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Green extractions to obtain value-added elephant grass co-products in an ethanol biorefinery

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ABSTRACT

The replacement of fossil fuels with renewable energy sources depends on the adoption of greener technologies and integrated processes within a biorefinery. In this study, organic molecules with high added value and fermentable sugars were obtained by combined extractions and enzymatic hydrolysis from elephant grass leaves and stems. Fatty acids, sterols, alcohols and phenolics were obtained in different concentrations using green extractions with supercritical carbon dioxide and pressurized liquids (PLE). The extract composition was mainly related to the solvent polarity and the best yield was achieved using water and ethanol as solvents in PLE (7.5% and 7.7% for leaves and stems, respectively). The extractions alone, without any other treatment, were able to increase the enzymatic digestibility of leaves by 50% due to the increase in the substrate wettability. The extractions also did not interfere with the biomass morphology, which allows the posterior use of the substrate for other applications. The results presented herewith showed that adopting extractions as the first step in an ethanol biorefinery is a promising way to achieve the biomass valorization and full use, together with more sustainable processes to obtain chemicals for the food, cosmetic and pharmaceutical industries.

1. Introduction

The production of chemicals and fuels from sources other than oil is a challenging but crucial scientific problem, aiming at more sustainable industrial processes. Lignocellulosic biomass is a promising platform for this purpose, especially due to its chemical composition and high availability (Rosales-Calderon and Arantes, 2019). In line with the renewable source of the raw materials, the production processes must be as green and sustainable as possible, with minimal waste generation, according to Green Chemistry principles (Herrero and Ibañez, 2018).

Several products can be obtained from biomass extracts, including phenolics, sterols, and hydrocarbons, which can be applied in coatings, polishes, detergents, anti-oxidants, nutraceuticals and cosmetics (Yu et al., 2019). The solid that remains after the extractions can be converted into chemicals, energy or biofuels via integrated processes. This approach can add value to the production chain and enable chemical industries to adopt cleaner production processes (Attard et al., 2018).

Conventional methods of extraction of plant compounds use volatile organic solvents, such as dichloromethane, chloroform, hexane, and toluene (Santos et al., 2015). However, several toxicological and environmental problems are related to the use of these solvents, which limits the applications of the produced extracts (Deswarte et al., 2006). The termed non-conventional methods are interesting substitutes to replace the traditional extraction procedures, due to the reduced use of synthetic and organic chemicals and shorter operation times. Some examples of these non-conventional techniques are the extractions using ultrasound (Vinatoru et al., 1997), pulsed electric field (Corrales et al., 2008), supercritical fluids (Santos et al., 2015) and pressurized liquids (Pereira et al., 2019).

Supercritical carbon dioxide ($scCO_2$) is an alternative to the use of non-polar toxic organic solvents in extraction processes. This method benefits from the compelling properties of CO_2, which is a non-inflammable, non-toxic, extensively available, recyclable and non-regulated solvent (Subramaniam et al., 1997). In supercritical

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conditions, CO_2 has low surface tension, high diffusivity and low viscosity, which enhances its extraction ability (Brunner, 2013). Its use has received attention especially in the food, pharmaceutical and cosmetic industries, due to the advantage of leaving no solvent residues. Considering the extraction of valuable organic compounds from lignocellulosic biomass, recent studies showed that $scCO_2$ extraction (SFE) is a promising technique that has been used to obtain extracts from residues of sugarcane (Attard et al., 2015a), date palm (Al Bulushi et al., 2018), wheat straw (Deswarte et al., 2006), maize (Attard et al., 2015b), giant miscanthus (Attard et al., 2016) and forestry biomasses (Fojtová et al., 2010). Applying SFE to these substrates allowed the extraction mainly of fatty acids and sterols, as well as *n*-policosanols and long-chain aldehydes.

However, the low polarity of CO_2 is a drawback for the extraction of polar compounds, an alternative technique being pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), enhanced solvent extraction (ESE) or high-pressure solvent extraction (HPSE) (Nieto et al., 2010). This method allows the use of polar solvents, including green solvents, such as water and ethanol. Due to the high pressure applied during the system operation, the solvent remains in the liquid phase beyond its normal boiling point, which facilitates the extraction (Azmir et al., 2013). The higher extraction temperature increases the solubility of the target compounds and decreases the viscosity and the surface tension of the solvent, enabling a faster and more efficient extraction with a reduced amount of solvent (Hayes, 2014). Like SFE, PLE is also an emerging technique, used mainly to prepare samples (Malvar et al., 2020) and extract bioactive compounds from plants, such as phenolics from grape marc (Pereira et al., 2019), parsley flakes (Luthria, 2008) and buriti shells (Rudke et al., 2019).

In a biorefinery, the remaining extracted solid can be used for other purposes, adding value to the production chain. This will also contribute to the sustainability of the process, minimizing the amount of residues. In the case of lignocellulosic substrates, the extracted solid has a rich chemical composition and can be converted into several products, including chemicals, biofuels, and materials. Extractions could thus be used as the first step in a complex and branched biorefinery scheme, generating multiple products (Arshadi et al., 2016). An example of this approach is the work developed by Attard and coworkers, who proposed the adoption of SFE for the extraction of valuable waxy compounds from maize stover before the production of 2G ethanol (Attard et al., 2015b).

Another possibility is the use of SFE and PLE in sequence to extract both non-polar and polar compounds. Currently, these two extraction procedures are normally performed as stand-alone methods, and only a few studies use them in an integrated way (Silva et al., 2018). Silva and coworkers evaluated the sequential use of SFE and PLE with ethanol to extract oil and pigments from turmeric rhizomes. The post-extraction solids contained *ca.* 80% of carbohydrates, mainly starch and dietary fibers, which were proposed to be used by the food industry as bioactive films and additives (Silva et al., 2018).

