untreated ("SFE Natural") *E. globulus* leaves.

The experimental data of the three previous extraction curves were analysed by GC-MS to determine the cumulative TTAs yield ($\eta_{\mathrm{TTA}}(t)$) and cumulative TTAs concentration in the extracts ($c_{\mathrm{TTA}}(t)$) plotted in Figures 5A and B, respectively. From such $\eta_{\mathrm{TTA}}(t)$ results, the final values obtained ranged from 0.24 wt.% ("SFE Dewaxed") to 0.64 wt.% ("SFE Natural"), while ground biomass ("SFE Ground") scored 0.62 wt.%, which is practically the same of untreated biomass. It is worth noting the consistency between the final η_{TTA} rendered by the natural biomass (0.64 wt.%) and the one obtained in Section 3.1 from the experimental optimization study (0.67 wt.%; run SFE6 in Table 3).

From Figure 5.A, the resemblance of $\eta_{\mathrm{TTA}}(t)$ for the natural biomass and the ground biomass is immediate, showing that in terms of TTAs recovery the reduction of particle size is not advantageous, i.e. does not increase the amount of extracted TTAs.

With respect to the dewaxed sample, its $\eta_{\mathrm{TTA}}(t)$ curve is systematically lower than that for untreated biomass, ending up in a TTAs yield 2.5 times smaller than the "SFE Natural" curve. Again, such underperformance can be explained by the impoverishment of the *E. globulus* leaves during waxes removal (according to Rodrigues et al. [10] the η_{TTA} of this preliminary step is 2.79 wt.%), instead of any disadvantage imparted by the dewaxing procedure upon the vegetal matrix. Summing up both contributions, a final TTAs yield of 3.05 wt.% is obtained, which is 4.8 times higher than that for "SFE Natural".

The cumulative TTAs concentration corresponding to the three biomasses is illustrated in Figure 5.B. At first glance, the untreated biomass ("SFE Natural") attained the highest $\mathrm{c}_{\mathrm{TTA}}$ values along time, from 3.0 wt.% ($t = 0.5$ h) up to 16.5 wt.% ($t = 6$ h). In turn, the ground biomass outcasts from this trend, exhibiting extracts with TTAs concentration between 1.2 wt.% (t= 0.5 h) and 10.5 wt.% ($t = 6$ h). This result owes to the fact that the higher η_{Total} of ground leaves combined with non-enhanced η_{TTA} gives rise to lower TTAs concentration (please note that $\mathrm{c}_{\mathrm{TTA}}(t) = \eta_{\mathrm{TTA}}(t)/\eta_{\mathrm{Total}}\,(t)$). Actually, after grinding the leaves more solutes are effectively extracted but not the TTAs specifically, leading to extracts where the target compounds become more diluted.

In what concerns the $\mathrm{c}_{\mathrm{TTA}}(t)$ of dewaxed leaves, its evolution with time resembles the results for untreated samples, thus revealing that despite poor in TTAs (lower η_{TTA} along

time) the dewaxed biomass produces extracts as rich in TTAs as the untreated one. This undoubtedly contrasts with data obtained for “SFE Ground”, and suggests that the TTAs are somehow concentrated at leaf surface, thus benefiting from processing non-ground biomass.

In the whole, the kinetic study performed here is revealing of the intricacy of this vegetal biomass for SFE, which offers different pathways depending on the major interest in bulk extracts, high TTAs recovery, or concentrated extracts in these bioactive compounds.

