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Sustainability-driven fractionation of hemp seed hulls into novel ingredients by the integrated high-pressure, ultrasound, and enzyme-assisted extractions

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ABSTRACT

Supercritical CO_2 (SFE-CO_2), pressurised liquid (PLE), ultrasound (UAE), and enzyme-assisted (EAE) extractions were applied for hemp seed hulls. Conventional and UAE with hexane and SFE-CO_2 recovered non-polar constituents. Consecutive PLE with acetone, ethanol, and water efficiently isolated higher polarity fractions. The antioxidant amounts after SFE-CO_2 and PLE were up to 16.5 mg GAE/g DW, 35.9 mg TE/g DW, and 77.4 mg TE/g DW for the TPC, ABTS, and ORAC assays, respectively. UAE with acetone before PLE increased the antioxidant potential of extracts. EAE at optimized parameters yielded 4.3–21.0 g/100 g DW of water-soluble substances, containing 17.3–50.1 mg GLU/g DW of reducing sugars. Enzymatic treatment increased the recovery of soluble constituents, the yield of sugars, and the antioxidant characteristics of supernatants and solid residues. The results on bioactivity indicate that all fractions can serve as sources of antioxidants and other nutrients, with potential applications in foods and nutraceuticals.

1. Introduction

Hemp (*Cannabis sativa* L.) is one of the earliest domesticated plant species, and it has been grown mainly for seeds, oil, and fibre since ancient times. However, commercial cultivation and processing of *C. sativa* became illegal in many countries after the endorsement of the UN Single Convention on Narcotic Drugs in 1961. Nowadays, industrial (fibre-type) hemp with low content of psychotropic tetrahydrocannabinol (THC $<$ 0.2 - 0.3 %) is officially legalized in many countries and has become an essential crop with the increasing cultivation areas and processing volumes. Applications of hemp in textile, insulation and building materials, the paper industry, horticulture, animal nutrition, food products, nutraceuticals, hygiene and cosmetics, medicine, agrochemistry, environment and energy production consist of $>$25,000 products (Crini et al., 2020). Furthermore, although industrial hemp has been studied as a multi-purpose crop (Dudziec et al., 2024), three main components of the plant are processed into numerous commercial products: fibre for textiles (Kirk et al., 2023) and composites (Müssig et al., 2020), inflorescence for non-psychoactive phytocannabinoids (Yang & Sun, 2024), and seed for oil and proteins (Tănase Apetroaei et al., 2024).

Processing of oilseed crops generates significant amounts of by-products, many of which are still discarded as waste or recycled for low-value applications, particularly due to the lack of scientific valorisation studies, which are necessary for developing innovative technological strategies to upcycle these by-products into more valuable materials sustainably. For instance, industrial hemp by-products have been studied as an ingredient in livestock rations (Altman et al., 2024; Mohamed et al., 2023), hemp threshing residues were biorefined into several valuable fractions containing cannabinoids, phenolics and dietary fibre (Kitrytė et al., 2018).

The outer coat of the hemp—the hull—has received much less attention than the seed. More recent research has revealed that hemp hulls may be a source of health-promoting bioactive compounds, among them *N-trans*-caffeoyltyramine and *N-trans*-feruloyltyramine, which were shown to shape the human gut microbiome *in vitro* (Martinez et al., 2024), to support gut health, liver function, and other physiological processes (Van Klinken et al., 2024). Five lignanamides isolated from the

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hemp seed hull ethyl acetate fraction inhibited soluble epoxide hydrolase, a therapeutic target for inflammation (Kim et al., 2023). Malayil et al. (2024) explored hemp hull biomass in an integrated C-5 biorefinery for producing xylose and activated carbon.

It should be noted that upcycling plant processing side-streams for the production of higher value novel ingredients for functional foods, food supplements, and food additives, as well as innovative packaging materials, is regarded as a promising trend in developing clean production processes and achieving the zero-waste society target set by the European Union till 2025 (Naziri et al., 2014). According to Facts and Factors (2025), the global industrial hemp market was valued at approximately USD 6.73 billion in 2023 and is expected to reach USD 26.42 billion by 2032, growing at a CAGR of roughly 16.4 %. Consequently, a significant increase in the side-stream volumes, including hemp seed hulls, may also be reasonably expected.

The methods for the extraction, fractionation, and purification of phytochemicals and other valuable constituents, which are present in the processing side-streams, may be conditionally divided into the traditional, more recently developed - commercialised, and emerging, which are still non-commercialised and/or their implementation upscaled at a minimal level (Galanakis, 2012, 2013; Wijngaard et al., 2012; Kammerer et al., 2014). Among more recent technologies, supercritical fluid extraction with CO_2 (SFE-CO_2), pressurised liquid (PLE), high hydrostatic pressure (HHPE), microwave (MAE), ultrasound (UAE) and enzyme (EAE) assisted extractions have been applied to numerous by-products for achieving cleaner and more sustainable utilisation of agro-food resources, particularly for upcycling and valorisation of by-products and waste (Okur & Okur, 2024).

The recovery of target constituents in a single-step conventional solvent extraction is limited by their affinity for solvent properties, particularly polarity; therefore, this type of extraction isolates only one fraction from the matrix and usually generates large amounts of residual by-products. Thus, modern circular economy strategies in the processing and valorisation of by-products have focused on the production of valuable materials for the food and nutraceutical industries using green chemistry principles and sequential extraction techniques. During the last decade, our research group has demonstrated that multi-step high-pressure (SFE-CO_2, PLE) and EAE of brewer's spent grain Kitrytė, Povilaitis at al., (2014), hemp threshing residues (Kitrytė at al., 2018), and small fruit pomace (Basegmez et al., 2017; Kitrytė at al., 2017, 2020) might be a promising strategy for the valorisation of by-products by their conversion into high-nutritional-value functional ingredients. SFE-CO_2 is a desirable green technology for the effective recovery of the lipophilic fraction, which consists mainly of triacylglycerols (Temelli, 2009). The method is desirable for replacing toxic organic solvents in the extraction of plant bioactives for functional foods and nutraceuticals. Moreover, although LCA of oil extraction from grape seed oil revealed that SFE-CO_2, when applied as a single process, was more expensive than hexane extraction, in the case of revalorization of defatted grape seeds, the CO_2-ethanol system showed the lowest global warming potential (Fonseca-Pérez et al., 2024). Consequently, higher-polarity compounds, such as polyphenolic antioxidants, can be re-extracted from the SFE--CO_2-defatted residue by PLE or other techniques, preferably using food-grade solvents of different polarities, *e.g.*, acetone, ethanol, and water. The optimization of extraction conditions for the recovery of the target phytochemicals by applying the consecutive high-pressure fractionation concept was reported for the recovery of lipids and phenolics from wheat and rye bran (Povilaitis & Venskutonis, 2015; Povilaitis et al., 2015), amaranth (Kraujalis & Venskutonis, 2013), and other plant materials.

High-pressure fractionation schemes may be expanded, and thereby the biorefining process potentially improved by subjecting plant material to ultrasound, microwave, or enzyme treatments, which have caused an increasing interest as innovative extraction methods of choice. Numerous industrial enzymes with different mechanisms of action are now available for the extraction of bioactive molecules (Hernández Becerra et al., 2021); however, challenges such as enzyme cost, enzyme inactivation, limited cellular disruption capacity, and process expense limit the wider industrial applications of this method. The integrated extraction techniques may be applied to mitigate EAE disadvantages, *e.g.*, by pairing EAE with other green methods such as UAE, MAE, SFE-CO_2, PLE, and others (Das et al., 2021). UAE is based on acoustic cavitation, which causes fragmentation, localised erosion, pore formation, increased shear forces, increased absorption, and swelling of the plant cellular matrix. It has therefore been widely tested for various materials, including fruit and vegetable processing by-products (Kumar et al., 2021).

To the best of our knowledge, a multi-step high-pressure UAE and EAE has not been reported for the sustainability-driven upcycling and valorisation of hemp seed hulls. Therefore, considering previously reported advantages of a comprehensive biorefining of various agri-food processing by-products into higher value ingredients, this study aimed to propose and to test a complex and innovative biorefining scheme for the development of a range of functional ingredients for foods and nutraceuticals from hemp hulls, and thereby to provide the input for the circular economy-based sustainability of the hemp processing sector. The following objectives were formulated for achieving this aim: (1) to evaluate the efficiency of conventional, SFE-CO_2, PLE, UAE and EAE techniques for the recovery of different polarity constituents; (2) to optimize the parameters of treatment with cellulolytic enzyme preparation Viscozyme L for the isolation of water-soluble components and reducing sugars and apply optimized EAE at different steps of biorefining; (3) to evaluate *in vitro* antioxidant properties of various recovered fractions. Such a systematic approach may facilitate the development of innovative biorefining schemes for fractionating industrial oilseed crop processing by-products into high-value ingredients with plausible food and nutraceutical applications.

2. Materials and methods

2.1. Materials

Dried hemp seed hulls were obtained from JSC "Agropro" (Vilnius, Lithuania) and ground prior to the analysis in an ultra centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.5 mm hole size sieve. Gallic acid (99 %), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX, 97 %), Folin & Ciocalteu's phenol reagent (2 M), 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), microcrystalline cellulose (20 μm), D-(+)-glucose (>99 %), 3,5-dinitrosalicylic acid (98 %), phenol (>99 %), potassium sodium tartrate tetrahydrate (Rochelle salt, 99 %) and Viscozyme L (cellulolytic enzyme mixture, ≥100 FBGU/g) were obtained from Sigma-Aldrich (Bornem, Belgium); sodium acetate (>99 %), citric acid (99 %, anhydrous), Na_2SO_3 (98.5 %, anhydrous) and NaOH (98 %, pellets) from Acros Organics (Geel, Belgium); fluorescein (FL) from Fluka Analytical (Bornem, Belgium); KCl, NaCl, $K_2S_2O_8$, Na_2HPO_4 and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt (ABTS) were from Merck (Darmstadt, Germany); Na_2CO_3 (98 %, anhydrous) from RPL (Grauwmeen, Belgium), KH_2PO_4 was from Jansen Chimica (Beerse, Belgium); CO_2 (99.9 %) from AGA (Vilnius, Lithuania), food grade ethanol from JSC Stumbras (Kaunas, Lithuania). All solvents were of analytical and HPLC-grade.

2.2. Conventional extraction techniques

The process of biorefining is summarized in Fig. 1. Briefly, Soxhlet extraction was performed from 20±0.1 g of ground hemp seed hulls in an automated extractor EZ100H (Behr Labor-Technik, Düsseldorf, Germany) using hexane (Sox-Hex) at 1 cycle per 5 min rate with a total time of 6 h (AOAC, 1995).

