LWT - Food Science and Technology 180 (2023) 114725

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

A preliminary multistep combination of pulsed electric fields and supercritical fluid extraction to recover bioactive glycosylated and lipidic compounds from exhausted grape marc

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ARTICLE INFO

Keywords:
Pulsed electric fields
Supercritical fluid extraction
Bioactive compounds
Exhausted grape marc
Process intensification mechanisms

ABSTRACT

This article reports the first multistep combination of pulsed electric field (PEF; 3 kV/cm, 100 kJ/kg, 2 Hz, 100 ms) and supercritical fluid extraction (SFE) with CO_2 (10–20 MPa, 25 mL/min [10% EtOH], 50 °C, 60 min) for exhausted grape marc (EGM). This current protocol was mainly created to recover bioactive glycosylated and lipidic compounds. In this regard, total antioxidant capacity (TAC) was enhanced up to 68% after PEF treatment compared to conventional soaking. However, re-extracting PEF-treated EGM after the application of SFE (PEF + SFE) boosted the efficiency by up to 87%. Several polyphenols (kaempferol, luteolin, scutellarin, and resveratrol, among others), together with other glycosylated structures, were identified by liquid chromatography coupled with mass spectrometry analysis. The bioactive lipidic compounds extracted by SFE, along with the carbohydrate fraction (free sugars) favourably extracted by PEF pre-treatment (mainly glucose, but also fructose and sucrose), were concurrently detected by nuclear magnetic resonance. The remaining solid fraction after treatment was also characterised. Different microscopic morphology was observed by scanning electron microscopy (SEM) on untreated, PEF, and PEF + SC–CO_2–treated EGM. Differential thermogravimetric (DTG) curves determined by thermogravimetric analysis (TGA) also suggested alternative and potential means for the valorisation of this matrix.

1. Introduction

Grape farming is one of the most important agricultural activities worldwide, thus making wine production a valuable industrial process. Many by-products are generated during this process, with most of them as lignocellulosic feedstocks that can be exploited for the production of high-added-value compounds (Portilla Rivera et al., 2021). Winemaking upgrading usually focusses on grape marc, obtained after the pressing step during vinification. These by-products are a good source of antioxidants, as well as a valuable starting point to produce biofuels and other fine chemicals (Barba et al., 2016; Ilyas et al., 2021; Muhlack et al., 2018). Furthermore, it is worth noting the importance of the exhausted grape marc (EGM) produced in distilleries. EGM still represents an underexplored feedstock, although its valuable energetic properties in the development of some bio-oils and bio-chars are well known (Ibn Ferjani et al., 2019, 2020). Moreover, some authors have reported the important bioactive compound (BAC) content found in EGM, mainly in the form of polyphenols (Salgado et al., 2014; Tacchini et al., 2019). Some of these polyphenols have important applications as food additives and nutraceuticals, as well as some potential benefits in humans, such as the ability to reduce oxidative stress in aging or Alzheimer disease, and reducing the risk of the appearance of some diseases such as diabetes, obesity, or Parkinson (de Araújo et al., 2021; Durazzo et al., 2019).

Polyphenols are usually recovered from agri-food biomass by

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https://doi.org/10.1016/j.lwt.2023.114725
Received 5 December 2022; Received in revised form 22 March 2023; Accepted 31 March 2023
Available online 2 April 2023

applying conventional methods, which are time-consuming and involve both a high volume of organic solvents and harsh conditions in terms of temperature and time. Moreover, these techniques can also enhance the extraction of unwanted compounds. Therefore, in this sense, there is a need for innovative and sustainable technologies, such as pulsed electric fields (PEF), supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), or subcritical water extraction (SWE), in full correspondence with the Principles of Green Chemistry (Chemat et al., 2020; Mariatti et al., 2021).

It should also be noted that, owing to their similar polarity, the recovery of polyphenols normally involves the extraction of freely accessible carbohydrates (sugars) from a matrix. Sugars are considered high-added-value compounds because they can be directly used as additives in the food sector. Moreover, they are valuable feedstocks for the synthesis of fine chemicals, such as levulinic acid (LA), 5-hydroxymethylfurfural (5-HMF), and furfural (FF) (Desir & Vlachos, 2022; Lau et al., 2021; Sun et al., 2021), considering bio-based platform chemicals with a wide range of applications in cosmetic or pharmaceutical sectors, among others.

Regarding these aforementioned innovative and enabling techniques, PEF can facilitate the extraction of BACs, in particular from wine by-products (Barba et al., 2015), by the application of an electrical current in the form of short pulses to a target matrix, thereby leading to cell damage with the corresponding formation of pores on the surface. Therefore, compared to conventional systems, this phenomenon, known as cell membrane electroporation, can improve the extraction process in a green and sustainable way. This innovative technique requires shorter working times and a lower energy consumption (micro- or milliseconds at moderate temperatures for PEF), allowing the preservation of thermolabile BACs compared to conventional extraction methods (Barba et al., 2016; Martí-Quijal et al., 2021).

Furthermore, solvents are usually employed near their critical pressure and temperature points during SFE. Under these conditions, they show intermediate properties between gases and liquids with a higher diffusion coefficient and lower viscosity, thus enhancing their penetration into the matrices (alKhawli et al., 2019; Quispe-Fuentes et al., 2022; Zhou et al., 2021). Moreover, it should be noted that the high pressures employed could also lead to the compaction of fibres. Therefore, a compromise between this efficient penetration and fibre compaction because of the pressurised conditions would facilitate the recovery of BACs.

The most commonly used treatment is supercritical (SC) fluid extraction (SFE) with CO_2 (SC–CO_2). This is mainly because of its excellent SC parameters (i.e., critical pressure and temperature), but also because it has several benefits in terms of availability, sustainability, and cost (da Silva et al., 2016; Pimentel-Moral et al., 2019). However, its low polarity can hinder the extraction of polyphenols from a matrix. For this reason, CO_2 is normally employed along with a doping polar co-solvent such as ethanol (EtOH). This can increase the polarity, thereby simultaneously facilitating high-phenolic solubility and green properties. Nonetheless, in some cases the ethanol flow used is low enough to make the extraction of some polyphenols such as anthocyanins or proanthocyanins difficult (Beres et al., 2017), thus making SFE extraction useful for other BACs, such as lipids (Talmaciu et al., 2016; Teixeira et al., 2021). Overall, despite being considered a sustainable and valuable alternative for the recovery of polyphenols, to the best of our knowledge, SFE is not yet commonly applied in combination with a PEF, although this technique has been reported in the academic literature (Ávila-Hernández et al., 2022).

Given all the above, in this work we developed the first sequential combination process using PEF + SC-CO_2 for use with EGM. This hybrid technique was significatively more efficient in terms of antioxidant extraction capacity compared to traditional methods, i.e., soaking. Moreover, the remaining solid fractions obtained after PEF and PEF + SC-CO_2 treatments suggested other alternative means for matrix valorisation, representing potential tools for achieving zero-waste with EGM.