A promising lignocellulosic substrate for fractionation is elephant grass, also known as Napier grass, which has been investigated to produce biofuels and biomaterials due to its high productivity, chemical composition, and adaptability to unfertile and dry soils (Lima et al., 2014). Elephant grass can be harvested four times a year in amounts that vary depending on the region, exceeding 50 Mg of dry biomass/ha in Puerto Rico (Sotomayor-Ríos et al., 1997) and Thailand (Rengsirikul et al., 2011) and 30 Mg of dry biomass/ha in the southern U.S. (Lamb et al., 2018). Other grass biomasses used for ethanol production show lower or similar productivity, such as giant miscanthus (11 Mg of dry biomass/ha in U.S. Southeast) (Dien et al., 2020), Bermuda grass (17 Mg of dry biomass/ha in U.S. Southern) (Anderson et al., 2008), and sugarcane bagasse (30 Mg of dry biomass/ha in Brazil) (Lima et al., 2014). Another advantage is that elephant grass is rich in extracts, with ethanol-extractable compounds reaching 12–20% on a dry mass basis (Nascimento and Rezende, 2018). The amount obtained from milled leaves extracted for 8 h is four times higher than the one obtained from sugarcane bagasse (*ca.* 3% of extracts) under similar conditions (Lima et al., 2014).

Previous studies have demonstrated the potential of elephant grass fractionation to produce biofuels and materials. For example, the yield of ethanol that can be extracted from elephant grass per hectare (11529.4 L/ha) is higher than that of other biomasses, such as sugarcane bagasse (8478.6 L/ha) and eucalyptus bark (7083.5 L/ha) (Lima et al., 2014). Cellulose nanocrystals prepared from elephant grass showed higher crystallinity and aspect ratio than those obtained from linter cotton and rice husks (Nascimento and Rezende, 2018), while the antioxidant activity of lignin extracted from this biomass was 8 times higher than that of corncob lignin, and 16 times higher than that of sugarcane bagasse lignin (Trevisan and Rezende, 2020).

Despite the potential of elephant grass to produce several products within a biorefinery, their extracts have been poorly exploited. To the best of our knowledge, there are no studies concerning the use of SFE or PLE alone or combined for elephant grass extraction. The only study reporting the characterization of elephant grass extractives used Soxhlet extraction with acetone as a solvent (Prinsen et al., 2012). The resulting extract was rich in fatty acids and sterols, which are molecules also found in SFE obtained from sugarcane bagasse (Attard et al., 2015a) and date palm residues (Al Bulushi et al., 2018).

In the present study, an integrated method to recover high value-added organic molecules from elephant grass was proposed using SFE and PLE, which are green extraction methods, followed by the use of the post-extraction solid to produce sugars that can be fermented into ethanol. These organic molecules have potential applications in the pharmaceutical, food and cosmetic industries, while ethanol production is a way to utilize the residual solid that remains after the extraction. This approach can enable the use of cleaner and sustainable methods to produce chemicals and fuels traditionally obtained from oil.

2. Material and methods

2.1. Materials

Elephant grass (*Pennisetum purpureum*) was kindly donated by Instituto de Zootecnia (Nova Odessa, SP, Brazil) and harvested 12 months after planting. The biomass was separated into leaves and stems, so that these parts could be individually studied. They were dried in a convection oven (Tecnal, TE-394/3) at 60 °C for 6 h, grounded in a knife mill (Arthur H. Thomas Co – Standard model 3) and passed through a 2 mm sieve. All the chemical reagents were used as received: CO_2 (99% purity) was obtained from White Martins; Ethanol (99.5% purity) from Synth; and Ethyl acetate (99.9% purity) from Panreac.

2.2. Extractions

Fig. 1 shows a schematic view of the four main extraction methods that were applied to the leaves and stems of elephant grass, together with the names of the corresponding extracts. SFE, PLE using water and ethanol as solvents and PLE using ethyl acetate were all applied directly to the *in natura* biomass, generating extracts referred to as SFE, PLE-WE and PLE-EA, respectively. PLE

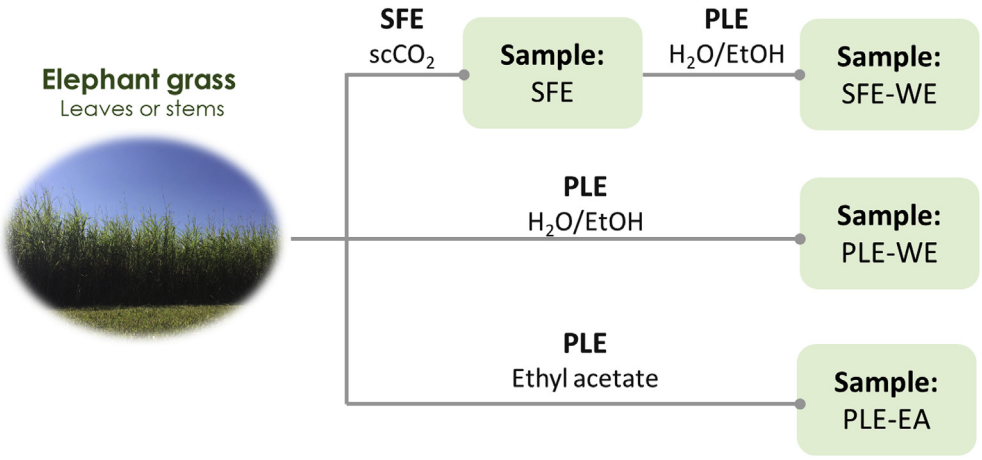

Fig. 1. Scheme of the four extraction methods applied to elephant grass in this study, and the corresponding names assigned to the extracts.

using water and ethanol was also applied to the solid that had been previously extracted via SFE, and the resulting sample was named SFE-WE. The extraction conditions will be further detailed in the next sections.

2.2.1. Supercritical carbon dioxide fluid extraction (SFE)

SFE was performed on elephant grass at the same conditions that were previously applied to sugarcane residues (Attard et al., 2015a): 40 °C and 350 bar using *ca.* 18 g of biomass and a solvent flow of 0.175 × 10^3 kg s^{-1}. Prior to the extractions, a kinetic assay was performed to define the amount of CO_2 necessary for elephant grass leaves and stems, and the obtained extracts were stored in the absence of light. More details on the process and on the kinetic assay are described in the Supplementary Material (Figs. S1–S2 and Table S1).