Solid-liquid extraction (SLE) was performed in a shaker from 20±0.1 g of hulls prior to or after SFE-CO2 using 80 mL of hexane or ethanol (solid:liquid ratio 1:4) at 60 °C, 800 rpm for 360 min. Afterwards, the

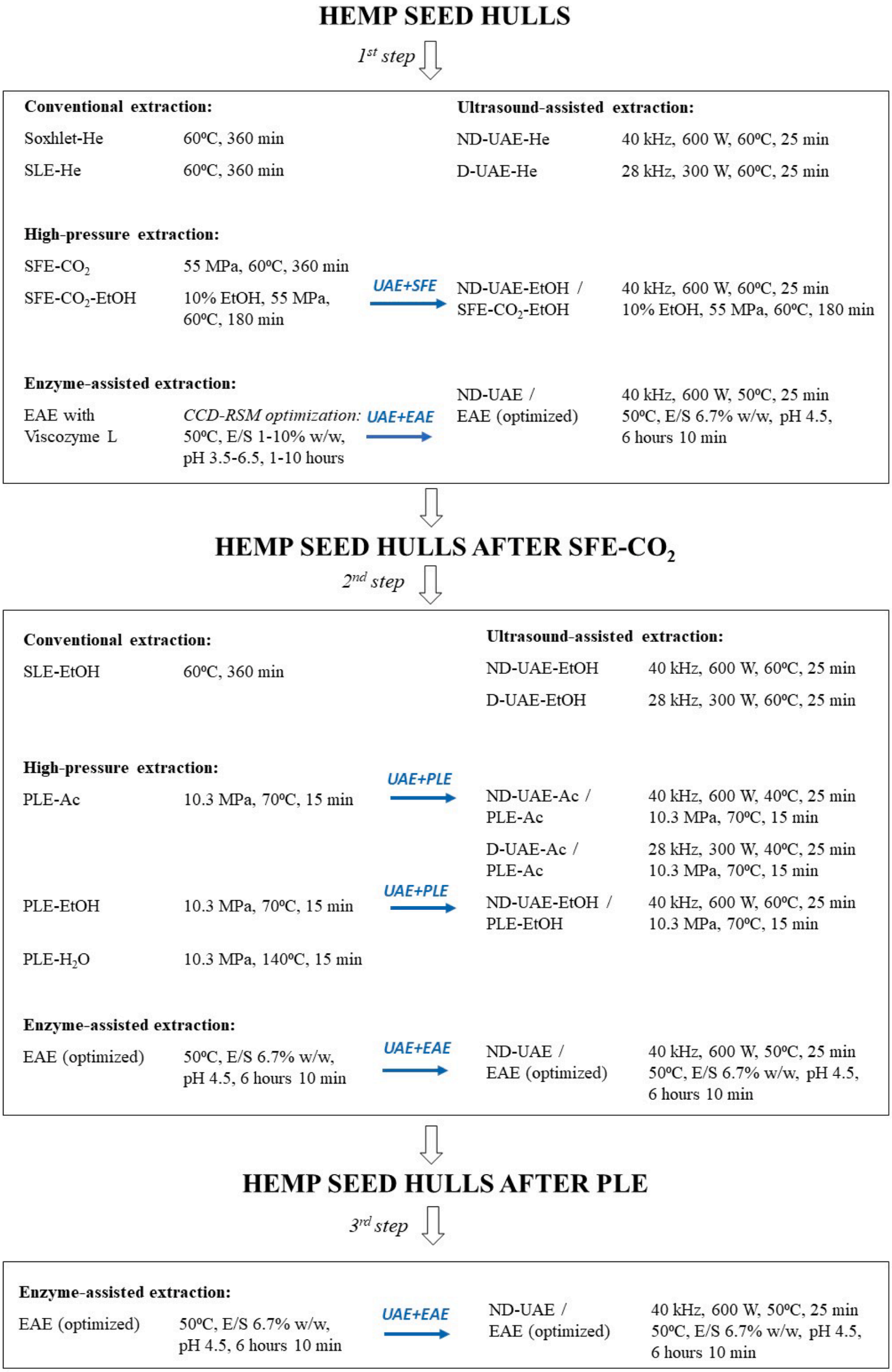

Fig. 1. Scheme of multistep high-pressure fractionation, ultrasonic treatment, and enzyme-assisted extraction of hemp seed hulls, and analysis of various products obtained.

samples were rapidly cooled, centrifuged at 9000 rpm for 10 min, and filtered. Soxhlet and SLE solvents were evaporated from the optically clear supernatants in a Rotavapor R–210 (Büchi, Flawil, Switzerland), followed by additional drying with a nitrogen flow for 15 min. The samples were kept at −18 °C. Sox-Hex, SLE-Hex and SLE-EtOH extract yields were expressed as g/100 g DW. The solid residues were dried at 50 °C.

2.3. High-pressure extraction methods

2.3.1. Supercritical CO_2 extraction (SFE-CO_2)

SFE-CO_2 was carried out in a Helix extractor (Applied Separation, Allentown, PA) from 200 ± 1.0 g ground hulls using a 500 mL stainless steel vessel (76 mm id, 114 mm length) at the following parameters: 60 °C, 55 MPa, static and dynamic extraction time 30 min and 360 min, respectively, CO_2 flow rate of 2–3 L/min. In addition, SFE-CO_2 with 10 % of a co-solvent ethanol was performed from 20±0.1 g ultrasound-treated and untreated hemp seed hulls, loaded in 50 mL stainless steel vessel (14 mm id, 320 mm length) under the following conditions: 60 °C, 55 MPa, static and dynamic extraction time 30 min and 180 min, respectively, CO_2 flow rate 2–3 L/min. SFE-CO_2 parameters were selected according to the previously optimised by Povilaitis and Venskutonis (2015). The extracts were collected in glass bottles, kept under the N_2 flow for 15 min to remove EtOH residues and stored at −18 °C. SFE-CO_2, SFE-CO_2-EtOH and ND-UAE-EtOH/SFE-CO_2-EtOH (ND-UAE explained in 2.4) extract yields were expressed as g/100 g DW.

2.3.2. Pressurized liquid extraction (PLE)

PLE was performed as described by Povilaitis et al. (2015) in an accelerated solvent extraction apparatus ASE 350 (Dionex Sunnyvale, *CA*, USA) from 7 ± 0.1 g of ultrasound-treated and untreated hull residue after SFE-CO_2, applying different polarity solvents, acetone (PLE-Ac), ethanol (PLE-EtOH), and water (PLE-H_2O). The sample was placed in a 66 mL stainless-steel cell (2.9 cm diameter). The PLE conditions were the following: 70 °C for PLE-Ac and PLE-EtOH, 140 °C for PLE-H_2O, 10.3 MPa, 5 min pre-heating time, 15 min static time (3 cycles × 5 min), 100 % of cell flush volume, 120 s purge time with N_2. Organic solvents were evaporated in a Rotavapor R–210, and water was removed by freeze-drying. The extracts were collected in glass bottles, flushed with N_2 for 15 min to remove residual solvent, and stored at −18 °C. PLE-EtOH, ND-UAE-EtOH/PLE-EtOH, PLE-Ac, PLE-Ac-EtOH, PLE-Ac-EtOH—H_2O, ND-UAE-Ac/PLE-Ac, and D-UAE-Ac/PLE-Ac extract yields were expressed as g/100 g DW. The SFE-CO_2 and PLE solid residues were collected and kept in the hermetically closed glass vessels.

2.4. Ultrasound-assisted extraction (UAE)

Ground hulls (20±0.1 g) before and after SFE-CO_2 were loaded into a 150 mL glass extraction flask, mixed with 80 mL hexane, ethanol, acetone, or citrate buffer (Fig. 1), and subjected to the indirect (ND-UAE) or direct (D-UAE) ultrasonic treatment. ND-UAE was performed in the GT SONIC 2300B ultrasonic bath at the following conditions: temperature 40 °C (acetone), 50 °C (citrate buffer) or 60 °C (hexane and ethanol), time 25 min, frequency of 40 kHz, and a maximum power of 600 W. The optimal position of the flask in the bath was determined by measuring the intensity of the excited ultrasonic waves (1.6 W/cm2) in the water using an immersion-type ultrasonic receiving transducer and the intensity meter YP0511A. Temperature stability was checked using a FLUKE 1523 Reference thermometer every 5 min (variations did not exceed ±2 °C over the entire sonication time). D-UAE of the hull samples was carried out with a high-intensity HCTS ultrasonic probe submergible horn (10 mm diameter, 1 cm deep, frequency 28 kHz, and a power 300 W). The intensity of the excited ultrasound by the active area of 0.79 cm^2 was 764 W/cm^2. The pulse-type operation mode (5 min on and 5 min off) was used to avoid temperature fluctuations caused by the high intensity of direct sonication. The sonication duration and the temperature of the treated suspensions were maintained at the same values as described for ND-UAE. After EAE, suspensions were stored at 4 °C for 1–2 h, centrifuged (9000 rpm, 10 min), and filtered. Organic solvents from the optically clear supernatants were evaporated in a Rotavapor R–210. The extracts were kept under a nitrogen flow for 15 min and stored at −18 °C before analysis. ND-UAE-He, D-UAE-He, ND-UAE-EtOH, and D-UAE-EtOH extract yields were determined gravimetrically (±0.001 g) and expressed as g/100 g DW. The solids were collected, dried at 50 °C, and kept in a dry place.

2.5. Enzyme-assisted extraction (EAE)

EAE parameters were selected according to the procedure reported by Kapasakalidis et al. (2009). Hemp seed hulls before SFE-CO_2 (Fig. 1) were treated with Viscozyme L preparation at 50 °C at varying enzyme/substrate ratio (1–10 % w/w, corresponding to 1–10 FBGU/g of hulls), pH (3.5–6.5), and time (1–10 h). The variables were selected as reported by Sørensen et al. (2005). The hulls (2.5 g) were loaded into a 50 mL polyethylene centrifugation tube, suspended in 25 mL of 50 mM sodium citrate buffer, followed by the addition of the enzyme to reach the settled E/S ratio and incubated in a temperature-controlled shaker (800 rpm) at various time combinations. The blanks, namely A (pomace+buffer), B (enzyme+buffer) and C (buffer) were prepared simultaneously. EAE was terminated by immersing the centrifugation bottle in boiling water for 10 min, rapid cooling, and 10 min centrifugation at 9000 rpm. Water-soluble and solid residue fractions were collected, freeze-dried, and kept at −18 °C.