2. Materials and methods

2.1. Chemicals

ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; CAS No. 30931-67-0), potassium persulfate ($K_2S_2O_8$; CAS No. 7727-21-1), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH; CAS No. 2997-92-4), fluorescein (CAS No. 2321-07-5), gallic acid (CAS No. 149-91-7), Folin-Ciocalteu reagent, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; CAS No. 53188-07-1), and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (98 atom %D, CAS No. 24493-21-8) were supplied by Sigma–Aldrich (Steinheim, Baden-Württemberg, Germany). Sodium carbonate (Na_2CO_3; CAS No. 497-19-8), sodium phosphate (Na_2HPO_4; CAS No. 7778-77-0), potassium phosphate (KH_2PO_4), and methanol-d_4 (99.80 atom %D, CAS No. 811-98-3) were purchased from VWR (Saint-Prix, France). Maleic acid (CAS No. 110–123 16-7) was obtained from Fluka. Absolute ethanol (CAS No. 64-17-5) was acquired from Thermo Fisher (Waltham, Massachusetts, USA). Ethanol 96% (CAS No. 64-17-5) was obtained from Guinama (La Pobla de Vallbona, Valencia, Spain).

2.2. Feedstock

EGM was provided by Alvinesa Natural Ingredients S.A. (Daimiel, Ciudad Real, Spain), after being collected at the beginning of the 2016–2017 grape harvest season. It was safely stored at −4 °C, then defrosted and finally used without any previous treatment. The moisture percentage measured for the raw matrix was 7.75 ± 0.35%.

2.3. Pulsed electric field-assisted extraction

PEF pre-treatment was carried out in a PEF-Cellcrack III equipment (German Institute for Food Technology [DIL], ELEA, Germany) located in the Faculty of Pharmacy at the University of Valencia, Spain. Forty grams of raw EGM were placed in a 900 mL treatment chamber with 400 mL of tap water. The PEF parameters were chosen according to previous studies (Martí-Quijal et al., 2021; Salgado-Ramos, Martí-Quijal, et al., 2022) as follows: specific energy, 100 kJ/kg EGM; field strength, 3 kV/cm; frequency, 2 Hz; pulse duration, 100 ms; number of pulses, selected considering the amount of biomass in order to maintain the constant level the above the specific energy; and pulse form, unipolar square wave pulse.

After the PEF application, the electrical conductivity of the water increased to 0.2585 S/m with respect to the initial 0.0918 S/m, as expected. The crude PEF-treated EGM + water was transferred to an Erlenmeyer flask containing 400 mL of ethanol (96% v/v) and was then stirred for 24 h at room temperature. For the sake of comparison, a conventional water/ethanol extraction without prior PEF application was concurrently performed in this context. Therefore, the PEF efficiency was evaluated compared to the traditional soaking (see Fig. 1). Thus, 10 g of raw EGM and 100 mL of a H_2O:EtOH mixture (50 mL EtOH + 50 mL H_2O) were mixed in an Erlenmeyer flask and was stirred at room temperature for 24 h. We used the extraction time previously reported by Lapornik et al. (2005) as optimal for achieving efficient polyphenol recovery from some winemaking wastes, such as grape marc.

After the extraction, solid–liquid separation was performed. Thus, the (i) PEF-treated EGM + EtOH/H_2O and (ii) untreated EGM + EtOH/H_2O mixtures were filtered, and the liquid fractions were analysed after few days at −4 °C by Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), and total polyphenol content (TPC) assays, as well as by nuclear magnetic resonance (NMR) and high-performance liquid chromatography–mass spectrometry (HPLC-MS). PEF-treated EGM was dried at a low temperature (60 °C) overnight for further SFE experiments. Next, part of this PEF-treated dried solid was blade milled in a Fritsch Pulverisette 14 Variable Speed Rotor Mill (Fritsch, Idar-Oberstein, Germany) operating for 1 min

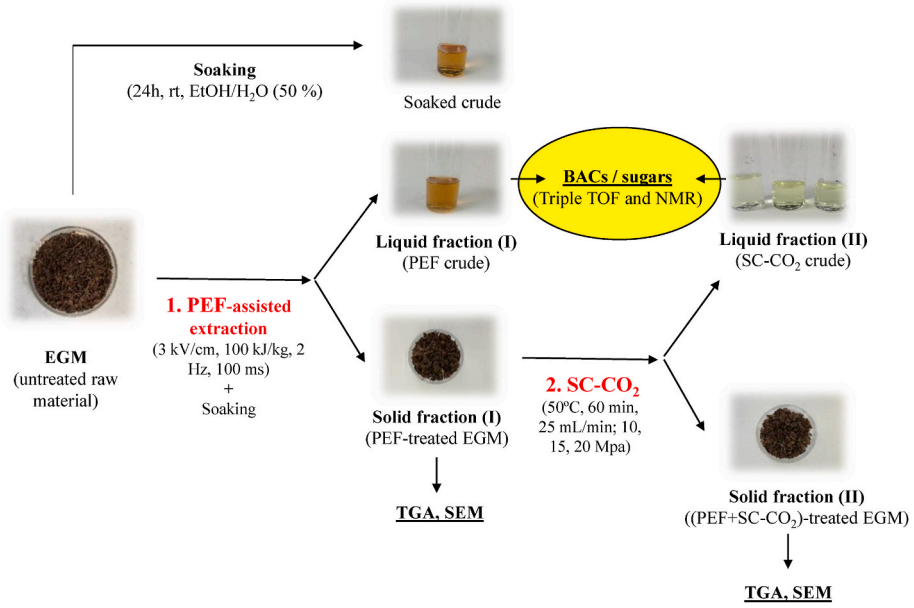

Fig. 1. Sequential combination of pulsed electric field (PEF)-assisted extraction and supercritical (SC) fluid extraction with CO_2 (SC–CO_2) to recover bioactive compounds (BACs) and sugars from exhausted grape marc (EGM). Abbreviations: rt: room temperature; SEM: scanning electron microscopy; TGA: thermogravimetric analysis; NMR: nuclear magnetic resonance.

at 20 000 rpm. Thus, a 1 mm particle size powder was obtained for subsequent thermogravimetric analysis (TGA) and scanning electron microscopy (SEM) analyses. For soaking, the solid fraction was directly discarded without being used further. The PEF and soaking experiments were performed in duplicate.

2.4. Supercritical fluid extraction

SFE was carried out using a supercritical extraction system (JASCO, Tokyo, Japan), located at the Faculty of Pharmacy in the University of Valencia (Spain).It comprised an isocratic CO_2 pump (PU-4387), with a flow range from 5 to 40 mL/min, adjustable in 0.01 mL/min increments, with a top pressure of 50 MPa and an extraction system for pulses; a refrigerating recirculating cooler (JULABO FL 1201) for pump cooling, with a temperature range of −20–40 °C, flow range of up to 23 L/min, and a cooling refrigeration capacity of 1.2 kW at 20 °C; an isocratic organic modifier pump (PU-4086, HPLC), with a flow ranging from 0.001 to 10 mL/min, adjustable in 0.01 mL/min increments, and with a top pressure of 70 MPa; a thermostatic oven for glass reaction (CO-4065), with a temperature range of 4–90 °C; a thermostatic system by Peltier and temperature transference by air flow; a pressure regulator (BP-4340); and 25 mL supercritical extraction glass (803559-25ML).