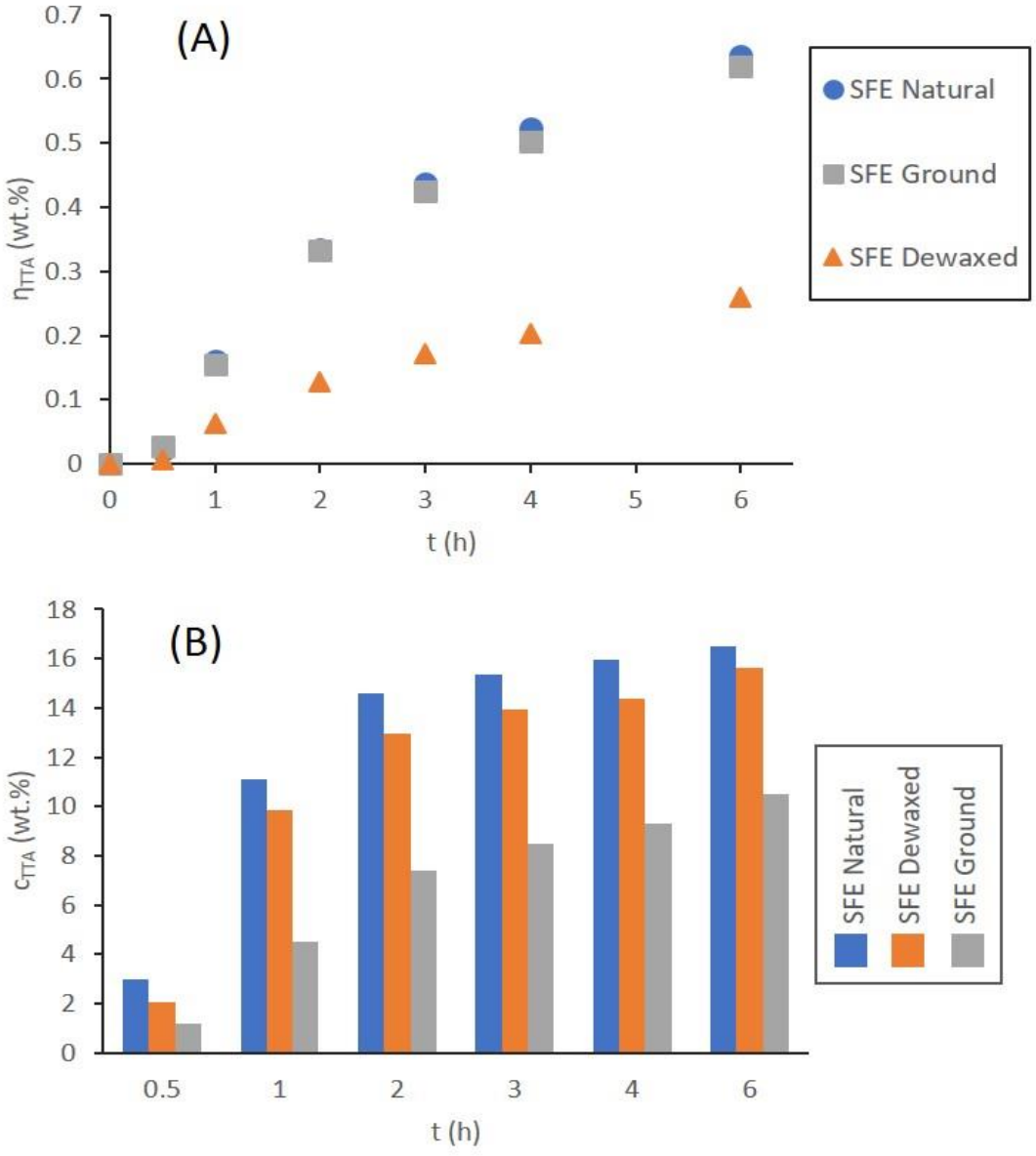

Figure 5 - (A) Cumulative TTAs extraction yield measured under the optimised conditions of run SFE6 (200 bar/5.0 wt.% EtOH) in the case of: pretreated (“SFE Dewaxed” and “SFE Ground”) and untreated (“SFE Natural”) *E. globulus* leaves. (B) Cumulative TTAs concentration for the corresponding extracts.

4.Conclusions

The SFE of *E. globulus* leaves was performed at different pressures (200, 250 and 300 bar) and ethanol contents (0.0, 2.5, 5.0 wt.%) with the objective to maximize the total and TTAs extraction yeild.

The optimised conditions correspond to 5.0 wt.% ethanol concentration, being essentialy independent of the extraction pressure in the range 200-300 bar. Under these condtions, the following yields were obtained: $\eta_{\mathrm{Total}} = 3.95$ wt.% and $\eta_{\mathrm{TTA}} = 0.67$ wt.%.