2.6. Determination of reducing sugar content

Reducing sugar content in EAE-derived supernatants and corresponding control samples was determined by applying the DNS (3,5-dinitrosalicilic acid) method (McCleary & McGeough, 2015) with slight modifications. A 1 % DNS reagent solution was prepared by dissolving 10 g DNS, 2 g phenol (liquid at 50 (C), 0.5 g sodium sulphite, and 10 g sodium hydroxide in the dist. H_2O and diluting to a final volume of 1 L. One mL of sample (5 mg/mL) was mixed with 3 mL of 1 % DNS reagent solution in the flat-capped plastic tubes and heated in a boiling water bath for 5 min to obtain a red-brown colour, followed by the addition of 1 mL of 40 % potassium sodium tartrate (Rochelle salt) solution, rapid cooling, and 30 min incubation in ambient temperature. 100 μL of optically clear supernatant was transferred to a 96-well transparent microplate, and the absorbance was measured at 540 nm in a FLUOstar Omega Reader (BMG Labtech, Offenburg, Germany). The RSC was expressed as glucose equivalents (mg GLU/g sample) using dose-response curves for L-glucose (0.312 – 5 mg/mL 50 mM citric acid buffer, pH=4.8).

2.7. In vitro antioxidant activity assessment

Total phenolic content (TPC) of hull extracts and EAE-derived supernatants was evaluated by the modified procedure of Singleton et al. (1999). The $ABTS^{\bullet+}$ scavenging was measured by the method of Re et al. (1999). Oxygen radical absorbance capacity (ORAC) of supernatants was evaluated using the procedure proposed by Prior et al. (2003). Antioxidant capacity of water-insoluble fractions (Fig. 1) was assessed by the QUENCHER method (Gökmen et al., 2009). Detailed description of the methods is available elsewhere (Kitrytė et al., 2014; Kitrytė, Kraujalienė et al., 2017).

2.8. Experimental design and statistical analysis

Response surface methodology (RSM) using a central composite design (CCD) was used to assess the effects of 3 independent variables on RSC in EAE-derived supernatants and to determine optimal EAE parameters. The model, consisting of 20 experimental runs with 8 factorial points, 6 axial points, and 6 centre points (Table 1), was established using the software Design-Expert trial version 8.0.7.1 (Stat–Ease Inc., Minneapolis, MN), as previously reported by Kraujalis and Venskutonis (2013). Standard statistical methods were applied for data handling as described elsewhere (Kitrytė, Kraujalienė et al., 2017).

3. Results and discussion

3.1. Yields of non-polar fractions of hemp seed hulls

During the 1st biorefining step (Fig. 1), 8.75 ± 0.26 – 14.07±0.20 g/100 g DW of lipophilic fractions were isolated from crude hulls with hexane by means of Soxhlet-He, SLE-He, ND-UAE, and D-UAE, and by SFE-CO_2 without and with a co-solvent ethanol (Table 2). The results indicate that the efficiency of SFE-CO_2 for extracting lipophilic constituents was 38 % lower than that of the Soxhlet and SLE techniques. The prolonged SFE-CO_2 time (up to 360 min) was based on the extraction kinetics data and could be reduced, as >90 % of the lipophilic compounds are recovered after 4.5 h of extraction. UAE with hexane at significantly shorter extraction time (25 *vs* 360 min) yielded on average 10.8 g/100 DW of oil, which amounted 77 %, 115 % and 124 % of total Soxhlet-He, SLE-He, and SFE-CO_2 yields. The results obtained by applying indirect and direct ultrasound treatments were not significantly different. Therefore, 25 min sonication could be used instead of the conventional 6-hour Soxhlet-He and SLE-He. The addition of 10 % ethanol as a co-solvent increased the yield of SFE-CO_2 by 23 %, thus achieving UAE-He yields in 180 min of extraction; however, with no significant changes due to the ultrasound treatment of hulls before SFE-CO_2 (Table 2).

Table 1
Central composite design matrix (levels of independent variables and variation levels in natural values) for the EAE optimisation using Viscozyme®L and hemp seed hulls prior SFE-CO_2, and values of observed responses (reducing sugar concentration RSC in EAE-derived supernatants, mg GLU/mL).

Levels and runs	Extraction variables in natural values			RSC[**]	Increase[***]	
	τ, h	pH	*E/S ratio,% w/w*[*]	*mg GLU/ mL*	*mg GLU/ mL*	*%*
Low level (−1)	**1.0**	**3.5**	**1.0**			
Medium Level (0)	**5.5**	**5.0**	**5.5**			
Max level (+1)	**10.0**	**6.5**	**10.0**			
1 (center)	5.5	5.0	5.5	5.18 ± 0.51	3.05	143.1
2 (factorial)	10.0	3.5	1.0	2.97 ± 0.13	1.60	116.6
3 (center)	5.5	5.0	5.5	5.25 ± 0.07	3.12	146.3
4 (center)	5.5	5.0	5.5	5.36 ± 0.15	3.23	151.1
5 (axial)	10.0	5.0	5.5	6.0 ± 0.14	3.17	111.8
6 (axial)	5.5	5.0	1.0	3.67 ± 0.05	1.65	77.4
7 (factorial)	10.0	6.5	1.0	2.31 ± 0.21	1.02	78.7
8 (axial)	5.5	3.5	5.5	5.43 ± 0.25	4.05	295.7
9 (center)	5.5	5.0	5.5	5.02 ± 0.09	2.89	135.6
10 (axial)	5.5	5.0	10.0	5.89 ± 0.53	3.76	176.2
11 (axial)	1.0	5.0	5.5	3.91 ± 0.13	2.20	128.3
12 (factorial)	10.0	3.5	10.0	6.84 ± 0.16	5.47	398.7
13 (center)	5.5	5.0	5.5	5.36 ± 0.18	3.23	151.5
14 (factorial)	1.0	3.5	10.0	3.84 ± 0.21	2.65	221.2
15 (factorial)	1.0	6.5	10.0	3.19 ± 0.18	2.12	198.4
16 (factorial)	1.0	3.5	1.0	2.46 ± 0.12	1.27	105.8
17 (factorial)	10.0	6.5	10.0	6.12 ± 0.34	4.83	374.6
18 (center)	5.5	5.0	5.5	5.24 ± 0.11	3.11	145.9
19 (factorial)	1.0	6.5	1.0	1.87 ± 0.07	0.81	75.4
20 (axial)	5.5	6.5	5.5	4.62 ± 0.31	3.12	208.0

E/S: enzyme/substrate ratio; *τ:* extraction time.

[*] corresponds to 1–10 FBGU/ g hemp seed hulls.

[**] RSC was calculated excluding the RSC of the *Blank B*:.

[***] calculated as the RSC difference between enzyme-treated sample and *Blank A*, excluding the RSC of the *Blank C*.

Comparing with some other reports, similar differences between Soxhlet-He and SFE-CO_2 were reported for the yields of hemp seeds oil, 30 and 22 %, respectively; however, the oil recovered under optimal SFE-CO_2 conditions (30 MPa, 40 °C, 4 h) was characterized by significantly higher oxidative stability and antiradical capacity, while ultrasound pre-treatment (20 Hz) of seeds prior SFE-CO_2 for 10 min only slightly increased oil yield (Da Porto et al., 2012a, 2012b, 2015).

Based on the above-mentioned literature data, SFE-CO_2 (55 MPa, 60 °C, 360 min) was chosen as the most appropriate technique for the recovery of lipophilic fraction from hemp seed hulls for this research too, thus avoiding (1) hexane removal after extraction, (2) elimination of problematic polar and bound lipids from the sample, (3) low extraction efficiency of target metabolites, (4) plausible degradation of valuable constituents upon solvent removal procedure, (5) high percentage of toxic solvent residues (maximum hexane residue limits in the foods is 1 mg/kg, as set by Directive 2009/32/EC).

3.2. Yields of polar fractions of hemp seed hulls after SFE-CO_2

Further, SLE, UAE PLE, and combined UAE/PLE were applied to isolate medium polarity acetone and hydrophilic ethanol and water-soluble constituents from the hemp seed hull residue after SFE-CO_2. The yields of EtOH extracts, calculated for the initial material (before SFE-CO_2), were from 1.58 ± 0.12 to 6.86 ± 0.53 g/100 g DW (Table 2) and decreased in the following manner: direct UAE < indirect UAE and SLE < PLE < PLE with indirect UAE pre-treatment. These results indicate that PLE was the most efficient method for the recovery of higher-polarity substances,

Different modifications of PLE-Ac recovered up to 5.62 ± 0.40 g/100 g DW. Indirect UAE slightly enhanced the efficiency at the first step of consecutive PLE; however, the differences between the UAE-treated and non-treated samples were not significant. In general, the effects of UAE were not so evident when using both ethanol and acetone, particularly when comparing direct and indirect treatments. The total yield of higher polarity substances was increased to 11.46 g/100 g DW by using a cascade PLE with acetone, ethanol, and water; the latter step (PLE-Ac-EtOH—H_2O) extracted an additional 4.59 ± 0.07 g/100 g DW of hydrophilic substances. In addition, consecutive extractions with different-polarity solvents yield 3 distinct fractions, which may be advantageous for their further application. It may be noted that, according to Directive 2009/32/EC, both acetone and ethanol are extraction solvents approved for all applications in compliance with GMP.

Chen et al. (2012) indicated that UAE with 10 different polar solvents, obtained by mixing methanol, ethanol and acetone with water (0, 50, 75, 100 %), yielded respectively 1.5–10.0 %, 0.5–8.4 % and 5.7–8.2 % of antioxidative active polar extracts from hemp seeds, which activity increased in the following order: ethanol > acetone > methanol. Xu et al. (2017) showed that UAE with 60 % ethanol at optimized parameters (40 °C, 400 W, 40 Hz, 9.8 min, ratio of solvent to solid 56.9:1

Table 2

Yields (g/100 g) of various non-polar and polar extracts and solid residues, obtained from hemp seed hulls applying conventional (Soxhlet and SLE), high pressure ($SFE-CO_2$ and PLE), and ultrasound-assisted (UAE) extraction techniques at various extraction conditions.