Experiments were performed after 2 days of applying PEF-assisted treatment. For this purpose, 2.5 g of PEF-treated EGM, without having been blade milled, was dried at 60 °C overnight and then introduced into the supercritical extraction glass. Absolute EtOH was used as a co-solvent. The remaining parameters were applied in accordance with the available literature (Talmaciu et al., 2016): pressure, 10, 15, or 20 MPa; total flow, 25 mL/min (10% EtOH, 2.5 mL/min, 90% CO_2); temperature, 50 °C; and time, 60 min. Similar to PEF pre-treatment, the liquid fraction was collected and stored for TEAC, ORAC, and TPC assays, as well as for NMR and HPLC-MS analyses. The solid fraction was dried at a moderate temperature (60 °C) overnight and then blade-milled for further TGA and SEM analyses. As for PEF and soaking, the tests were performed in duplicate for SFE.

2.5. Antioxidant assays and phenolics profile

The total antioxidant capacity (TAC) was measured by two different methods, TEAC and ORAC, both according to previous studies (Martí-Quijal et al., 2021; Salgado-Ramos, Martí-Quijal, et al., 2022). TEAC was performed in a PerkinElmer UV/Vis Lambda 2 spectrophotometer, and ORAC was carried out in a Wallac 1420 VICTOR 3 plate reader (both from PerkinElmer, Jügesheim, Germany). Trolox was used as the standard for both assays, therefore the results are shown in terms of μmol Trolox equivalents/g dry weight (μmol TE/g DW). The TPC was determined by the Folin–Ciocalteu method in a PerkinElmer UV/Vis Lambda 2 spectrophotometer (PerkinElmer, Jügesheim, Germany), also according to the aforementioned studies. Gallic acid was selected as the standard and the results were expressed in terms of milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g DW). All the TEAC, ORAC, and TPC assays were performed in triplicate. The mean was calculated directly from the experimental data, and standard deviations were calculated by processing the data in an Excel file with view to further statistical analysis.

The phenolics' profile characterization and quantification was carried out by HPLC-MS and was performed based on a previously described method (Roselló-Soto et al., 2019). An Agilent 1260 Infinity instrument (Agilent, Waldbronn, Germany) with a Waters C18 1.7 μm column (2.1 × 50 mm) Acquity UPLC BEH.C18 column (Waters, Cerdanyola del Vallès, Spain) was used for the separation of the main phenolic compounds in the samples, while a TripleTOF™ 5600 liquid chromatography with tandem mass spectrometry (LC/MS/MS) system (AB SCIEX, Foster City, CA, USA) was employed for their identification. For the quantification, we prepared an external calibration curve, using a representative polyphenol from each group of phenolic compounds potentially found in the samples, following the procedure described by Szczepańska et al. (2022). These phenolic compounds were phenolic acids (gallic acid); flavonoids (flavones: apigenin; flavonols: kaempferol; flavanones: naringenin; and flavanols: catechin); stilbenes (resveratrol); isoflavonoids (genistein); and phenylethanoids (hydroxytyrosol). These analyses were also performed by triplicate.

2.6. Nuclear magnetic resonance analyses

Liquid crudes, obtained after PEF and PEF + SFE treatments, were analysed by NMR according to the method proposed by Salgado-Ramos, Martí-Quijal, et al. (2022). The dried crudes were dissolved in

deuterated methanol (MeOD; 600 μL), passed through nylon syringe filters (13 mm diameter and 0.45 μm pore size), and finally introduced into a 5 mm NMR tube. ^{1}H NMR spectra were recorded on a Bruker Ascend™ 500 spectrometer (Bruker Corporation, Billerica, MA, USA) operating at a frequency of 500.16 MHz for the ^{1}H nucleus, with the probe temperature adjusted to 25 °C. Chemical shifts were referred at 0 ppm with the addition of 20 μL of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP) solution to the tube along with the reference for the SFE experiments.

2.7. Scanning electron microscopy

SEM images were recorded on a ZEISS Gemini500 high resolution scanning electron microscope (HRSEM, ZEISS, Oberkochen, Germany) fitted with an Oxford EDS 80 mm^2 detector, operating from 3 pA to 20 nA for electrical current intensity and 0.02–30 kV, (Salgado-Ramos, Martí-Quijal, et al., 2022); magnification ranged between 50 and 2,000, 000 ×.

2.8. Thermogravimetric analysis

Thermograms (TG) and differential thermogravimetric (DTG) analyses were recorded on a Q50 thermogravimetric analyser (TA Instruments, New Castle, Delaware, USA). The analysis was carried out according to our previous work (Salgado-Ramos, Martí-Quijal, et al., 2022) by increasing the temperature from 25 to 800 °C, at a heating rate of 10 °C/min and using N_2 as an inert gas.

2.9. Statistical analysis

The results of TPC, TEAC, and ORAC assays were analysed using Student *t*-tests to compare the control samples (soaking) to the PEF-treated samples. For the latter, along with the combined PEF + SFE sample results, a one-way ANOVA test followed by a Tukey post-test was applied. A probability value of $p < 0.05$ was considered significant, with $n = 3$, and the results were expressed as the mean ± the standard deviation (*SD*). All the statistical analysis were performed using GraphPad Prism 8.0.2 software (GraphPad Software, San Diego, CA, USA).

3. Results and discussion

3.1. Recovery of bioactive glycosylated and lipidic compounds from exhausted grape marc

To the best of our knowledge, in this current work, PEF and SFE with SC-CO_2 were sequentially applied to EGM for the first time (Fig. 1). We then analysed liquid fractions (LFs) I and II obtained from the PEF and SFE treatments, respectively, using different antioxidant assays. First, the activity of PEF crudes was evaluated with respect to soaking (Fig. 2a), which was used as control for the sake of comparison. The conditions used for the PEF-assisted extraction were the same as those from our previous studies (Martí-Quijal et al., 2021; Salgado-Ramos, Martí-Quijal, et al., 2022). In turn, we conducted a rapid pressure optimisation (10, 15, and 20 MPa) for the SFE experiments, according to the work by Talmaciu et al. (2016).

As shown in Fig. 2a, the activity for PEF crudes (LF I) was significantly higher than that of traditional methods (soaking), especially for the TAC when measured using ORAC and TEAC assays ($p < 0.01$ and $p < 0.001$, respectively). These results represented an increase from 34.65 μmol TE/g DW by soaking to 40.54 μmol TE/g DW by PEF for TEAC, and from 218.07 μmol TE/g DW by soaking to 366.47 μmol TE/g DW by PEF for the ORAC assay. The high sensitivity of ORAC relative to TEAC and with respect to BACs other than polyphenols, such as peptides with conformational changes after the application of PEF (Lin et al., 2017; Zhang et al., 2019), could have led to this lower significance ($p < 0.01$) and to these higher values for antioxidants. In contrast, for TPC, other extracted compounds such as sugars could have interfered in the assays, thus leading to insignificant results compared to conventional soaking ($p > 0.1$). In fact, as shown in Fig. 2a, the TPCs were similar for soaking and PEF (20.60 and 21.03 mg GAE/g DW, respectively).