2.2.2. Pressurized liquid extractions (PLE)

PLE was performed in an accelerated solvent extractor (Dionex ASE 350, Thermo Scientific) using two types of solvents: 1) a mix of water and ethanol (1:1 v/v) (extracts SFE-WE and PLE-WE shown in Fig. 1, depending on the substrate) or 2) ethyl acetate (extract PLE-EA shown in Fig. 1). In each extraction, *ca.* 5 g of substrate were used and all extractions were performed at 100 °C in 3 cycles of 15 min each. The system was preheated for 5 min and purged with N_2 for 120 s. The pressure and the rate of solvent were 1500 psi (approximately 103 bar), and 70 mL/min, respectively. After the extractions, the solvent was evaporated under a N_2 flow and the extract was stored in the absence of light.

2.2.3. Soxhlet extractions

Soxhlet extractions were performed for comparison purposes with SFE and PLE and to determine the total of extractable compounds in elephant grass. Firstly, the extraction was performed using cyclohexane for 8 h to determine the total amount of non-polar compounds. After that, extraction with water and ethanol (1:1 v/v) as solvents was carried out for 24 h, using the solid that remained after the cyclohexane extraction to determine the total amount of polar extractives.

2.3. Enzymatic hydrolysis

The post-extraction solids underwent enzymatic hydrolysis using a Cellic CTec 2 cocktail (Novozymes) in citrate buffer (pH = 5) at 50 °C. The enzyme load used was 25 mg/g of substrate in a 1:40 solid:liquid ratio. After the hydrolysis (72 h), the system was heated to 95 °C for 5 min to denature the enzymes, and then centrifuged. An aliquot of 200 μl was collected from the sample supernatants and used to quantify glucose, xylose and arabinose via high performance liquid chromatography (HPLC).

2.4. Characterizations

2.4.1. Extract identification

All the extracts were analyzed via gas chromatography – mass spectrometry (GC-MS) and had to be dissolved prior to the analysis. The SFE extracts were dissolved in dichloromethane, the PLE-WE and SFE-WE extracts were dissolved in methanol, and the PLE-EA extracts were dissolved in ethyl acetate. In the case of PLE-WE and SFE-WE, only part of the total extractives could be dissolved in methanol, because some compounds that were soluble in water/ethanol under high pressure became insoluble as the pressure decreased. These samples were then centrifuged to separate the insoluble part and only the soluble fraction was analyzed via GC-MS. Prior to injection, all the extracts were filtered once more (0.22 μm of porosity).

The equipment used to analyze the extracts was an Agilent 7890 gas chromatograph coupled with an Agilent 5975C mass detector, using a HP-5MS column (30 m × 0.25 mm x 0.25 μm) and a single quadrupole analyzer. The compounds were ionized via electron impact (EI) and helium was used as the carrier gas at a rate of 1.0 mL min^{-1}. The column was heated at 10 °C.min^{-1} to 150 °C, kept at this temperature for 5 min, then heated to 300 °C at 5 °C.min^{-1}, and kept at constant temperature for another 5 min. The mass range analyzed was 30–550 u, and the data were processed with the Chemstation software. The compound identification was performed by comparing them with the standards of the library of the National Institute of Standards and Technology (NIST).

2.4.2. Sugar quantification

Sugar quantification was carried out in a high-performance liquid chromatography (HPLC) coupled to a refractive index detector. A BIORAD HPX87H column was used at 45 °C, with H_2SO_4 5 mmol.L^{-1} as mobile phase. The sugars were quantified according to a calibration curve using standards of glucose, xylose and arabinose.

2.4.3. Morphological analysis

The substrate morphology was analyzed before and after the extractions using a scanning electron microscope equipped with a field emission gun (FESEM-FEI Quanta 250), operating at 5 kV.

Dried samples were sputter coated with iridium prior to the analysis (Baltec, Oerlikon-Balzers) at 11.3 mV for 120 s. At least 20 images of each sample were obtained to ensure reproducibility.

2.4.4. Contact angle measurements

The static contact angles of water droplets (3 μL) deposited on the leaves *in natura* and after the Soxhlet extraction were measured using a Krüs EasyDrop goniometer and the Drop Shape Analysis software. The upper and lower surfaces of the unmilled leaves were analyzed by performing 3 measurements on each side.

3. Results and discussion

3.1. Extraction yields and extract composition

The sequential application of SFE and PLE is a strategy to fractionate and extract the maximum components possible from elephant grass. Due to the affinity of $scCO_2$ to non-polar compounds, such as hydrocarbons and fatty acids (Santos et al., 2015), the integration of SFE with PLE using polar solvents is an effective approach to optimize the extraction of compounds with different polarities, using green and non-toxic methods. Aiming at a cleaner production, the extractions in this study were performed using only solvents that follows the Safety, Health and Environment criteria (SHE) (Prat et al., 2015). In addition, to reduce the spending on the solvents used in SFE, a kinetic assay was performed to optimize the extraction time of both parts of elephant grass (leaves and stems) (Fig. S2 in the Supplementary Material).

Fig. 2 shows the extraction yields for leaves and stems, using four different extraction procedures. PLE with water and ethanol was the most efficient among the methods evaluated for both leaves and stems. Both PLE applied directly to *in natura* biomass (PLE-WE) or after SFE (SFE-WE) resulted in extraction yields between 7.5% and 8.0% for leaves, and 6.3% and 7.8% for stems. The use of water and ethanol in PLE led to higher yields in comparison to the use of ethyl acetate (PLE-EA), which corresponded to 3.8% and 1.4% of the total extracted from the leaves and stems, respectively.