Sample	**Extraction conditions**				**Yield***			
					Extract		***Solid residue***	
	Pressure, MPa	Frequency, kHz	Temperature, °C	Time, min	*g/100 g*	g/100 g DW	*g/100 g*	g/100 g DW
Non-polar fractions from hemp seed hulls (crude):								
Hexane extraction:								
Soxhlet-He	0.1**	-$^{n.a}$	68***	360	-$^{n.a}$	14.07±0.20 g	-$^{n.a}$	85.93±1.22^{a}
SLE-He	0.1	-$^{n.a}$	60	360	-$^{n.a}$	9.40±0.08^{ef}	-$^{n.a}$	90.60±0.77^{a}
ND-UAE-He	0.1	40	60	25	-$^{n.a}$	10.91±0.01^{f}	-$^{n.a}$	89.09±0.82^{a}
D-UAE-He	0.1	28	60	25	-$^{n.a}$	10.76±0.18^{f}	-$^{n.a}$	89.24±1.49^{a}
$SFE-CO_2$ extraction:								
$SFE-CO_2$	55	-$^{n.a}$	60	360	-$^{n.a}$	8.75±0.26^{e}	-$^{n.a}$	91.25±2.71^{a}
$SFE-CO_2$-EtOH (10 %)	55	-$^{n.a}$	60	180	-$^{n.a}$	10.78±0.27^{f}	-$^{n.a}$	89.22±2.23^{a}
ND-UAE-EtOH/$SFE-CO_2$-EtOH(10 %)	0.1/55	40/-$^{n.a}$	60/60	25/180	-$^{n.a}$	10.35±0.33^{f}	-$^{n.a}$	89.65±2.86^{a}
Higher polarity fractions from solid hemp seed hull residues after $SFE-CO_2$:								
Et-OH extraction:								
SLE-EtOH	0.1	-$^{n.a}$	60	360	2.67±0.21^{ab}	2.44±0.19^{a}	97.38±7.66^{a}	88.86±6.99^{a}
ND-UAE-EtOH	0.1	40	60	25	1.73±0.13^{a}	1.58±0.12^{a}	98.27±7.38^{a}	89.67±6.73^{a}
D-UAE-EtOH	0.1	28	60	25	3.03±0.08^{ab}	2.76±0.07^{a}	96.97±2.56^{a}	88.49±2.34^{a}
PLE-EtOH	10.3	-$^{n.a}$	70	15	6.14±0.46^{de}	5.60±0.42^{c}	93.86±7.03^{a}	85.65±6.41^{a}
ND-UAE-EtOH/PLE-EtOH	0.1/10.3	40/-$^{n.a}$	60/70	25/15	7.52±0.58^{e}	6.86±0.53^{d}	92.48±7.13^{a}	84.39±6.51^{a}
Acetone extraction:								
PLE-Ac	10.3	-$^{n.a}$	70	15	5.52±0.48^{d}	5.04±0.44^{c}	94.48±8.22^{a}	86.21±7.50^{a}
ND-UAE-Ac/PLE-Ac	0.1/10.3	40/-$^{n.a}$	40/70	25/15	6.16±0.44^{de}	5.62±0.40^{c}	93.84±6.70^{a}	85.30±6.11^{a}
D-UAE-Ac/PLE-Ac	0.1/10.3	28/-$^{n.a}$	40/70	25/15	4.03±0.40^{bc}	3.68±0.37^{b}	95.97±9.53^{a}	87.57±8.70^{a}
Consecutive PLE extraction:								
PLE-Ac	10.3	-$^{n.a}$	70	15	5.52±0.48^{d}	5.04±0.44^{c}	94.48±8.22^{a}	86.21±7.50^{a}
PLE-Ac-EtOH	10.3	-$^{n.a}$	70	15	2.01±0.11^{a}	1.83±0.10^{a}	92.47±7.24^{a}	84.37±6.61^{a}
PLE-Ac-EtOH-H_2O	10.3	-$^{n.a}$	140	15	5.03±0.08^{cd}	4.59±0.07^{c}	87.44±4.66^{a}	79.79±4.25^{a}

[*] : Fraction yield expressed as g/100 g of hemp seed hulls residue after $SFE-CO_2$ or g/100 g DW of starting material prior $SFE-CO_{2;}$.

[**] 0.1 MPa – standard atmospheric pressure.

[***] hexane boiling point

-$^{n.a}$: not applicable; Ac: acetone; D-UAE: direct ultrasound-assisted extraction; EtOH: ethanol; He: hexane; ND-UAE: indirect ultrasound-assisted extraction; PLE: pressurised liquid extraction; $SFE-CO_2$: supercritical carbon dioxide extraction; SLE: conventional solid-liquid extraction. Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$).

mL/g) recovers higher amounts of the antiradical compounds from the *Limonium sinuatum* flowers during the remarkably shorter extraction time as compared to the traditional maceration and Soxhlet extraction. Similarly, 70 % ethanol extraction with ultrasound (61 °C, 400 W, 23.7 min, solvent-to-solid ratio 21.7:1 mL/g) enhances the recovery of anthocyanins and phenolics from blueberry wine pomace (He et al., 2016). Nevertheless, careful selection of appropriate ultrasonic treatment parameters is essential (He et al., 2016; Wang et al., 2016; Xu et al., 2017), since sonication can potentially degrade target compounds (Da Porto et al., 2015).

3.3. *Optimization of EAE parameters and model analysis*

So far as EAE depends on various factors (enzyme concentration, substrate characteristics, time, selected responses, and others) and their interactions, the process should usually be optimized (Guan & Yao, 2008; Vaštag et al., 2010). In this research, commercially available Viscozyme L, which is mixture of cellulolytic enzymes, was used for EAE optimization before $SFE-CO_2$ (Fig. 1). The effects E/S ratio (1–10 % w/w), buffer pH (3.5–6.5) and time (1–10, hours) at constant temperature of 50 °C on the reducing sugar concentration (RSC) in EAE supernatants (mg GLU/mL) were analysed by using RSM and CCD, enabling simultaneous assessment of multiple factors and their interactions with fewer experiments than a classical one-factor-at-a-time optimization approach (Cannavacciuolo et al., 2024). The experimental ranges of independent variables for EAE optimization were selected based on the manufacturer-reported activity and stability profile of Viscozyme L, showing optimal performance at pH 3.3–5.5 and 40–50 °C, while remaining active up to pH 6.5 and approximately 60 °C. In this study, the EAE temperature was fixed at 50 °C, following the previous report by Kapasakalidis et al. (2009), which suggested that Viscozyme L exhibits higher catalytic efficiency at 50 °C than at 40 °C. The remaining independent EAE variables (pH, E/S ratio, and time) were varied across the enzyme's feasible operating range, covering practically relevant low-to-high levels for CCD–RSM modelling.

Experimental data show that under different conditions, supernatants contained 1.87–6.84 mg GLU/mL of reducing sugars after EAE, corresponding to a 75–399 % increase in reducing sugar concentration compared with samples without enzymatic treatment (Table 1). The analysis of variance (Table 3) confirmed that the model was significant,

Table 3

Analysis of variance of the regression parameters for response surface quadratic model.

Source	SS	df	MS	F	*p*-value
Model	37.50	9	4.17	119.02	< 0.0001*
Time (τ, *hours*)	8.03	1	8.03	229.40	< 0.0001*
pH	1.17	1	1.17	33.49	0.0002*
ES ratio (*E/S, % w/w*)	15.86	1	15.86	453.11	< 0.0001*
τ *pH*	0.002381	1	0.002381	0.068	0.7996**
τ *E/S*	3.11	1	3.11	88.92	< 0.0001*
pH *E/S*	0.001682	1	0.001682	0.048	0.8309**
τ^2	0.68	1	0.68	19.37	0.0013*
pH^2	0.49	1	0.49	14.06	0.0038*
E/S^2	1.23	1	1.23	35.16	0.0001*
Residual	0.35	10	0.035		
Lack of fit	0.27	5	0.054	3.33	0.1061**
Pure error	0.081	5	0.016		
Total SS	37.85	19			

[*] significant.

[**] not significant; SS: sum of square; df: degree of freedom; MS: mean square; F: Fisher value.

and the non-significant lack-of-fit ($p > 0.05$) indicated that it adequately represented the experimental data. Additionally, the high determination coefficient ($R^2 = 0.9908$), together with the close agreement between the adjusted ($R^2 = 0.9824$) and predicted ($R^2 = 0.9561$) values and a low coefficient of variation (4.13 %), further demonstrates that the model fits the data well and is highly reproducible. Predicted (calculated using the regression model) and experimental RSC values after EAE are plotted in Fig. 2A.

The impact of significant model factors ($p < 0.05$) on reducing sugar content (RSC) decreased in the following order: $E/S > \tau >$ linear interaction between E/S and $\tau >$ quadratic effect of $E/S >$ pH $>$ quadratic effects of τ and pH. In contrast, the remaining linear interactions between τ and pH as well as between pH and E/S were not significant ($p > 0.05$), suggesting limited mutual influence under the studied enzymatic-assisted extraction (EAE) conditions. 3D plots in Fig. 2B-D represent the RSC as a function of 2 independent variables with the third factor fixed at the zero level (Table 1), as well as the interactions between the tested factors and their importance on the selected response in EAE supernatants. Among the significant variables, the E/S ratio exerted the most pronounced effect on RSC, with higher enzyme concentrations facilitating more extensive hydrolysis of glycosidic bonds and thereby enhancing sugar release. Extraction time was the next most influential factor, as prolonged reaction durations allowed the enzyme to interact more completely with the substrate, resulting in greater solubilization and liberation of reducing sugars. The significant linear E/S and τ interaction indicates a positive synergistic relationship between these two factors, indicating that the efficiency of enzymatic hydrolysis depends on a balanced combination of enzyme dosage and hydrolysis duration. As illustrated in Fig. 2D (E/S *vs.*τ), higher E/S ratios were most effective when paired with sufficiently long EAE times. This response surface plot shows a dome-shaped region, with the highest RSC values achieved at moderate-to-high enzyme dosages (6–10 % w/w