For the SFE (LF II), the best results for TAC and TPC were achieved at 15 MPa (5.63 μmol TE/g DW for TEAC, 41.97 μmol TE/g DW for ORAC, and 0.65 mg GAE/g DW by TPC; Fig. 2b), which agrees with the work by Talmaciu et al. (2016). These results were notable, because they indicate that a prior PEF step could considerably increase the antioxidant extraction from the PEF-treated EGM (solid fraction I, Fig. 1). This difference was significant for the TEAC ($p < 0.001$) and TPC ($p < 0.0001$) assays at both 10 and 20 MPa (TEAC: 1.79 and 2.86 μmol TE/g DW at 10 and 20 MPa; ORAC: 37.03 and 34.12 μmol TE/g DW at 10 and 20 MPa; and TPC: 0.52 and 0.33 mg GAE/g DW at 10 and 20 MPa, respectively). Of note, the experiments performed at 10 MPa were 60% more efficient compared to those done at 20 MPa ($p < 0.0001$; 0.52 vs. 0.33 mg GAE/g DW). This fact could perhaps be explained by the fact that EtOH is not as compressible as CO_2 under supercritical conditions, thereby decreasing its effectiveness for extraction at high pressures.

According to the results reported above, TAC was enhanced by up to 68% after PEF-assisted extraction when compared to conventional soaking (i.e., 366.47 μmol TE/g DW by PEF and 218.07 μmol TE/g DW by soaking in the ORAC assay), thus highlighting the application of this 'enabling' technique to EGM. Nonetheless, the benefit of re-extracting the PEF-treated EGM with SC-CO_2 was especially observed when combining LF I (PEF crudes) + LF II (SFE only for 15 MPa experiments; Fig. 3). In this regard, the efficiency of BAC recovery was boosted by up to 87% in terms of TAC, corresponding to an increase by ~30% with respect to PEF treatment application alone.

The results obtained for TEAC were also noteworthy, with the

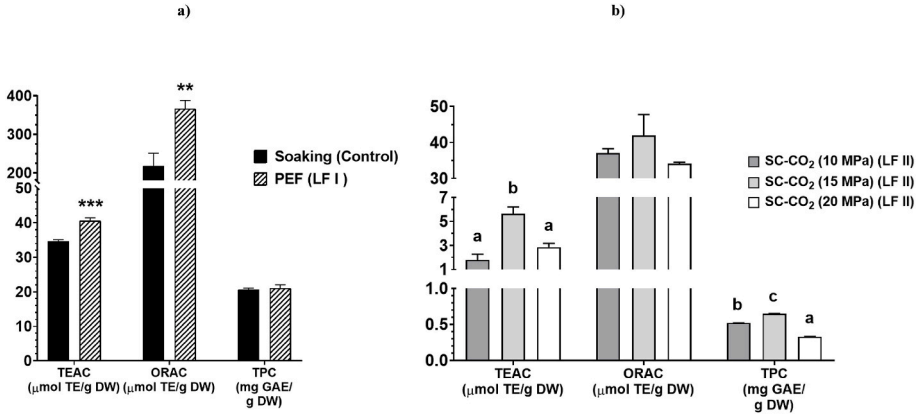

Fig. 2. Radical scavenging activity by means of antioxidants (TEAC and ORAC) and total polyphenol content (TPC) assays in terms of μmol Trolox equivalents/g dry weight (μmol TE/g DW) and mg gallic acid equivalent/g dry weight (mg GAE/g DW), respectively, for pulsed electric field (PEF)-assisted extraction and soaking as a control (a) (TEAC: $p < 0.001$; ORAC: $p < 0.01$; TPC: no significant differences) and supercritical (SC) fluid extraction with CO_2 (SC–CO_2) at different pressures (b). Different letters in the same graph indicate statistically significant differences. Abbreviations: LF I and LF II: liquid fraction I and II, respectively (see Fig. 1).

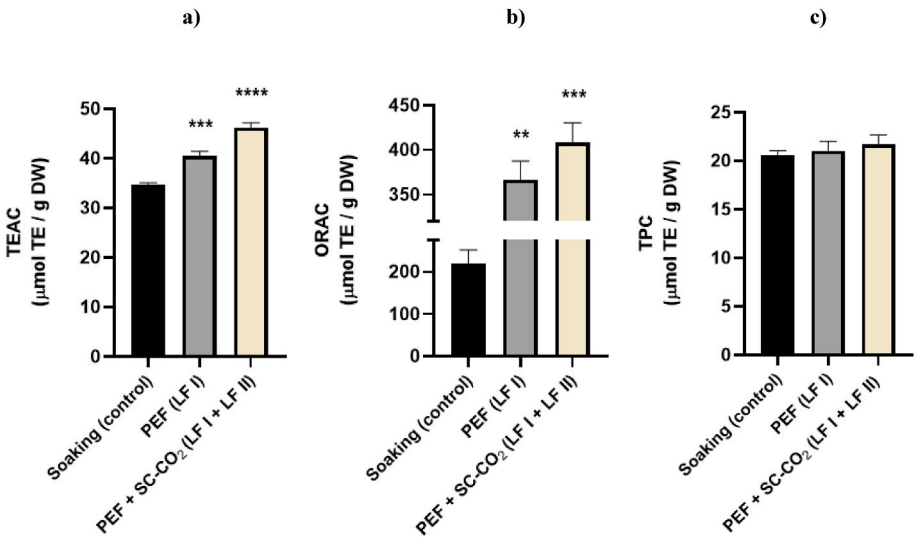

Fig. 3. Radical scavenging activity by means of antioxidants (TEAC (a), ORAC (b)) and total polyphenol content (TPC (c)) assays in terms of µmol Trolox equivalents/g dry weight (µmol TE/g DW) and mg gallic acid equivalent/g dry weight (mg GAE/g DW), respectively, for pulsed electric field (PEF)-assisted extraction, PEF + supercritical (SC) fluid extraction with CO_2 (PEF + SC-SCO_2) at 15 MPa and using soaking as a control. ****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$. Abbreviations: LF I and LF II: liquid fraction I and II, respectively (see Fig. 1).

application of SC-CO_2 after PEF increasing the values obtained compared to PEF alone (46.17 vs. 40.54 µmol TE/g DW, respectively, $p = 0.0004$) and this increase being higher and more significant than those for soaking (34.65 µmol TE/g DW, $p < 0.0001$; Fig. 3a). In contrast, for the ORAC values, no significant differences were found after PEF + SC-CO_2 compared to PEF alone (408.44 vs. 366.47 µmol TE/g DW, respectively, $p = 0.199$), although the values were significantly increased compared to soaking (218.07 µmol TE/g DW, $p = 0.0003$; Fig. 3b), thus intensifying the biomass valorisation. Finally, no significant changes were observed after SC-CO_2 for TPC (Fig. 3c).