The yields obtained using PLE-WE were *ca.* 9 times higher than those obtained using SFE for leaves, and 45 times higher for stems. The PLE-WE yields were also 2 times higher for leaves and 4 times higher for stems when ethyl acetate was used as solvent (PLE-EA). The better performance of PLE in terms of extraction yields is mostly due to the polar nature of most components in elephant grass, being also influenced by temperature, pressure and the diffusivity of the compounds in the solvent (Pronyk and Mazza, 2009).

The total amount of polar compounds extracted via Soxhlet using water and ethanol (1:1 v/v) for 24 h was 17.2 ± 0.2% for leaves and 12.6 ± 0.6% for stems. In turn, the non-polar compounds extracted via Sohxlet using cyclohexane for 8 h totalized 3.0 ± 0.2% for leaves and 1.6 ± 0.2% for stems. Considering that these are the maximum extractable amounts of each compound class, 28% of the total extractable non-polar compounds in the leaves and 10% of the total in the stems were obtained with SFE. On the other hand, 44% of the polar compounds in the leaves and 61% of the compounds in the stems were obtained with PLE-WE, compared to the total obtained in the Soxhlet extraction. PLE can also be performed with a variety of other solvents; however, it is important to highlight that high extraction yields could be obtained in this work with food grade and environmentally-friendly solvents (water, ethanol and ethyl acetate) (Calvo et al., 2007).

The relatively low extraction yields obtained with SFE (0.85% w/w for elephant grass leaves and 0.17% w/w for stems) are similar to those obtained for sugarcane residues (1.6% w/w for leaves and 0.5–0.8% w/w for bagasse) (Attard et al., 2015a). Despite the relatively low yields, $scCO_2$ is a green and food grade alternative for the extraction of non-polar components that would be more acceptable for applications in food and beverages (Attard et al., 2015a). Most of the conventional non-polar solvents, such as hexane, are toxic for the nervous system (Deswarte et al., 2006), and are listed as hazardous air pollutants in the Clean Air Act of the Environmental Protection Agency (EPA) (DeSimone, 2002). Conventional extractions are also time-consuming and require significant amounts of toxic solvents, resulting in great volumes of extraction waste to be treated.

A major bottleneck in SFE extraction is the high energy demand to keep the solvent in supercritical condition. One alternative to compensate this problem is the use of the biomass that remains after the extractions to generate energy for the biorefinery, as proposed by Attard et al. (Attard et al., 2015). Another interesting proposal is the one adopted in the present study, i.e., the use of the post-extraction solid to generate other products with added value, followed or not by the final burning of the residues to release energy. The identification of the extracted compounds that can be obtained from elephant grass as co-products will be discussed in the next sections.

Considering leaves and stems, a total of 83 compounds were identified among the extracts via GC-MS. The complete list of compounds is detailed in Table S2 (Supplementary Material), and they were grouped into 9 chemical classes: acids; alcohols and phenolics; aldehydes; amides; esters; hydrocarbons; ketones;

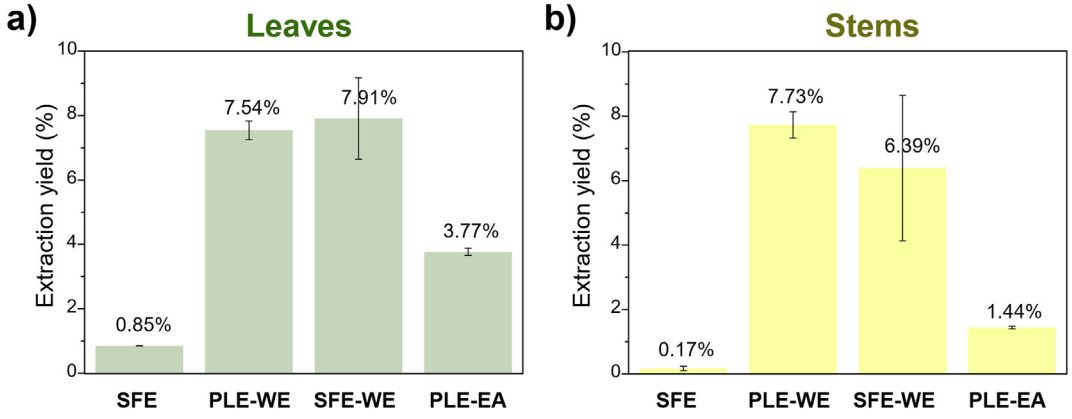

Fig. 2. Extraction yields for leaves (a) and stems (b) obtained after different extraction procedures. SFE: $scCO_2$ extraction; PLE-WE: PLE using water and ethanol; SFE-WE: Sequential extraction using SFE, followed by PLE-WE; PLE-EA: PLE using ethyl acetate. The extractions were carried out in duplicate, and the error bars correspond to the standard deviations.

sterols and "others". The main compounds identified were acids, alcohols and phenolics, hydrocarbons and sterols, and their percentages are depicted in Fig. 3. As expected, the extract composition is related to the solvent polarity. For example, fatty acids were mainly extracted via SFE from both leaves and stems (up to 21% for leaves and 18% for stems), while alcohols and phenolics were mainly extracted via PLE-WE and SFE-WE (up to 27% for leaves and 35% for stems) due to the use of polar solvents. The extracts obtained using ethyl acetate are more complex because this solvent is able to extract both polar and non-polar compounds. PLE-EA extracts were composed of fatty acids (up to 9% of the extracts obtained from both leaves and stems), alcohols and phenolics (*ca.* 15% for leaves and 25% for stems) and also sterols (*ca.* 16% for both).

The sequential extraction using SFE and PLE allows the fractionation of the extracts, reducing the cost and the time required to separate the extracted mixtures. This characteristic can be used to obtain acids, hydrocarbons and sterols from elephant grass via SFE, by separating them from alcohols and phenolics that would be obtained in the subsequent PLE extraction. The four main classes of compounds identified among the elephant grass extracts (acids; alcohols/phenolics; hydrocarbons; and sterols) are secondary metabolites, facilitating the plant interactions with the environment. In humans and animals, these compounds have pharmacological effects and can be used by the pharmaceutical, food and cosmetic industries (Azmir et al., 2013).