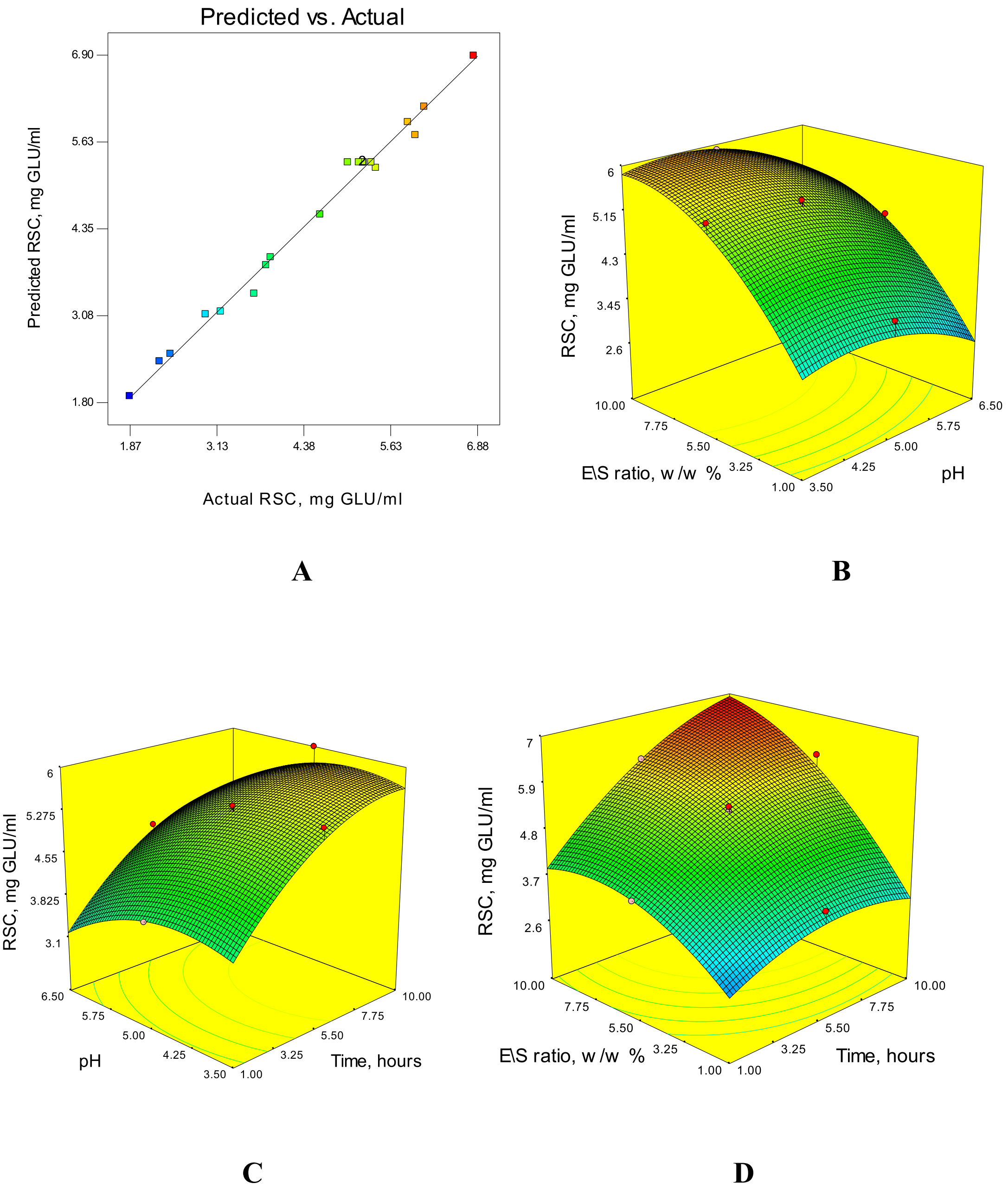

Fig. 2. A: Comparison between predicted and observed reducing sugar concentration (RSC, mg GLU/mL); B, C, D: Response surface 3D plots showing the effects of independent variables (E/S ratio, extraction pH and time) on RSC in EAE supernatants after EAE using Viscozyme® L and hemp seed hulls prior SFE-CO_2.

relative to the substrate mass) and prolonged hydrolysis (>6 h). In contrast, at low E/S ratios, even extended EAE time led to limited sugar release, confirming that enzyme availability was the limiting factor under these conditions. The slight curvature of the 3D plot near the maximum region indicates that excessively high enzyme concentrations or overly long hydrolysis do not further enhance RSC, consistent with the significant quadratic effects E/S^2 and τ^2, and suggests that beyond specific enzyme concentration and EAE time, the rate of sugar release plateaus, likely due to substrate saturation or product accumulation. Although less influential than E/S and τ, pH also affected RSC, indicating the enzyme's dependence on protonation conditions for catalytic efficiency. As shown in Fig. 2B (E/S *vs.*pH), RSC decreased with increasing pH, reaching its maximum under strongly acidic conditions (pH < 4.6). Similarly, Fig. 2C (pH *vs.*τ) shows that lower pH values, combined with longer extraction times, enhance RSC. However, the curvature along the time axis indicates diminishing effect beyond approximately 8 h, likely due to substrate depletion or the limited availability of hydrolysable sites. The empirical relationship between the dependent and the independent variables in coded factors is given in the equation, allowing direct comparison of factor effects by direction and magnitude within the selected EAE operability region:

$$RSC = 5.32 + 0.90 \times (\tau) - 0.34 \times (pH) + 1.26 \times (E/S) - 0.017 \times (\tau \times pH) + 0.62 \times (\tau \times E/S) - 0.014 \times (pH \times \tau) \\ -0.50 \times (\tau^2) - 0.42 \times (pH^2) - 0.67 \times (E/S^2)$$

Thus, optimal EAE parameters for the highest RSC in supernatants, minimizing extraction time and E\S ratio, were the following within the selected operability region: E/S ratio 6.71 % w/w (6.71 FBGU/g hulls), pH 4.55, extraction time 6 h 10 min. The yields and RSC of water-soluble (*SF*) and insoluble (*NF*) fractions of optimized EAE and control samples at different biorefining steps are listed in Table 4. Under optimal EAE conditions, crude hemp seed hulls (prior SFE-CO_2) yielded 19.6 g/100 g DW of water-soluble fraction. Significantly lower amounts of water-soluble constituents were recovered from enzyme-treated hemp seed hull residues after SFE-CO_2 (1.4-fold decrease in global yield, as compared to the sample before SFE-CO_2), and after polar constituent extraction via PLE with acetone, ethanol, and water (1.8–4.5-fold decrease). Nevertheless, compared with the control sample and expressed as an increase in soluble component yield, EAE efficiency followed the opposite trend. Viscozyme-assisted EAE resulted in a yield increase of 145–277 % for the residues after various steps of PLE with acetone, ethanol, and water, while enzyme treatment of hemp seed hulls before and after SFE-CO_2 was not so efficient and augmented soluble-component recovery only by 18–53 %, as compared to hulls after incubation in solely 50 mM sodium citrate buffer. UAE prior EAE had a significant positive effect and assisted in extracting a 1.7-fold higher amount of SF-Blank fraction only from the hemp seed hull residue after PLE-Ac. In most cases no significant differences were noted for ultrasound-treated and non-treated samples. The amounts of solid residues after EAE at various biorefining steps of hemp seed hulls varied from 75 to 80 g per 100 g of starting plant material.

RSC analysis showed that enzyme addition augmented sugar content from 116 to 333 mg GLU per g supernatant. When recalculated per gram of starting plant material, this corresponded to values of 17−50 mg GLU, with the highest value obtained for the SF-Viscozyme fraction from crude hemp seed hulls. In most cases, introducing UAE prior EAE was noticed to decrease RSC. Nevertheless, in all cases, the RSC (mg GLU/g DW) of EAE extracts remained 1.7–22.4-fold higher as compared to the enzyme-untreated control samples, suggesting that Viscozyme L-assisted EAE with or without UAE may cause some degradation of cell walls constituting polysaccharides and release of reducing sugar components from hemp seed hulls. In agreement with the above-discussed results, higher enzyme-treatment efficiency (in comparison to control samples) was achieved by applying EAE on hemp seed hull residues after high-pressure fractionation with polar solvents (Fig. 1), as compared to the materials before and after SFE-CO_2. It may be explained by the partial degradation of cell-wall polysaccharides upon removal of polar compounds under increased pressure, while the enzyme may contribute to the release of monosaccharides.

Significant increase in RSC *via* cellulase-induced cell-wall polysaccharide hydrolysis was also reported for some other by-products in the juice and wine industry, *e.g.,*grape skins (Arnous & Meyer, 2010) and pomace (Chamorro et al., 2012), black currant pomace (Kapasakalidis et al., 2009), Earlier, optimization of oat bran protein extraction using Viscozyme L was conducted by Guan and Yao (2008). Authors reported a lower optimal E/S ratio (3 FBGU/g of bran) and shorter optimal incubation time (2.8 h) as compared to the results of our study, which could be explained by the different starting materials utilized and response factors (target fractions) selected for EAE optimization.

3.4. In vitro antioxidant potential assessment

In vitro antioxidant potential evaluation of extracts, EAE supernatants and control samples was evaluated as a radical scavenging capacity by $ABTS^{\bullet+}$ decolourisation method, which is based on electron transfer, ORAC assay, which is using of a more relevant to biosystems radical source (Prior et al., 2003), and as a total phenolic content (TPC), which is determined with Folin-Ciocalteu's reagent. Although the latter assay is commonly used to determine TPC in natural products (Singleton et al., 1999), different compounds, *e.g.*, reducing sugars, may also reduce the Folin-Ciocalteu reagent. Therefore, this assay can also be applied to measure the reducing capacity of the whole sample (Prior et al., 2005).

As presented in Table 5, the TPC ranged from 43.10 ± 0.66 to 48.15 ± 1.77, and from 50.37 ± 0.70 to 246.29 ± 4.65 mg GAE/g of non-polar and polar hemp seed hull extracts, respectively. Considering the extracts' yield of the extracts (Table 2), this amounted from 1.94 ± 0.04 to 6.12 ± 0.03 mg GAE/g DW plant material. As obtained in ABTS and ORAC assays, polar SLE, UAE, and PLE extracts showed up to 73-fold higher radical scavenging capacity ($TEAC_{ABTS} = 47.76 \pm 2.52 - 613.28 \pm 15.79$ and $TEAC_{ORAC} = 328.86 \pm 21.20 - 1236.23 \pm 20.29$ mg TE/g), as compared to non-polar fractions ($TEAC_{ABTS} = 8.42 \pm 0.61 - 28.72 \pm 2.62$; $TEAC_{ORAC} = 67.42 \pm 5.95 - 225.15 \pm 10.81$ mg TE/g). When recalculated per gram of unextracted hemp seed hulls, the central portion of antioxidants was recovered from hemp seed hulls by PLE with acetone, ethanol, and water, with or without UAE treatment: 2.41 ± 0.13 − 20.60 ± 0.79, and 7.05 ± 0.42 − 27.86 ± 0.80 mg TE/g DW for ABTS and ORAC assays, respectively. Significantly higher recovery of radical scavengers in case of SFE-CO_2 was achieved by adding of a co-solvent ethanol; on average, $TEAC_{ABTS} = 3.0$ and $TEAC_{ORAC} = 21.7$ mg TE/ g DW. These values for hexane extracts obtained at the 1st step of biorefining *via* SLE, Soxhlet and UAE were 2-fold lower: on average, $TEAC_{ABTS} = 1.1$ and $TEAC_{ORAC} = 10.3$ mg TE/g DW, as compared to SFE-CO_2-EtOH with or without UAE pre-treatment. It may be observed that ultrasound treatments before PLE with acetone significantly increased TPC and ABTS values, whereas ORAC increased only with indirect UAE. These tendencies have not been observed in the case of other methods applied in this study. The highest activity of EtOH and H_2O extracts is in agreement with the results of Chen et al. (2012), who

Table 4

Yield (g/100 g DW), reducing sugar content (RSC, mg/g and DW), TPC (mg GAE/g and DW), ABTS$^{\bullet+}$ and oxygen radical scavenging capacity (TEAC, mg TE/g and DW) of water-soluble (SF) and non-soluble (NF) fractions and control samples, obtained after optimized EAE (E/S 6.7 % w/w, 50 °C, pH 4.55, 6 h 10 min) and indirect UAE (40 kHz, 50 °C, 25 min) of hemp seed hulls and solid residues after SFE-CO_2 and PLE.