Overall, this work showed the potential of EGM PEF + SFE treatment for enhancing BACs recovery and progressed the state of the art for biomass upgrading and process intensification. In addition, in view of the ongoing focus on zero-waste-based approaches, this allowed the concurrent development of an alternative valorisation approach for EGM (for instance, for the solid fraction remaining after both treatments). Nonetheless, this type of enhancement will still require further refinement and study. In this sense, an alternative method involving increasing the EtOH flow, which concurrently leads to an increase in the polarity of the system, has been proposed. These new conditions might enhance both the recovery of polyphenols and the efficiency of the protocol. In parallel, another option would be to directly perform SFE from EGM without the prior PEF step, in order to observe whether SFE treatment is also efficient by itself or if either an increase the EtOH flow or application of PEF pre-treatment would be indeed necessary for better recovery.

The LC-MS data provided further information about the type of polyphenols present in each liquid fraction. In this regard, the phenolic profiles were obtained according to the molecular weight by triple time-of-flight (TOF) mass spectrometry analysis (Table 1). PEF crudes mainly showed the presence of glycosylated polyphenols: flavonols (quercetin 3-*O*-(6-acetyl-glucoside) or kaempferol 3-*O*-galactoside), flavons (6-hydroxyluteolin-7-*O*-rhamnoside), and/or anthocyanins such as petunidin 3-*O*-arabinoside. Indeed, the presence of these glycosylated phenolics agrees with the literature for EGM (Tacchini et al., 2019). Furthermore, other non-glycosylated structures were also present in PEF crudes, including, once again, flavonols (quercetin and kaempferol) which can reduce the risk of type 2 diabetes (Rienks et al., 2018), flavons (luteolin and scutellarein) which have several benefits helping to mitigate the risk of developing ovarian cancer, amnesia, and depression (Mohammadi et al., 2016; Salehi et al., 2019), as well as some flavanols such as catechin and epicatequin which have been shown, for instance, to be useful anti-obesity agents (Akhlaghi et al., 2018).

For SFE, the abundance of antioxidants in PEF-treated extracts (mg/mL) was low in these crudes (LF II) compared to PEF treated EGM, in agreement with the moderate antioxidant capacity and TPC observed in the former (Fig. 2b). As previously mentioned, this fact could be attributed to the efficiency of the prior PEF extraction. Unfortunately, no data is available in the literature to compare these LC-MS analysis results. Nevertheless, the presence of BACs in these fractions would tend to confirm the high efficiency of the developed protocol. It should also be noted that the characteristics of supercritical fluids can vary with pressure and/or temperature, thus also modulating the solubility of compounds and, therefore, their further extraction. This fact would explain

Table 1
Triple TOF-LC-MS-MS characterization for the antioxidants liquid fractions obtained by pulsed electric field (PEF)-assisted extraction and supercritical fluid extraction (SFE) from exhausted grape marc (EGM).

	Compound	Formula	Intensity	RT [a] (min)	mg/L
PEF	Quercetin 3-*O*-(6-acetyl-glucoside)	$C_{21}H_{20}O_{12}$	2 407 906	11.49	450.45
	Kaempferol 3-*O*-galactoside	$C_{21}H_{20}O_{11}$	2 077 633	12.13	388.67
	6-hydroxyluteolin-7-*O*-rhamnoside	$C_{21}H_{20}O_{11}$	2 077 633	12.13	388.67
	Quercetin	$C_{15}H_{10}O_7$	908 702	13.05	170.00
	Petunidin 3-*O*-arabinoside	$C_{21}H_{21}O_{11}$	535 687	12.12	100.22
	(+,-)-Catechin, (+,-)-epicatechin	$C_{15}H_{14}O_6$	512 045	8.99	95.80
	Kaempferol, luteollin, scutellarein	$C_{15}H_{10}O_6$	426 144	13.8	79.73
SFE (10 MPa)	Quercetin-3-*O*-glucuronide	$C_{21}H_{18}O_{13}$	52 209	10.8	9.78
	Quercetin, morin, 6-hydroxyluteolin	$C_{15}H_{10}O_7$	18 224	12.94	3.42
	Kaempferol, luteolin, scutellarein	$C_{15}H_{10}O_6$	17 192	13.78	3.23
SFE (15 MPa)	Kaempferol, luteolin, scutellarein	$C_{15}H_{10}O_6$	25 186	13.92	4.72
	Trans-resveratrol and *cis*- resveratrol	$C_{14}H_{12}O_3$	1362	12.91	0.27
SFE (20 MPa)	Kaempferol, luteolin, scutellarein	$C_{15}H_{10}O_6$	24 123	13.94	4.53
	Quercetin, morin, 6-hydroxyluteolin	$C_{15}H_{10}O_7$	14 882	13.23	2.80
	Quercetin-7-*O*-glucoside	$C_{21}H_{20}O_{12}$	5273	11.68	1.00

[a] RT: retention time.

the higher abundance of glycosylated structures observed at low pressures (10 MPa), where 3-*O*-glucuronide was predominant, in contrast to pressures of 15 and 20 MPa, where only 1 mg/mL of quercetin-7-*O*-glucoside was detected.

However, for non-glycosylated structures, this difference was not significant, as observed, for instance, for kaempferol, luteolin, or scutellarein (3.23, 4.72, and 4.53 mg/mL at 10, 15, and 20 MPa, respectively) or quercetin, morin, or 6-hydroxyluteolin (3.42 and 2.80 mg/mL at 10 and 20 MPa, respectively). Finally, apart from these previously mentioned flavonols (quercetin, kaempferol, and morin) and flavones (luteolin, 6-hydroxyluteolin, and scutellarein), the presence of non-flavonoid structures such as resveratrol (*cis* and *trans*) is also noteworthy. These compounds are useful as agents that are protective against brain aging and inflammation in ocular diseases and age-related diseases (Silva et al., 2019), among others.

Furthermore, as previously reported, SFE can be considered a valuable approach for the extraction of other high-added-value compounds such as lipids (de Aguiar Saldanha Pinheiro et al., 2021; Tacchini et al., 2019; Teixeira et al., 2021). Indeed, some lipidic compounds are considered bioactive ingredients for common foodstuffs such as oil, dried nuts, or cacao, and they can be also employed to obtain biodiesel by transesterification with low-molecular-weight alcohols, suggesting an alternative way for valorisation (Carmona-Cabello et al., 2021; Singh et al., 2021). Moreover, this pathway could be especially useful for EGM because this type of waste contains a high proportion of non-polar components (above 20 g/100 g dry weight), according to the reported literature (Salgado et al., 2014).

In our experiments, SFE liquid fractions were characterised by ^{1}H NMR (Fig. 4), allowing us to clearly observe the lipidic profile present in the crudes, assigned based on the results of previous studies (Lucas--Torres et al., 2014). According to the academic literature, the main lipids present in EGM are palmitic, myristic, and lauric acid as saturated fatty acids, and oleic and linoleic as unsaturated fats (Tacchini et al., 2019). Moreover, the presence of the latter in EGM can be corroborated by NMR by the presence of signals around 5.5 ppm related to olefinic hydrogens. Also of note is the presence of acylglycerols in this crude, detectable by signals between 5.5 and 4.5 ppm in the spectrum, corresponding to glycerine chains. Finally, NMR analysis also confirmed, once again, that after SFE polyphenols are present in a very low concentration in this fraction, with the corresponding aromatic signals being undetectable between 8 and 6 ppm in the spectrum. In this case, the internal standard maleic acid was added to make quantification possible.