The most abundant compounds identified in SFE extracts were fatty acids (Table S2). Some examples are linoleic acid (3.1% for leaves and 4.1% for stems), α-linolenic acid (8.1% for leaves), palmitic acid (7.1% for leaves and 6.1% for stems) and oleic acid (5.8% for stems). More than 50% of the fatty acids extracted from elephant grass via SFE were unsaturated, being classified as Omega-3, Omega-6 and Omega-9 (Fig. 4). Fatty acids have a hypocholesterolemic effect on serum cholesterol in humans (de Iongh et al., 2015), and α-linolenic acid also has cardioprotective effects (de Lorgeril et al., 1994). Moreover, unsaturated acids can be used by the food, cosmetic and pharmaceutical industries as platform molecules in lipase- and phospholipase-catalyzed processes to obtain several biotransformed products (Gill and Valivety, 1997).

Fatty acids and glycerol are obtained from the hydrolysis of triacylglycerols, which are energy sources for the plants, and components of cell membranes (Heldt and Piechulla, 2010). Glycerol can be converted into triacylglycerols or glycerol 3-phosphate, and then subjected to gluconeogenesis or glycolysis. In the food and pharmaceutical industries, this molecule is used as solvent, emulsifier and humectant (Quispe et al., 2013). Glycerol was extracted when a mixture of water and ethanol was applied directly to the biomass or after SFE. The PLE-WE extracts contain *ca.* 18% of glycerol for both leaves and stems, while this compound represents 27.1% of the SFE-WE extracts obtained from the leaves, and 20.0% of those obtained from the stems.

Phenol and coniferyl alcohol, used in lignin synthesis (Lora and Glasser, 2002), have applications as antioxidant agents and UV protectors, being especially useful for the dermo-cosmetic industries. Phenol was identified only in the extracts of leaves (2.7% in PLE-WE and 4.0% in SFE-WE), while coniferyl alcohol was identified in both parts of elephant grass, especially in the stems (up to 7.4% in the PLE-WE extracts). The use of the natural compounds present in elephant grass is thus a way to minimize the negative impact of the current sunscreen formulations on the marine environment (Trevisan and Rezende, 2020).

The sterols identified here are plant hormones, synthesized from isoprenoids, and responsible for controlling the plant development through stimulation of the unfolding of leaves and xylem differentiation, among other processes (Heldt and Piechulla, 2010). Sitosterol and β-sitosterol have potential applications as anti-inflammatory and anti-cancer compounds; in the metabolism of cholesterol by reducing the plasma LDL-cholesterol levels with minimal side-effects; and to treat atherosclerosis (Bradford and Awad, 2007). Sterols were one of the main compounds among the SFE and PLE-EA extracts due to their low polarity. Sitosterol makes up 4.0% of the SFE extracts obtained from the leaves, and 7.5% of those obtained from the stems, while among the PLE-EA extracts, it represents 7.6% and 7.5% for leaves and stems, respectively. Tocopherol, popularly known as vitamin E, was found in the leaves (2.8% via SFE and 3.2% via PLE-EA); this compound has prominent antioxidant properties that protects the cells against free radicals, and can be used in skin creams (McVean and Liebler, 1997).

The application of these compounds requires the separation and purification of the molecules. A method that has been widely used for this purpose is the fractionation of target compounds of essential oils via fractional distillation under a vacuum (Perini et al., 2017). This approach was used by Perini and coworkers to separate compounds of orange essential oil. Due to the different boiling points, the terpenes were firstly evaporated and then separated from the oxygenated compounds, which remained at the bottom of the column (Perini et al., 2017). Another alternative, especially in the laboratory, is the use of preparative HPLC methodologies (Russo

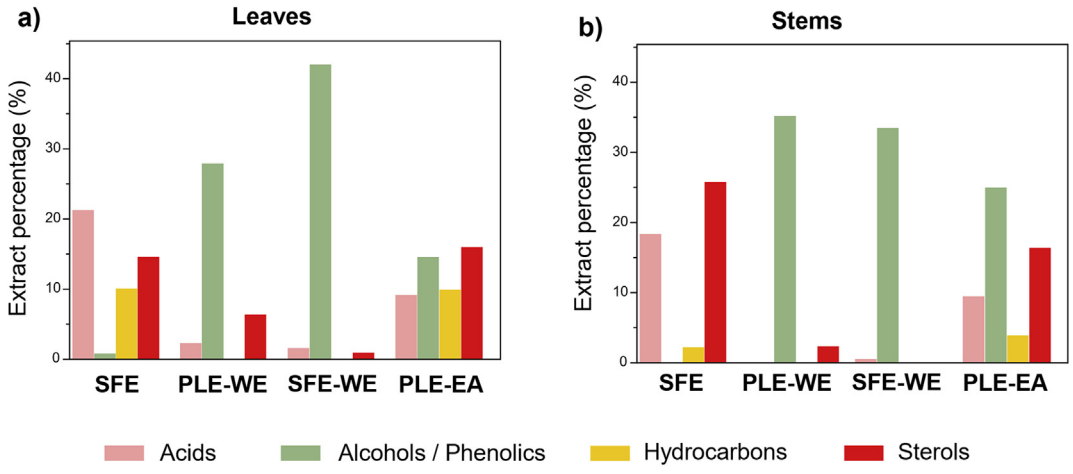

Fig. 3. Percentage composition of the extracts for leaves (a) and stems (b), as estimated via GC-MS. SFE: $scCO_2$ extraction; PLE-WE: PLE using water and ethanol; SFE-WE: Sequential extraction using SFE, followed by PLE-WE; PLE-EA: PLE using ethyl acetate.