Sample	Prior SFE-CO_2		After SFE-CO_2		After SFE-CO_2-PLE-EtOH:		After SFE-CO_2-PLE-Ac:		After SFE-CO_2-PLE-Ac-EtOH:		After SFE-CO_2-PLE-Ac-EtOH—H_2O	
	EAE	*UAE/EAE*	*EAE*	*UAE/EAE*	*EAE*	*UAE/EAE*	*EAE*	*UAE/EAE*	*EAE*	*UAE/EAE*	*EAE*	*UAE/EAE*
Yield of water-soluble fractions:												
SF-Viscozyme												
g/100 g	-$^{n.a}$	-$^{n.a}$	15.33 ±0.33^{e}	6.48±0.60^{ab}	10.23 ±0.34^{cd}	11.02 ±1.01^{cd}	12.25 ±0.89^{cde}	13.64 ±1.78^{de}	9.33 ±1.11^{bc}	-$^{n.a}$	5.44±0.24^{a}	-$^{n.a}$
g/100 g DW	19.62 ±0.09^{e}	21.01 ±3.01^{e}	13.99 ±0.30^{d}	6.24±0.55^{b}	8.76 ±0.29^{bc}	9.44 ±0.87^{bcd}	10.56 ±0.77^{bcd}	11.76 ±1.53^{cd}	7.87 ±0.94^{b}	-$^{n.a}$	4.34 ±0.19^{a}	-$^{n.a}$
SF-Blank												
g/100 g	-$^{n.a}$	-$^{n.a}$	12.94 ±0.71^{d}	2.85 ±0.07^{ab}	3.05 ±0.14^{ab}	3.23 ±0.07^{b}	3.37 ±0.03^{b}	5.83 ±1.00^{c}	3.81 ±0.12^{b}	-$^{n.a}$	1.44 ±0.08^{a}	-$^{n.a}$
g/100 g DW	12.83 ±0.78^{c}	13.87 ±2.46^{c}	11.81 ±0.65^{c}	2.60±0.06^{a}	2.61 ±0.12^{a}	2.77 ±0.06^{a}	2.90±0.02^{a}	5.02±0.86^{b}	3.21±0.10^{ab}	-$^{n.a}$	1.15±0.19^{a}	-$^{n.a}$
Yield of water-non soluble fractions:												
NF-Viscozyme												
g/100 g	-$^{n.a}$	-$^{n.a}$	84.67 ±1.82^{a}	93.52±8.66^{a}	89.77 ±2.98^{a}	88.98 ±8.16^{a}	87.75 ±6.22^{a}	86.36 ±11.27^{a}	90.67 ±10.79^{a}	-$^{n.a}$	94.56 ±4.17^{a}	-$^{n.a}$
g/100 g DW	80.38 ±0.34^{a}	78.99 ±11.31^{a}	77.26±1.66^{a}	85.34±7.90^{a}	76.89 ±2.56^{a}	76.21 ±6.89^{a}	75.65 ±5.37^{a}	74.45 ±9.72^{a}	76.51 ±9.10^{a}	-$^{n.a}$	75.43 ±3.33^{a}	-$^{n.a}$
NF-Blank												
g/100 g	-$^{n.a}$	-$^{n.a}$	87.06±4.78^{a}	97.15±2.34^{a}	96.95 ±4.45^{a}	96.77 ±2.10^{a}	96.63 ±0.86^{a}	94.17 ±16.15^{a}	96.19 ±3.03^{a}	-$^{n.a}$	98.56 ±5.48^{a}	-$^{n.a}$
g/100 g DW	87.17 ±5.30^{a}	86.13 ±15.27^{a}	79.44±4.36^{a}	88.65±2.18^{a}	83.04 ±3.81^{a}	82.88 ±1.80^{a}	83.30 ±0.74^{a}	81.18 ±13.93^{a}	81.17 ±2.56^{a}	-$^{n.a}$	78.62 ±4.37^{a}	-$^{n.a}$
Reducing sugar content (RSC):												
SF-Viscozyme												
mg Glu/gSF	240.61 ±13.13^{cd}	116.25 ±5.49^{a}	252.22 ±19.87^{cde}	251.31 ±14.37^{cde}	217.26 ±11.20^{c}	165.26 ±9.88^{b}	332.67 ±20.55 *g*	258.51 ±10.89^{de}	311.70 ±25.51^{fg}	-$^{n.a}$	281.85 ±26.31^{ef}	-$^{n.a}$
mg Glu/ gDW	50.06 ±2.78^{f}	25.35 ±1.42^{bc}	38.32 ±3.09^{de}	18.70 ±1.12^{a}	21.52 ±1.15^{ab}	17.28 ±1.09^{a}	39.41 ±2.49^{e}	33.53 ±1.45^{d}	28.48 ±2.40^{c}	-$^{n.a}$	18.82 ±1.84^{a}	-$^{n.a}$
SF-Blank												
mg Glu/g SF	148.10 ±5.75^{e}	68.18 ±2.75^{a}	180.02 ±7.87^{f}	120.00 ±8.78^{d}	66.99 ±4.23^{a}	88.02 ±1.81^{b}	148.92 ±5.43^{e}	127.39 ±6.15^{d}	105.67 ±7.00^{c}	-$^{n.a}$	61.30 ±3.89^{a}	-$^{n.a}$
mg Glu/ gDW	19.39 ±0.82^{f}	6.86 ±0.42^{e}	22.12±1.01 *g*	3.10±0.36^{c}	0.96 ±0.12^{a}	1.95 ±0.08^{b}	4.72 ±0.24^{d}	6.48 ±0.40^{e}	3.16 ±0.33^{c}	-$^{n.a}$	<0.1^{a}	-$^{n.a}$
***In vitro* antioxidant capacity of water-soluble fractions:**												
TPC:												
SF-Viscozyme												
mg GAE/g SF	12.24 ±0.28^{f}	6.63 ±0.45^{a}	10.61 ±0.19^{de}	9.62 ±0.09^{bc}	9.99 ±0.21^{bcd}	9.28 ±0.50^{b}	11.23 ±0.19^{e}	10.82 ±0.07^{e}	10.11 ±0.20^{cd}	-$^{n.a}$	6.35 ±0.12^{a}	-$^{n.a}$
mgGAE/ gDW	2.40±0.06 *g*	1.39 ±0.05^{e}	1.48±0.03^{f}	0.60±0.01^{b}	0.88 ±0.00^{c}	0.88 ±0.05^{c}	1.19 ±0.02^{d}	1.21 ±0.01^{d}	0.80 ±0.02^{c}	-$^{n.a}$	0.35 ±0.01^{a}	-$^{n.a}$
SF-Blank												
mg GAE/g SF	9.14±0.29 *g*	6.61 ±0.36^{d}	7.07±0.06^{e}	7.32±0.08^{e}	4.33 ±0.10^{a}	4.90 ±0.13^{b}	6.58 ±0.11^{d}	6.11 ±0.14^{c}	7.81 ±0.15^{f}	-$^{n.a}$	4.13 ±0.03^{a}	-$^{n.a}$
mgGAE/ gDW	1.17 ±0.04^{h}	0.92 ±0.05 *g*	0.83±0.01^{f}	0.19±0.00^{c}	0.11 ±0.00^{b}	0.14 ±0.00^{bc}	0.19 ±0.00^{c}	0.31 ±0.01^{e}	0.25 ±0.00^{d}	-$^{n.a}$	0.05 ±0.00^{a}	-$^{n.a}$
$TEAC_{ABTS}$:												
SF-Viscozyme												
mg TE/g SF	43.68 ±1.68^{d}	22.40 ±2.03^{a}	40.17 ±0.51^{d}	35.32 ±0.58^{c}	41.65 ±0.53^{d}	41.65 ±0.49^{d}	42.68 ±1.78^{d}	39.32 ±0.36^{d}	42.38 ±2.13^{d}	-$^{n.a}$	28.90 ±0.26^{b}	-$^{n.a}$
mg TE/g DW	8.57±0.33 *g*	5.09 ±0.85^{ef}	5.62±0.07^{f}	2.20±0.04^{b}	3.65 ±0.05^{c}	3.93 ±0.05^{cd}	4.51 ±0.19^{de}	4.62 ±0.04^{de}	3.34 ±0.17^{c}	-$^{n.a}$	1.25 ±0.01^{a}	-$^{n.a}$
SF-Blank												
mg TE/g SF	33.17 ±0.62^{f}	21.32 ±0.41^{b}	25.09 ±0.53^{de}	26.94 ±0.96^{e}	19.16 ±1.67^{a}	22.73 ±0.55^{bc}	24.36 ±0.61^{cd}	22.55 ±0.76^{bc}	36.41 ±0.22 *g*	-$^{n.a}$	23.13 ±0.32^{bc}	-$^{n.a}$
mg TE/g DW	4.26 ±0.08^{f}	2.96 ±0.06^{e}	2.96±0.06^{e}	0.70±0.02^{c}	0.50 ±0.04^{b}	0.63 ±0.02^{c}	0.71 ±0.02^{c}	1.13 ±0.04^{d}	1.17 ±0.01^{d}	-$^{n.a}$	0.27 ±0.00^{a}	-$^{n.a}$
$TEAC_{ORAC}$:												
SF-Viscozyme												
mg TE/g SF	61.17 ±3.30^{ef}	37.88 ±2.03^{b}	63.47 ±3.22^{f}	56.23 ±5.43^{de}	43.56 ±3.43^{c}	39.20 ±2.02^{b}	62.51 ±3.22^{ef}	45.60 ±1.23^{c}	55.42 ±1.26^{d}	-$^{n.a}$	23.06 ±0.21^{a}	-$^{n.a}$
mg TE/g DW	12.00 ±0.65^{h}	7.96 ±0.43^{f}	8.88±0.45 *g*	3.51±0.34^{b}	3.82 ±0.30^{bc}	3.70 ±0.19^{bc}	6.60 ±0.34^{e}	5.36 ±0.14^{d}	4.36 ±0.10^{c}	-$^{n.a}$	1.00 ±0.01^{a}	-$^{n.a}$
SF-Blank												
mg TE/g SF	62.83 ±2.95^{f}	33.19 ±1.61^{c}	49.42 ±3.36^{de}	52.43 ±1.54^{e}	24.06 ±1.43^{a}	27.22 ±2.04^{ab}	34.81 ±4.11^{c}	36.43 ±1.76^{c}	45.56 ±1.37^{d}	-$^{n.a}$	31.61 ±2.03^{bc}	-$^{n.a}$
mg TE/g DW	8.06±0.38 *g*	4.60 ±0.22^{e}	5.84±0.40^{f}	1.36 ±0.04^{cd}	0.63 ±0.04^{a}	0.75 ±0.06^{ab}	1.15 ±0.12^{bc}	1.83 ±0.09^{d}	1.46 ±0.04^{cd}	-$^{n.a}$	0.36 ±0.02^{a}	-$^{n.a}$
***In vitro* antioxidant capacity of water-insoluble fractions:**												
TPC:												