Furthermore, apart from lipids and polyphenols, other valuable metabolites such as sugars can be efficiently extracted from EGM. According to the literature, sugars are present in EGM in a low proportion (Salgado et al., 2014) and in this work it was possible to recover them by applying PEF, thus further highlighting the efficiency of this protocol. For these experiments, the main sugars identified by ^{1}H NMR were hexoses, mainly glucose, although sucrose and fructose were also present (Fig. 5), in accordance with previous studies for other types of biomass (Lucas-Torres et al., 2016; Salgado-Ramos, Martí-Quijal, et al., 2022). Sugars are considered valuable metabolites in the food sector as additives, and they are also present in a wide range of common foodstuffs. Moreover, they are valuable feedstocks for obtaining platform chemicals useful in chemistry sector such as LA, 5-HMF, or FF, thus suggesting another valorisation route for EGM. Apart from sugars, signals related to polyphenols were also observed in the NMR spectrum of the liquid fraction obtained after PEF (Fig. 5). Once again, compared to SFE experiments, these signals were more intense, in full concordance with the results found for both antioxidant assays and the phenolics profile. Therefore, overall, the sequential combination of PEF + SC-CO_2 can be considered an innovative and green process intensification strategy which allowed the recovery of valuable metabolites from EGM. These were mainly polyphenols, but also included lipids (selectively recovered by SC-CO_2) and sugars (PEF), thus establishing a three-way valorisation route for this by-product.

3.2. Analysis of the recovered solid fraction after PEF and PEF + SFE treatment

The remaining solid fractions obtained after PEF and PEF + SFE treatments were characterised by SEM and TGA (Figs. 6 and 7) to evaluate their composition after the application of these protocols, and to propose other potential and feasible valorisation routes for EGM. The application of process intensification strategies usually leads to valuable changes in the physical structure of samples (Mesquita et al., 2021; Teixeira et al., 2021), for instance, by breaking the cores of intact cells, usually converting the raw material into products with reduced particle size and enhancing certain characteristics as the result of the mechanisms that take place during the extraction (Sovová & Stateva, 2011). These mechanisms are mainly influenced by several chemical and physical processes, such as diffusivity, density, or viscosity variations, among others, thus favouring the recovery of the metabolites by

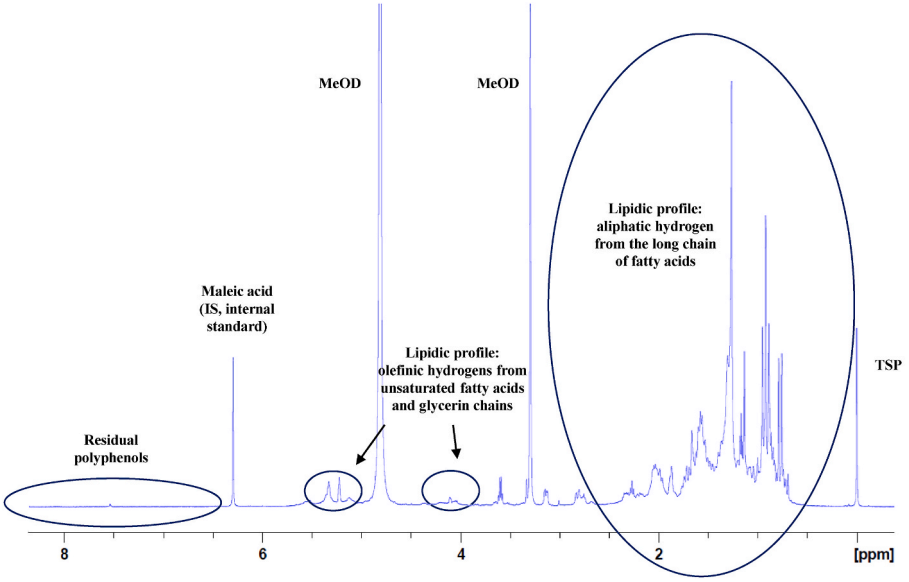

Fig. 4. ^{1}H NMR spectrum (MeOD, 500 MHz) for the liquid fraction obtained after supercritical fluid extraction (SFE) from PEF-treated exhausted grape marc (EGM).

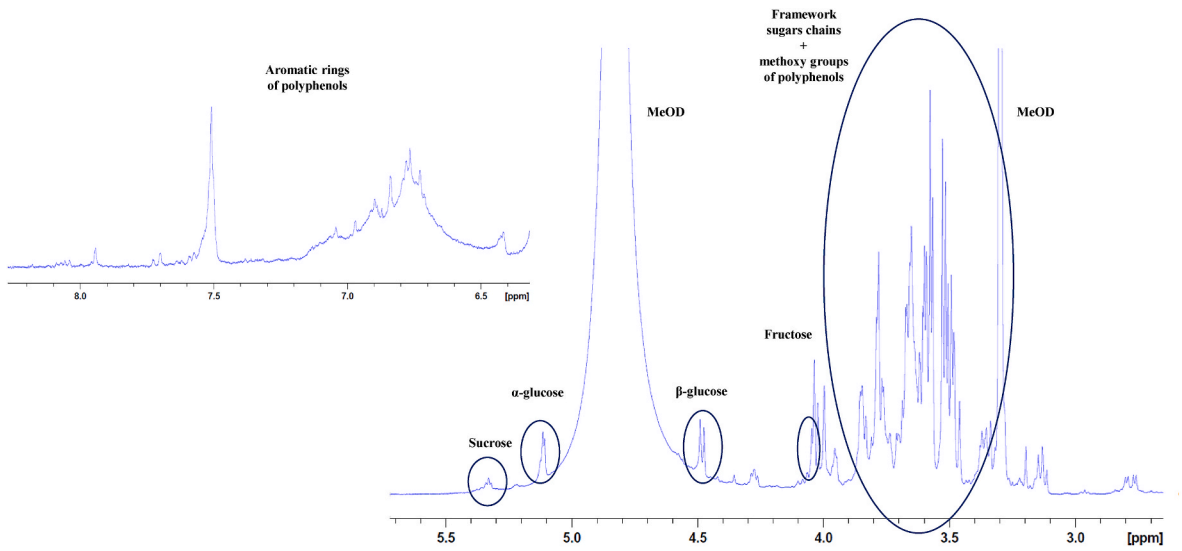

Fig. 5. ^{1}H NMR spectrum (MeOD, 500 MHz) for the liquid fraction obtained after PEF treatment from exhausted grape marc (EGM).