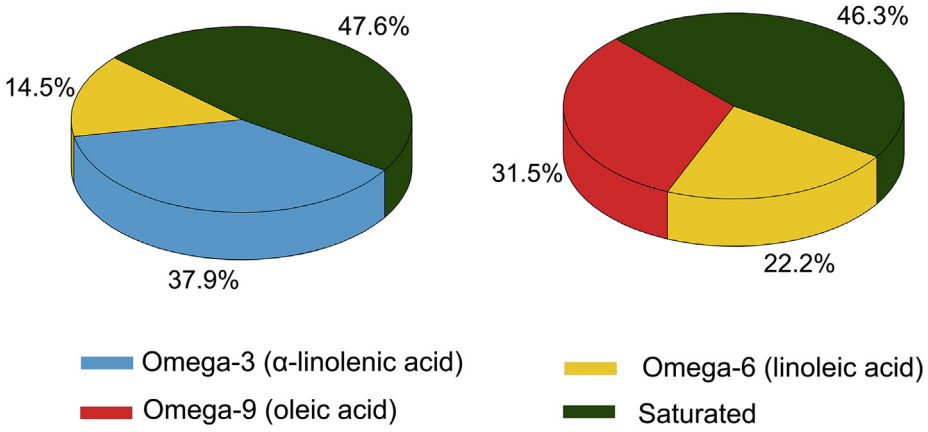

Fig. 4. Percentage of saturated and unsaturated acids (Omega-3, Omega-6 and Omega-9) identified in the SFE extracts of leaves (a) and stems (b).

et al., 2012). In this case, the compounds will be adsorbed into the column and elute at different times, which will allow their separation. A similar method was used to adsorb free fatty acids from microalgae oil using aminopropyl-functionalized mesoporous silica (Valenstein et al., 2012).

The modulation of temperature and pressure in SFE is also an alternative to obtain extracts that are richer in specific classes of compounds. Al Bulushi and coworkers obtained extracts from date palm residues that were richer in alcohols when SFE was performed at 240 bar and 70 °C, while extracts that were richer in hydrocarbons were obtained by performing it at 80 bar and 40 °C (Al Bulushi et al., 2018). However, this approach does not exclude further purification to obtain a pure target compound.

3.2. Use of post-extraction solids

Currently, economic perspectives indicate that stand-alone extraction technologies are not feasible (Attard et al., 2018). In most biomasses, extractives represent less than 10% or 20% of the total dried weight, which means that the non-utilization of the post-extraction solids would be counter-productive in terms of sustainability and profit. Analogously, extraction and use of the resulting extractives can also contribute to a more valuable production of 2G ethanol, a process that has also been facing cost problems. Considering this, the post-extraction solid was submitted to enzymatic hydrolysis here aiming at the conversion of cellulose into fermentable sugars and then into bioethanol. The total amount of sugars released by hydrolysis included glucose, xylose and arabinose, and the results are presented in Fig. 5. The samples that underwent extraction with ethyl acetate were not subjected to hydrolysis, since this solvent is more toxic than water, ethanol or sc-CO_2, and because its extraction results were not outstanding in comparison to SFE, PLE-WE and SFE-WE.

After SFE and SFE-WE, the amount of sugars released increased by 40–50% in the leaves: *in natura* leaves released 124.3 ± 2.7 mg of sugars/g of biomass, while after SFE-WE, they released 198.2 ± 20 mg/g (Fig. 5). Considering the cost of hydrolyzing enzymes and the importance of this step to the production of 2G ethanol, 40–50% of improvement in the leaves using only extraction is quite remarkable. This increase is higher than in other strategies for the utilization of post-extraction solids proposed in the literature. For example, SFE improved sugar release by 20% in giant miscanthus (Attard et al., 2016) and enhanced the conversion of cellulose into glucose from 13.1% to 18.7% in sugarcane bagasse (Qi et al., 2017). Another study showed that ethanol production increased by 40% in maize stover samples extracted via SFE, hydrolyzed, and then fermented (Attard et al., 2015b).

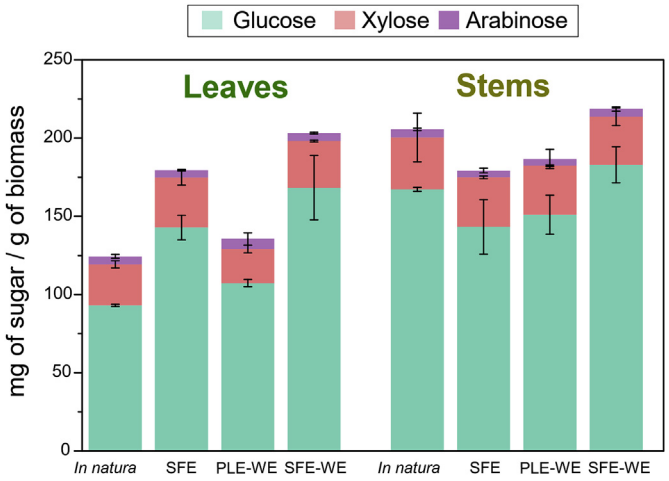

Fig. 5. Sugars released after 72 h of enzymatic hydrolysis by leaves and stems before (*in natura*) and after the extractions. SFE: $scCO_2$ extraction; PLE-WE: PLE using water and ethanol; SFE-WE: Sequential extraction using SFE, followed by PLE-WE; PLE-EA: PLE using ethyl acetate.

The improvement in sugar release after the SFE and SFE-WE extractions is also similar to the one achieved in an experimental design wherein an organosolv treatment was applied to elephant grass leaves (Rezende et al., 2018). Organosolv treatments with 40% ethanol (v/v) in water under different experimental conditions (using sulfuric acid as a catalyst or not, at temperatures from 160 to 200 °C, with reaction times from 30 to 90 min and in the presence of a pretreatment step using diluted sulfuric acid or not) improved the sugar release of elephant grass under enzymatic hydrolysis by up to 50%. Although the time of enzymatic hydrolysis was lower than in this study (12 h), it is important to consider that the organosolv treatment is more energy-demanding than the extractions due to the higher temperature and time, and it does not allow obtaining extracts that can be repurposed. This result reinforces the importance of this increase in sugar release resulted from SFE and SFE-WE.