(*continued on next page*)

Table 4 (*continued*)

Sample	Prior SFE-CO$_2$		After SFE-CO$_2$		After SFE-CO$_2$-PLE-EtOH:		After SFE-CO$_2$-PLE-Ac:		After SFE-CO$_2$-PLE-Ac-EtOH:		After SFE-CO$_2$-PLE-Ac-EtOH—H$_2$O	
	EAE	*UAE/EAE*	*EAE*	*UAE/EAE*	*EAE*	*UAE/EAE*	*EAE*	*UAE/EAE*	*EAE*	*UAE/EAE*	*EAE*	*UAE/EAE*
NF-Viscozyme												
mg GAE/gNF	9.35 ± 0.39^{e}	7.73 ± 0.35^{d}	4.00 ± 0.13^{c}	3.82 ± 0.22^{c}	1.32 ± 0.12^{a}	1.11 ± 0.09^{a}	1.96 ± 0.07^{b}	1.68 ± 0.07^{ab}	1.39 ± 0.14^{a}	$-^{n.a}$	1.68 ± 0.15^{ab}	$-^{n.a}$
mgGAE/gDW	7.51 ± 0.31^{e}	6.10 ± 0.28^{d}	3.09 ± 0.10^{c}	3.26 ± 0.19^{c}	1.01 ± 0.09^{a}	0.85 ± 0.07^{a}	1.48 ± 0.06^{b}	1.25 ± 0.05^{ab}	1.06 ± 0.10^{a}	$-^{n.a}$	1.27 ± 0.12^{ab}	$-^{n.a}$
NF-Blank												
mg GAE/gNF	3.26 ± 0.14^{d}	5.05 ± 0.44^{e}	2.69 ± 0.18^{c}	2.54 ± 0.03^{c}	1.04 ± 0.12^{a}	1.34 ± 0.09^{ab}	1.22 ± 0.13^{ab}	1.54 ± 0.09^{b}	1.22 ± 0.07^{ab}	$-^{n.a}$	1.31 ± 0.13^{ab}	$-^{n.a}$
mgGAE/gDW	2.84 ± 0.12^{d}	4.35 ± 0.38^{e}	2.14 ± 0.14^{c}	2.25 ± 0.03^{c}	0.86 ± 0.10^{a}	1.11 ± 0.07^{ab}	1.02 ± 0.11^{ab}	1.25 ± 0.07^{b}	0.99 ± 0.06^{ab}	$-^{n.a}$	1.03 ± 0.10^{ab}	$-^{n.a}$
TEAC$_{ABTS}$:												
NF-Viscozyme												
mg GAE/gNF	50.62 ± 0.25^{d}	49.41 ± 1.39^{d}	20.90 ± 0.28^{b}	19.01 ± 0.90^{b}	27.56 ± 1.59^{c}	18.97 ± 1.19^{b}	27.34 ± 1.26^{c}	15.84 ± 0.61^{a}	19.21 ± 0.99^{b}	$-^{n.a}$	29.70 ± 1.07^{c}	$-^{n.a}$
mgGAE/gDW	40.69 ± 0.20^{d}	39.03 ± 1.09^{d}	16.15 ± 0.22^{b}	16.23 ± 0.77^{b}	21.19 ± 1.22^{c}	14.45 ± 0.91^{b}	20.88 ± 0.95^{c}	11.79 ± 0.45^{a}	14.70 ± 0.76^{b}	$-^{n.a}$	22.40 ± 0.81^{c}	$-^{n.a}$
NF-Blank												
mg GAE/gNF	27.13 ± 2.41^{f}	44.33 ± 1.07 *g*	17.15 ± 1.11^{bc}	14.75 ± 0.44^{a}	17.47 ± 1.14^{bc}	20.46 ± 0.44^{de}	17.79 ± 0.86^{cd}	20.77 ± 0.73^{e}	10.83 ± 0.90^{a}	$-^{n.a}$	26.55 ± 1.27^{f}	$-^{n.a}$
mgGAE/gDW	23.65 ± 2.10^{f}	38.19 ± 0.92 *g*	13.62 ± 0.88^{bc}	13.08 ± 0.39^{b}	14.51 ± 0.95^{bc}	16.96 ± 0.36^{e}	14.82 ± 0.72^{cd}	16.86 ± 0.60^{e}	8.79 ± 0.73^{a}	$-^{n.a}$	20.87 ± 1.00^{f}	$-^{n.a}$

*Expressed as g/100 g of hemp seed hulls residue prior each step of extraction or g/100 g DW of starting material prior SFE-CO$_2$; the yields of enzyme-treated and *Blank A* samples were calculated excluding the weight of the freeze-dried supernatants from the *Blank B* (*enzyme*+*buffer*) and *Blank C* (*buffer*). **expressed as mg/g SF, NF or DW of water-soluble fraction, non-soluble fraction and starting material prior SFE-CO$_2$, respectively; Ac: acetone; D-UAE: direct ultrasound-assisted extraction; EtOH: ethanol; He: hexane; ND-UAE: indirect ultrasound-assisted extraction; PLE: pressurised liquid extraction; SFE-CO$_2$: supercritical carbon dioxide extraction; SLE: conventional solid-liquid extraction. Different superscript letters within the same line indicate significant differences (one way ANOVA and Tukey's test. $p < 0.05$).

Table 5
TPC (mg GAE/g and DW), ABTS$^{\bullet+}$ and oxygen radical scavenging capacity (TEAC, mg TE/g and DW) of various non-polar and polar extracts, obtained from hemp seed hulls applying conventional (Soxhlet and SLE), high pressure (SFE-CO$_2$ and PLE), and ultrasound-assisted (UAE) extraction techniques.

Sample	**TPC**		**TEAC$_{ABTS}$**		**TEAC$_{ORAC}$**	
	mg GAE/g extract	*mg GAE/g DW**	*mg TE/g extract*	*mg TE/g DW**	*mg TE/g extract*	*mg TE/g DW**
Non-polar fractions from hemp seed hulls (crude):						
Hexane extraction:						
Soxhlet-He	43.53 ± 0.20^{a}	6.12 ± 0.03^{l}	12.86 ± 1.45^{a}	1.81 ± 0.20^{ab}	90.06 ± 4.98^{a}	12.67 ± 0.70^{cd}
SLE-He	43.10 ± 0.66^{a}	4.05 ± 0.06^{e}	8.42 ± 0.61^{a}	0.79 ± 0.06^{a}	118.9 ± 12.0^{a}	11.18 ± 1.13^{bcd}
ND-UAE-He	44.17 ± 0.83^{a}	4.82 ± 0.09^{ghi}	8.73 ± 0.82^{a}	0.89 ± 0.09^{a}	67.42 ± 5.95^{a}	7.36 ± 0.65^{a}
D-UAE-He	46.65 ± 0.94^{ab}	5.02 ± 0.10^{ij}	9.52 ± 1.00^{a}	1.02 ± 0.11^{a}	94.15 ± 7.79^{a}	10.13 ± 0.84^{bc}
SFE-CO$_2$ extraction:						
SFE-CO$_2$	44.68 ± 2.55^{ab}	3.91 ± 0.22^{de}	19.16 ± 1.41^{ab}	1.68 ± 0.12^{ab}	104.3 ± 11.4^{a}	9.13 ± 1.00^{ab}
SFE-CO$_2$+EtOH (10 %)	48.02 ± 0.56^{ab}	5.17 ± 0.06^{j}	27.32 ± 2.45^{ab}	2.97 ± 0.26^{cd}	225.1 ± 10.8^{b}	24.27 ± 1.17^{i}
ND-UAE-EtOH/SFE-CO$_2$+EtOH(10 %)	48.15 ± 1.77^{ab}	4.98 ± 0.18^{hij}	28.72 ± 2.62^{ab}	2.97 ± 0.30^{cd}	186.2 ± 11.1^{b}	19.27 ± 1.14^{fg}
Polar fractions from solid hemp seed hull residues after SFE-CO$_2$:						
Et-OH extraction:						
SLE-EtOH	151.1 ± 2.4^{h}	3.69 ± 0.06^{d}	530.5 ± 23.0^{h}	12.94 ± 0.56^{i}	457.0 ± 36.4^{e}	11.15 ± 0.89^{bcd}
ND-UAE-EtOH	123.0 ± 2.5^{fg}	1.94 ± 0.04^{a}	400.1 ± 8.3^{f}	6.32 ± 0.13^{f}	446.2 ± 26.4^{e}	7.05 ± 0.42^{a}
D-UAE-EtOH	109.0 ± 1.7^{e}	3.01 ± 0.05^{c}	317.6 ± 23.8^{e}	8.77 ± 0.66 *g*	437.1 ± 12.6^{e}	12.07 ± 0.35^{cde}
PLE-EtOH	55.88 ± 1.17^{c}	3.13 ± 0.07^{c}	79.59 ± 2.34^{c}	4.46 ± 0.13^{e}	373.9 ± 15.7^{d}	20.94 ± 0.88^{gh}
ND-UAE-EtOH/PLE-EtOH	46.74 ± 1.53^{ab}	3.21 ± 0.11^{c}	48.90 ± 2.52^{bc}	3.35 ± 0.17^{cd}	328.9 ± 21.2^{c}	22.56 ± 1.45^{hi}
Acetone extraction:						
PLE-Ac	50.37 ± 0.70^{bc}	2.54 ± 0.04^{b}	47.76 ± 2.52^{bc}	2.41 ± 0.13^{bc}	353.4 ± 15.0^{c}	17.81 ± 0.75^{f}
ND-UAE-Ac/PLE-Ac	81.35 ± 3.03^{d}	4.57 ± 0.17^{fg}	197.53 ± 4.59^{d}	11.10 ± 0.31^{h}	477.7 ± 11.7^{e}	26.85 ± 0.66^{j}
D-UAE-Ac/PLE-Ac	128.1 ± 1.1 *g*	4.71 ± 0.04^{fgh}	307.8 ± 33.1^{e}	11.30 ± 1.22^{h}	387.0 ± 2.7^{d}	14.24 ± 0.10^{e}
Consecutive PLE extraction:						
PLE-Ac	50.37 ± 0.70^{bc}	2.54 ± 0.04^{b}	47.76 ± 2.52^{bc}	2.41 ± 0.13^{bc}	353.38 ± 14.97^{c}	17.81 ± 0.75^{f}
PLE-Ac-EtOH	246.3 ± 4.6^{e}	4.51 ± 0.09^{f}	613.3 ± 15.8^{i}	11.22 ± 0.29^{h}	1236.23 ± 20.29^{g}	22.62 ± 0.37^{hi}
PLE-Ac-EtOH—H$_2$O	120.7 ± 5.4^{f}	5.54 ± 0.25^{k}	448.7 ± 17.3 *g*	20.60 ± 0.79^{j}	606.94 ± 17.34^{h}	27.86 ± 0.80^{j}

[*] expressed as mg/g DW of starting material prior SFE-CO$_2$; Ac: acetone; D-UAE: direct ultrasound-assisted extraction; EtOH: ethanol; He: hexane; ND-UAE: indirect ultrasound-assisted extraction; PLE: pressurised liquid extraction; SFE-CO$_2$: supercritical carbon dioxide extraction; SLE: conventional solid-liquid extraction. Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$).

reported radical scavenging capacity (DPPH-IC$_{50}$ and ABTS-IC$_{50}$) of acetone, ethanol, and methanol extracts, isolated from *C. sativa 'Bama'* cultivar.