In terms of biomass pretreatments, grinding enhanced η_{Total} up to 5.90 wt.% at *t* = 6 h, while a dewaxing pretreatment lowered η_{Total} down to 1.66 wt.%. With respect to TTAs, the untreated biomass achieved the best results, namel, $\eta_{\mathrm{TTA}} = 0.64$ wt.% and $c_{\mathrm{TTA}} = 16.5$ wt.% for *t*=6 h. The grinding of the biomass gave rise to $\eta_{\mathrm{TTA}} = 0.62$ wt.% at *t*=6 h, very near the value for untreated leaves, which proves that more solutes distinct from TTAs were being removed. The dewaxing pretreatment yielded less TTAs, but presented TTAs concentration in the extracts similar to the untreated one (15.6 wt. % for “SFE Dewaxed”) maintaing a TTAs rich extract.

In the whole, the initial optimization results were confirmed by the kinetic extraction curves, denoting the importance of pressure and cosolvent on SFE, where cosolvent (ethanol) assumed a leading role within the range of conditions studied. The pretreatments presented interesting results, showing that, from the dewaxing and “SFE Dewaxed” results, the TTAs might be located in external parts of the leaf and, the usefulness of the grinding is not directed to TTAs extarction.

5. Acknowledgements

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IV. Final conclusions and future work

This work intended to study the potential of *E. globulus* leaves as a source of extracts rich in bioactive triterpenic acids, namely ursolic, oleanolic, betulinic and betulonic acids. Accordingly, Soxhlet extractions with dichloromethane, ethanol and methanol were firstly performed, as well as supercritical fluid extraction (SFE) using carbon dioxide, and FTIR-ATR, GC-MS and SEM techniques were employed to characterize the biomass and the produced extracts, and to compare them through a Cluster Analysis. Moreover, an experimental optimization was specifically performed in the case of SFE, aiming at higher TTAs recoveries. Finally, SFE kinetic curves were measured using *E. globulus* leaves with and without preliminary pretreatment of biomass, namely, grinding or dewaxing pretreatments.

From the initial extractions of *E. globulus* leaves, total extraction yield (η_{Total}) values were significantly dependent on the chosen methods and solvents, with a maximum of 30.34 wt.% being obtained for methanol (Soxhlet), and the minimum of 1.52 wt.% for SFE (200 bar/40 ºC/5.0 wt.% EtOH). The best SFE result at this stage represented a lower recovery (in relation to the dichloromethane Soxhlet yield) when compared to data from literature for *E. globulus* bark, which could be due to the gross granulometry of the studied leaves and also to cuticular waxes present at leaves surface. Through the removal of this layer, a wax rich extract is produced, which exhibits also high TTAs yield and concentration: 2.79 wt.% and 66.8 wt.%, respectively. However, the further extraction of dewaxed leaves leads to lower TTAs yields in comparison to the Soxhlet extraction of untreated biomass with the same solvent.

Concerning the experimental optimization of SFE of eucalypt leaves, the conditions that maximize TTAs extraction ($\eta_{\mathrm{TTA}} = 0.64$ wt.%) and extract concentration ($c_{\mathrm{TTA}} = 16.5$ wt.%) correspond to carbon dioxide modified with 5.0 wt.% ethanol, pressures in the range 200-300 bar, flow rate of 12 g_{CO2} min^{-1} and 40 ºC. These results were not improved by the pretreatments implemented here: (i) Biomass grinding increases the global amount of extractives but maintains TTAs yield (0.62 wt.%), thus resulting in more diluted extracts ($c_{\mathrm{TTA}} = 10.5$ wt.%). (ii) The dewaxing pretreatment presented interesting results as the obtained TTAs content was similar to untreated leaves ($c_{\mathrm{TTA}} = 15.6$ wt.%), but the respective yield was lower ($\eta_{\mathrm{TTA}} = 0.26$ wt.%). However, if the TTAS yields of dewaxing (preliminary

stage) and SFE (subsequent stage) are summed, a global η_{TTA} of 3.05 wt.% is obtained, which largely overcomes the yields obtained for the straightforward SFE of non-treated biomass.

In the whole, this thesis provides useful information for the valorisation of *E. globulus* leaves in terms of extraction methods, solvents and biomass pretreatment.

In terms of future work, the characterization of this biomass can be further refined, either towards individual TTAs profiles, but also regarding other families of molecules such as phenolic compounds. The interaction of wax layer constituents and removal procedures can also benefit from further investigation. Finally, at SFE level, harsher experimental conditions should also be tested, not only cosolvent content, since its effect has proven to be determinant for the performance of this technology, but also pressure and temperature.