Other traditional treatments can be used to improve the biomass enzymatic digestibility. Applying H_2SO_4 in concentrations between 5 and 20% w/w to elephant grass leaves improved glucose release from 67 mg/g of biomass (*in natura*) to 330 mg/g (enzymatic hydrolysis performed using 15 FPU/g Celluclast 1.5 L and 15 U/g β-glucosidase for 48 h) (Santos et al., 2018). Similarly, the glucose released by elephant grass treated with steam explosion and water

washing increased from *ca.* 100 mg/g of biomass to 248.34 mg/g of substrate after 48 h of enzymatic hydrolysis using 10 FPU/g of substrate of an enzyme produced from this same plant (Scholl et al., 2015).

The use of sequential treatments to delignify the biomass is also a good strategy to obtain a cellulose-rich solid that improves enzymatic digestibility in ethanol production (Rezende et al., 2018) or enables the production of cellulosic materials (Nascimento and Rezende, 2018). Applying alkaline treatments to elephant grass improved *ca.* 4 times the amount of sugars in enzymatic hydrolysis (using an enzyme cocktail with a 4:1 ratio of Celluclast 1.5 L and Novozymes 188 for 12 h) (Rezende et al., 2018). Nanocellulose was also isolated from elephant grass after a sequential treatment using diluted acid and diluted alkaline solutions (Nascimento and Rezende, 2018). Other alternatives include pyrolysis of the post-extraction solid to produce char and oils or to generate energy for the biorefinery (Collazzo et al., 2017).

In the stems, the extractions had no positive influence on sugar release. Nonetheless, this can still be considered a good result, since it indicates that the extracts can be removed and used for different applications, without any disadvantages for ethanol production. There are two possible approaches to the post-extraction stems: using them to generate energy for the biorefinery, which would reduce the cost of the processes and the energy demand (Attard et al., 2015); or applying sequential treatments to produce fermentable sugars or other products with high added value, such as organosolv, acid, alkaline or steam explosion.

The improvement in sugar release after SFE could be related to morphological or compositional changes caused by the extraction. However, the sample morphology was not changed by the extractions (Fig. 6). The leaves subjected to SFE, PLE-WE or SFE-WE have a very similar morphology to that of the sample *in natura*. Images of the extracted leaves at higher magnification are shown in Fig. S3 of the Supplementary Material, confirming the absence of changes. Similar results were obtained for the stem samples, as they also showed no morphological modifications (Fig. S4). Better hydrolysis yields due to conventional pretreatments (acid, alkali, organosolv, and so on) are often associated with very clear morphological alterations, characterized by the exposure of cellulose fibers to the enzyme (Rezende et al., 2011).

As shown in Fig. 6, neither SFE nor PLE are enough to expose cellulose or to create any other noticeable morphological changes in the substrate. The improvement in hydrolysis efficiency must thus be a consequence of compositional changes. Extractions are not expected to alter the compositional profile of structural sugars and lignin in the plant cell wall, as observed in previous works (Nascimento and Rezende, 2018). However, due to the removal of non-polar compounds, they can have a strong influence on hydrolysis by increasing the biomass wettability. Non-polar compounds, such as waxes and oils, in the plant outermost layers, restrain the transport of aqueous solutions through the biomass and slow down enzymatic hydrolysis (Djajadi et al., 2017). Indeed, contact angle measurements (Fig. S5) showed a significant difference in the hydrophobicity of the leaves before (contact angle higher than 100°) and after the extraction of non-polar compounds (angle between 33 and 75°). This indicates that the biomass wettability is improved by the removal of waxes, and that this is the most likely cause of the improvement in hydrolysis yields. In the stems, where the amount of extractable hydrophobic compounds is smaller than in the leaves, the impact of the SFE extraction on wettability, and thus on the hydrolysis improvement, is less pronounced.

Fig. 7 shows a summary of the products that can be obtained from elephant grass leaves or stems. The green extractions, especially using PLE, were effective to obtain them prior to enzymatic hydrolysis, and the combination of SFE and PLE allowed

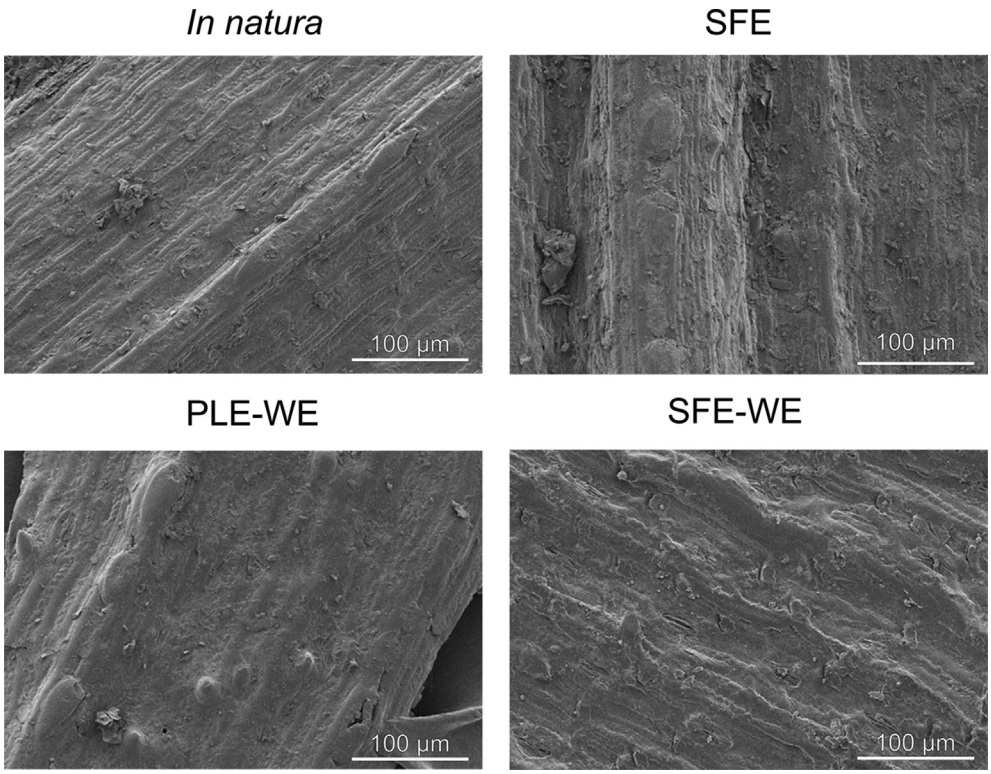

Fig. 6. FESEM images of the leaves before and after the extractions. SFE: $scCO_2$ extraction; PLE-WE: PLE using water and ethanol; SFE-WE: Sequential extraction using SFE, followed by PLE-WE; PLE-EA: PLE using ethyl acetate.