TPC, TEAC$_{ABTS}$ and TEAC$_{ORAC}$ values of supernatants from enzyme-treated hemp seed hulls were in the range of 6.35 ± 0.12 – 12.24 ± 0.28 mg GAE/g SF (0.35 ± 0.01 – 2.40 ± 0.06 mg GAE/g DW), 22.40 ± 2.03 – 43.68 ± 1.68 mg TE/g SF (1.25±0.01 – 8.57±0.33 mg TE/g DW) and 23.06 ± 0.21 – 63.47 ± 3.22 mg TE/g SF (1.00 ± 0.01 – 12.00 ± 0.65 mg TE/g DW), respectively. The values (in mg/g DW) of corresponding control samples, SF-Blank, were up to 6-fold lower. Kapasakalidis et al.

Table 6

TPC (mg GAE/g and DW) and ABTS$^{\bullet+}$ scavenging capacity (TEAC, mg TE/g and DW) of crude hemp seed hulls and solid residues after the SFE-CO_2 (55 MPa, 60 °C, 360 min), PLE (10.3 MPa, 70–140 °C, 15 min) and the indirect UAE (40 kHz, 40–70 °C, 25 min) prior to the selected steps of PLE.

Sample	TPC		$TEAC_{ABTS}$	
	mg GAE/g	*mg GAE/g DW**	*mg TE/g*	*mg TE/g DW**
Hemp seed hulls (crude)	-$^{n.a}$	4.80 ±0.21^{d}	-$^{n.a}$	28.43 ±1.15^{f}
Solid hemp seed hulls residues after:				
SFE-CO_2	3.93 ±0.40^{c}	3.58±0.36^{c}	24.02 ±1.04^{e}	21.92 ±0.95^{e}
SFE-CO_2 and PLE-EtOH	1.49 ±0.11^{a}	1.28±0.10^{a}	8.90 ±0.56^{a}	7.62 ±0.48^{ab}
SFE-CO_2 and ND-UAE-EtOH/PLE-EtOH	4.04 ±0.23^{c}	3.41±0.20^{c}	15.16 ±0.85^{cd}	12.80 ±0.71^{c}
SFE-CO_2 and PLE-Ac	3.74 ±0.11^{c}	3.22±0.10^{c}	16.96 ±0.71^{d}	14.62 ±0.61^{d}
SFE-CO_2 and ND-UAE-Ac/PLE-Ac	1.00 ±0.06^{a}	0.85±0.05^{a}	7.80 ±0.68^{a}	6.65 ±0.58^{a}
SFE-CO_2 and PLE-Ac-EtOH	2.33 ±0.14^{b}	1.97 ±0.11^{b}	13.38 ±0.42^{c}	11.29 ±0.35^{c}
SFE-CO_2 and PLE-Ac-EtOH-H_2O	2.82 ±0.28^{b}	2.25 ±0.22^{b}	10.99 ±1.02^{b}	8.76 ±0.81^{b}

* expressed as mg/g DW of starting material prior SFE-CO_2; Ac: acetone; D-UAE: direct ultrasound-assisted extraction; EtOH: ethanol; He: hexane; ND-UAE: indirect ultrasound-assisted extraction; PLE: pressurised liquid extraction; SFE-CO_2: supercritical carbon dioxide extraction; SLE: conventional solid-liquid extraction. Different superscript letters within the same column indicate significant differences (one way ANOVA and Tukey's test, $p < 0.05$).

(2009) reported positive effects of 3 commercial pectinases and 1 protease on cell-wall degradation and on increased release of soluble phenolic antioxidants. The treatment of black currant pomace with cellulolytic enzymes increased TPC and antioxidant activity of supernatants and methanol extracts in several applied assays; however, at the enzyme concentration of 0.16 w/w and 50 °C, antioxidant activity decreased, possibly due to the thermal degradation of liberated phenolic compounds and/or modified activity at longer EAE process (Kapasakalidis et al., 2009).

Assuming that the solid residues at the various phases of biorefining may contain residual bound phytochemicals, antioxidant characteristics of water-insoluble fractions were assessed by using the QUENCHER method (Gökmen et al., 2009). The activities of raw (untreated) hulls were 4.8 ± 0.21 mg GAE/g DW and 28.4 ± 1.15 mg TE/g DW (Table 6) and after each step of fractionation decreased in the following manner in all assays: after SFE-CO_2 (~25 % decrease in TPC and $TEAC_{ABTS}$ values) > after PLE-Ac (~33 and 49 % decrease in TPC and $TEAC_{ABTS}$ values, respectively) > PLE-Ac-EtOH (~60 % decrease in TPC and $TEAC_{ABTS}$ values) > PLE-Ac-EtOH—H_2O (~53 and 69 % decrease in TPC and $TEAC_{ABTS}$ values, respectively). Looking at the results of corresponding extracts in Table 5, the sum of antioxidant activity values after SFE-CO_2 and consecutive PLE was 16.5 mg GAE/g DW, 35.9 mg TE/g DW, and 77.4 mg TE/g DW in TPC, ABTS and ORAC assays, respectively; thus, higher than the values determined for the solid material by the QUENCHER method.

TPC of solid residues from EAE samples varied from 0.85 ± 0.07 to 7.51 ± 0.31 mg GAE/g DW, while $TEAC_{ABTS}$ varied from 13.08 ± 0.39 to 40.69 ± 0.20 mg TE/g DW when recalculated for the material DW (Table 4). Generally, the levels of the determined TPC and TEAC of enzyme-treated residues constituted 18 − 156 % and 48 − 141 % of the corresponding antioxidant values, determined for the hemp hulls before SFE-CO_2 (Table 6). Higher activity of hemp seed hull residues after EAE, as compared to the starting material, may be partially explained by the enzyme-enhanced release of cell-wall-bound water non-soluble radical scavenging compounds from plant matrix and/or the increase in the availability of the antioxidant sites in the bound molecules during the longer EAE at lower pH. Albeit, *NF/SF* ratios after EAE were still >1 in all cases, showing that a considerable part of antioxidants remained in the solid residues; however, these residues also could be further treated as a source of antioxidant dietary fibre for food applications. To the best of our knowledge, this type of biorefining approach has not been reported for the hemp seed hulls previously. However, various extraction methods have been applied to the hulls from various other plant sources. UAE, EAE and other novel techniques were applied for buckwheat hull, and the highest DPPH$^{\bullet}$-scavenging values were obtained using high pressure, microwave, and UAE, while in the case of FRAP no clear pattern was determined (Noore et al., 2022). Kan et al. (2023) reported that the TPC, DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging capacity values of free phenolic compounds of buckwheat hulls were significantly higher than that of bound phenolic compounds. PLE using a mixture of ethanol and citric acid solutions was applied to black bean hulls; IC_{50} values in the DPPH and ABTS assays were 149 μg/mL and 135 μg/mL, respectively. Salgado-Ramos et al. (2023) applied sequential extraction with pulsed electric fields and supercritical CO_2 to almond hull biomass and determined that, compared to traditional soaking, the efficiency for total antioxidant capacity and recovery of polyphenols was higher up to 77 % and 20 %, respectively. However, direct comparisons with our data with the previously reported are somewhat complicated because other studies used different values for expressing antioxidant properties, *e.g.*, in percentages of the scavenged radicals, effective concentrations (IC50), etc.

4. Conclusions and perspectives

The results of this study show that 8.9 − 14.1 g/100 g DW of non-polar constituents could be extracted from hemp seed hulls by applying conventional, UAE and SFE-CO_2 extractions. Consecutive PLE with acetone, ethanol, and water is most efficient for polar fraction isolation, amounting to 11.5 g/100 g DW of starting plant material. The results of this study show that hemp seed hull polar and non-polar extracts exert particular *in vitro* antioxidant properties. The total amount of antioxidants after SFE-CO_2 and consecutive PLE was equal, respectively, to 16.5 mg GAE/g DW, 35.9 mg TE/g DW, and 77.4 mg TE/g DW for TPC, ABTS, and ORAC assays, which was higher than the initial activity of plant material. In addition, indirect UAE with acetone before PLE could further increase the total antioxidant activity of the extractable portion by 2.0 mg of GAE and by 8.9 mg of TE. Optimization of enzyme-assisted extraction was performed to recover the highest amount of reducing sugars from hemp seed hulls. The process at the optimal EAE parameters (E/S 6.71 % v/w, 50 °C, pH 6.5, 6.1 h) yielded 4.3–21.0 g/100 g DW of water-soluble constituents, containing 17.3–50.1 mg GLU/g DW of reducing sugars. Compared with the control samples, enzyme treatment significantly increased the recovery of total soluble constituents and the yield of reducing sugars, resulting in up to a 2-fold increase in the *in vitro* antioxidant properties of supernatants and solid residues after EAE. The antioxidant potential of solid residues from ultrasound and enzyme-treated hemp seed hulls indicates that all fractions could be utilized as a source of nutritionally valuable ingredients for food applications. Due to a negative attitude toward commercially used synthetic additives, the fractions isolated from hemp seed hulls could serve as a natural source of bioactive phytochemicals and antioxidants for the plausible isolation of functional food, nutraceutical, and pharmaceutical ingredients. For future studies, the identification of the most sustainable and investment-worthy processes for the valorisation of hem hulls should be performed.

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CRediT authorship contribution statement

Vaida Kitrytė-Syrpa: Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Data curation. **Darius Povilaitis:** Visualization, Methodology, Investigation, Formal analysis. **Michail Syrpas:** Writing – original draft, Methodology, Investigation, Formal analysis. **Renaldas Raišutis:** Validation, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Petras Rimantas Venskutonis:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

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

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Data availability

The authors declare that the data supporting the findings of this study are available within the paper.

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