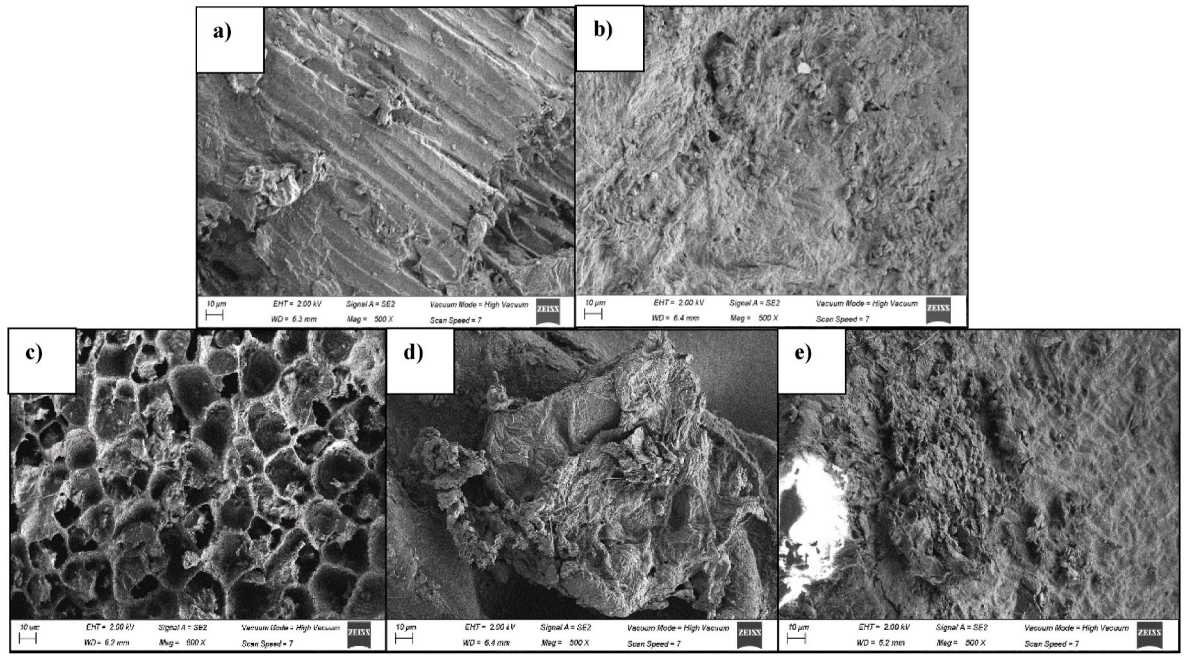

Fig. 6. SEM images for exhausted grape marc (EGM): untreated EGM (a); pulsed electric field (PEF)-treated EGM (b); PEF + supercritical fluid extraction (SFE)-treated EGM at 10 MPa (c), 15 MPa (d), and 20 MPa (e). 500 × magnification.

applying these innovative protocols as compared to traditional systems (Osorio-Tobón, 2020; Teixeira et al., 2021).

Regarding the above, the morphology of these solids was studied by SEM (Figs. 6× and 500 × magnification). The untreated EGM (Fig. 6a) mainly showed a well-compacted structure with organised fibres in the matrix (typical for the cellulose framework) prior to all treatment types. In this current work, the subsequent application of PEF clearly led to surface cell damage (Fig. 6b), with the corresponding formation of large pores or micropores, which could enhance the extraction of intracellular metabolites. Indeed, these results also agree with some previously reported work (Koubaa et al., 2016; Li et al., 2021).

The application of SFE after PEF treatment led to some differences in the remaining solids obtained in the experiments after applying different pressures. For instance, at 10 MPa the pores formed after PEF treatment were still not closed (Fig. 6c), with porous structures that could facilitate the extraction of metabolites being observed. However, an increased pressure (up to 15 MPa) partially closed these pores (Fig. 6d), with this solid also showing a damaged structure because of the prior PEF treatment. Concurrently, the application of high pressures in supercritical conditions could lead to compaction of the fibres present in the matrix, as observed by SEM (Mesquita et al., 2021). Overall, the fact that the best antioxidant capacity and TPC was observed at 15 MPa could be explained by the best point of compromise between the presence of porous structures and the fibre compaction caused by the pressurised conditions. Antioxidant capacity could not be observed for experiments at 20 MPa (Fig. 6e). In this case, some microspheres were formed on the solid surface. Overpressure can favour oversaturation, with the corresponding formation of these microspheres from the first nucleation, thus hindering the extraction of metabolites and explaining why this pressure had the lowest antioxidant capacity.