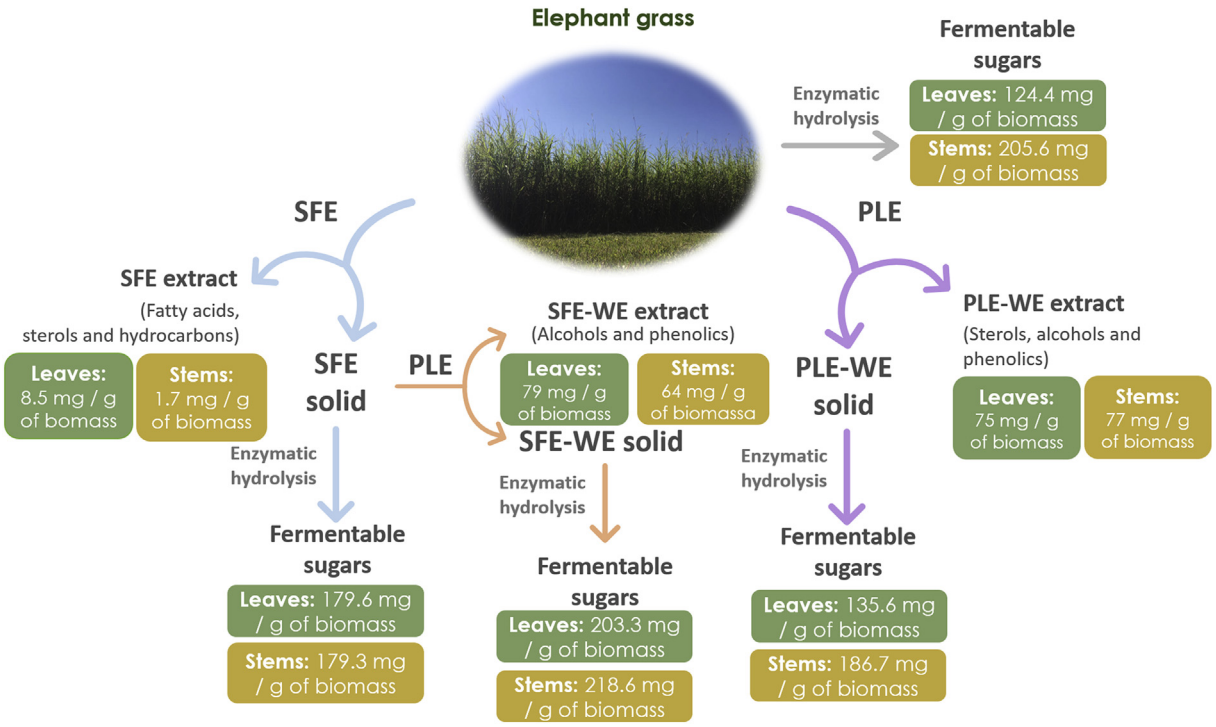

Fig. 7. Flowchart of the products that can be obtained from elephant grass leaves and stems. SFE: $scCO_2$ extraction; PLE-WE: PLE using water and ethanol; SFE-WE: Sequential extraction using SFE, followed by PLE-WE.

fractionating fatty acids, sterols and hydrocarbons via SFE, followed by alcohols and phenolics via PLE.

Although in this study, applying PLE directly to non-treated leaves and stems did not improve the amount of sugars released, extractions can be combined with other pretreatment strategies (acid, alkaline, steam explosion treatments, etc.), as previously mentioned. In any case, extractions are important to preserve the extracts before the biomass processing to generate fuels or materials. If only the chemical treatments were applied, the extractives would solubilize in the liquid fraction and be mixed with other byproducts, which would consequently make their recovery harder, and lead to possible degradation.

4. Conclusions

This study demonstrates the potential of using extraction methods based on food-grade and environmentally friendly solvents as a first step in a biorefinery to produce ethanol, materials and chemicals from elephant grass. Besides highlighting the compelling properties of elephant grass, which is a relatively unexplored biomass, this work also presents an approach that can be extended to other biomasses to reach a more integral use of bio-derived compounds via cleaner methodologies. Higher extraction yields (6.3–8%) could be obtained using only PLE or PLE combined with SFE. An advantage of this combination is the possibility of extracting non-polar (i.e. fatty acids and sterols) and polar components (alcohols and phenolics) from the biomass separately. The different extractions studied did not hinder the hydrolysis step in ethanol production. On the contrary, the hydrolysis yields increased up to 50% in the leaves after SFE and SFE-WE, which could be assigned to the improvement in the substrate wettability. The extractions do not change the morphology or the substrate chemical composition, which are important aspects concerning further processing steps to obtain biofuels and biomaterials. Based on these positive results, future studies on this system considering other pretreatment methods to improve the hydrolysis efficiency within the context of a biorefinery may be conducted.

CRediT authorship contribution statement

Eupídio Scopel: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Luana Cristina dos Santos:** Methodology, Validation, Formal analysis, Writing - review & editing. **Matheus Rodrigues Bofinger:** Methodology, Validation, Writing - review & editing. **Julian Martínez:** Resources, Funding acquisition, Writing - review & editing. **Camila Alves Rezende:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2020.122769.

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