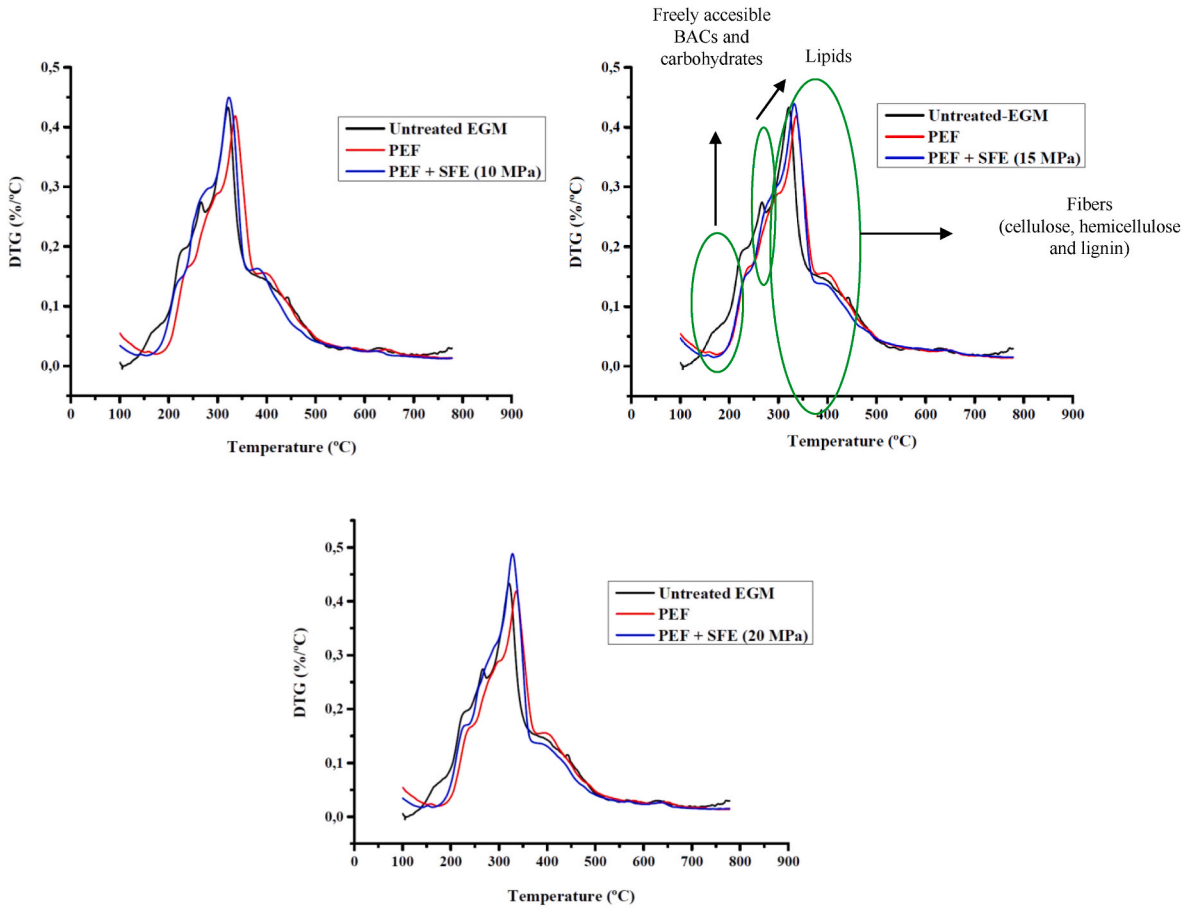

Fig. 7. Differential thermogravimetric (DTG) curves for exhausted grape marc (EGM): untreated EGM (black), pulsed electric field (PEF)-treated EGM (red) and PEF + SFE (supercritical fluid extraction)-treated EGM (blue) at 10 MPa (a), 15 MPa (b), and 20 MPa (c). Abbreviations: BACs: bioactive compounds; DTG: derivative thermogram. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

In parallel to SEM analysis, untreated, PEF, and PEF + SFE-treated EGM were also analysed by TGA. DTG curves provided the information about the EGM composition, thus allowing us to correlate each peak in this curve to each fraction present in the biomass (Fig. 7). The assignments were made based on our previous studies, evaluating other biomass such as melon rind or almond hulls (Lucas-Torres et al., 2016; Salgado-Ramos, Martí-Quijal, et al., 2022). As shown in Fig. 7, compared to the untreated EGM, insignificant differences were found in the remaining solids obtained after PEF and PEF + SFE treatments. However, this difference seemed to be slightly more notable for the solid obtained after SFE at 15 MPa (Fig. 7b, blue line), which also agrees with the antioxidant capacity and TPC observed for these samples.

The decomposition peak at around 200 °C, related to the polar extractives (containing polyphenols and free sugars), and the lignocellulosic framework (250–500 °C), which remained practically unaltered after the extraction of BACs and sugars, should also be noted. These characteristics make this fraction more amenable to further transformation into fine chemicals with many industrial applications, thus suggesting a further means of EGM valorisation. For instance, cellulose and hemicellulose are considered a valuable starting point for the production of fine chemicals such as LA, 5-HMF, or FF, respectively (Mika et al., 2018). In contrast, lignin is normally exploited to develop chemicals such as vanillin, syringaldehyde, or *p*-hydroxybenzaldehyde, with many applications in drugs manufacturing, flavouring, or cosmetics (Salgado-Ramos, Mariatti, et al., 2022; Tabasso et al., 2019). The fibre fraction could be also transformed by applying sustainable and emerging protocols such as microwaves, thereby also making this process more sustainable, in line with a zero-waste-based approach.

4. Conclusions

To the best of our knowledge, this article describes the first multi-step combination PEF + SFE via CO_2 (PEF + SC-CO_2) developed and applied to EGM reported in the scientific literature to date. This sustainable, process-intensified protocol mainly led to the recovery of bioactive glycosylated and lipidic compounds. With this in mind, the application of PEF pre-treatment enhanced the TAC by up to 68% compared to conventional soaking. However, the subsequent of application of PEF + SC-CO_2 boosted this efficiency by up to 87%. Furthermore, the LC-MS phenolic profile analyses revealed the presence of a wide range of polyphenols in EGM, most notably, flavons, flavanols, and glycosylated structures. NMR analysis also showed the potential of PEF + SFE to selectively recover free sugars by PEF (mainly glucose, but also sucrose and fructose) and lipidic high-added-value components by SC-CO_2. Finally, we characterised the remaining solid fraction after PEF + SFE treatment. SEM analysis showed notable differences in terms of morphology between the untreated, PEF, and PEF + SFE treated biomass, whereas TGA analyses suggested other potential valorisation routes for EGM, with the aim of achieving the desired zero-waste.

Funding

This research was funded by the Consejería de Educación, Cultura y Deporte of Junta de Comunidades de Castilla-La Mancha (Spain) and the Ministerio de Ciencia, Innovación y Universidades (projects SBPLY/17/180501/000522, "Valorización energética de residuos agroindustriales: obtención de precursores de biocombustibles y evaluación de los efectos

de sus emisiones en la contaminación atmosférica" and "RTI2018-099503-8-I00-Formación, caracterización y reactividad química de aerosoles en la atmósfera", respectively), which were both co-financed by the Fondo Europeo de Desarrollo Regional (FEDER). Manuel Salgado-Ramos wishes to thank the post-PhD program of the Universidad de Castilla-La Mancha for the requalification of the Spanish University System from the Ministry of Universities of the Government of Spain, modality "Margarita Salas - Complementaria" (MS2022), financed by the European Union, Next Generation EU. This study forms part of the AGROALNEXT program (AGROALNEXT/2022/060 - Desarrollo y optimización de procesos innovadores y sostenibles de extracción de aceite y proteínas a partir de microalgas, insectos, residuos y subproductos agroalimentarios: Evaluación de propiedades biológicas (EXTRAOLIOPRO)) and was supported by MCIN with funding from European Union NextGenerationEU (PRTR-C17.I1) and the Generalitat Valenciana.

CRediT authorship contribution statement

Manuel Salgado-Ramos: Methodology, Formal analysis, Writing – original draft, preparation, Writing – review & editing. **Francisco J. Martí-Quijal:** Methodology, Formal analysis, Writing – original draft, preparation, Writing – review & editing. **Alberto J. Huertas-Alonso:** Methodology, Formal analysis. **M. Prado Sánchez-Verdú:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Andrés Moreno:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Francisco J. Barba:** Conceptualization, Resources, Funding acquisition, 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.

Data availability

The authors do not have permission to share data.

Acknowledgements

Alberto J. Huertas-Alonso would like to express their gratitude to the Junta de Comunidades de Castilla-la Mancha (Spain) for their pre-PhD fellowship grants. Francisco J. Martí-Quijal would like to acknowledge the pre-PhD scholarship program grant awarded to him from the University of Valencia "Atracció de Talent". All the authors would like to acknowledge the Generalitat Valenciana (Spain) for financial support (IDIFEDER/2018/046—Procesos innovadores de extracción y conservación: pulsos eléctricos y fluidos supercríticos) through European Union ERDF (European Regional Development Fund) funds. Andrés Moreno, María Prado Sánchez-Verdú, Manuel Salgado-Ramos, Francisco J. Barba, and Francisco J. Martí-Quijal are members of the CYTED network "P320RT0186 - Aprovechamiento sostenible de recursos biomásicos vegetales iberoamericanos en cosmética (BIOLATES)". In addition, the authors are grateful for the technical support from the mass spectrometry group in the Central Service for Experimental Research (SCSIE) at the Universitat de València for their help in the analysis of the triple TOF-LC-MS-MS data.

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