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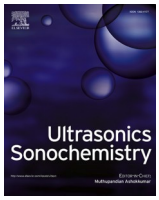

Association of enzymatic and optimized ultrasound-assisted aqueous extraction of flavonoid glycosides from dried *Hippophae rhamnoides* L. (Sea Buckthorn) berries

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ABSTRACT

The main purpose of the present study was to determine the effect of associating an optimized ultrasound-assisted extraction (UAE) protocol with enzyme-assisted extraction (EAE) in aqueous media, using the dried berries of *Hippophae rhamnoides* L. (sea buckthorn) as plant material. A specialized software was used for the determination of potential optimal extraction parameters, leading to the development of four optimized extracts with different characteristics (UAE ± EAE). For these extracts, buffered or non-buffered solutions have been used, with the aim to determine the influence of adjustable pH on extractability. As enzymatic solution, a pectinase, cellulase, and hemicellulase mix (2:1:1) has been applied, acting as pre-treatment for the optimized protocol. The highest extractive yields have been identified for non-buffered extracts, and the E-UAE combination obtained extracts with the highest overall *in vitro* antioxidant activity. The HPLC-MS^n analysis demonstrated a rich composition in different types of isorhamnetin-*O*-glycosides, as well as some quercetin-*O*-glycosides, showing a high recovery of specific flavonol-type polyphenolic species. Moreover, we have tentatively identified two flavanols (i.e., catechin and epigallocatechin) and one flavone derivative (i.e., luteolin).

1. Introduction

Hippophae rhamnoides L, synonym with *Elaeagnus rhamnoides* (L.) A. Nelson (commonly "sea buckthorn"), a representative species in the Elaeagnaceae family, is well-known for its nutritional value and medicinal potential. Although it originates in the cold-temperate regions of Asia and Europe, ethnopharmacological evidence suggests wide medicinal applications around the world [1–3]. This claim is promoted by pharmacological studies, which showed that sea buckthorn displays antibacterial, antifungal, anti-sebum and wound healing effects, being a valuable cosmeceutical ingredient [2] with great potential in dermatology [4,5]. Moreover, sea buckthorn can act as medicinal plant with immunomodulation capacity, displaying *in vivo* anti-inflammatory activity [6], with additional anti-atherosclerosis and cardioprotective effects [7].

The fruits (or the berries), as well as the leaves of this species, are rich in compounds with phenolic backbone (such as isorhamnetin, quercetin and hydroxycinnamic acid derivatives), alkaloids, phytosterols, and carotenoids (such as lutein, zeaxanthin and β-carotene) [8], yet the quantitative profile is different among sea buckthorn varieties [9] and

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for specimens collected from various locations [10]. Moreover, the lipidic composition is subjected to variations according to the cultivar and the region of origin, and its oleosome commonly includes fatty acids (such as palmitoleic acid), carotenoids, sterols and tocopherols, in different proportions [11]. The oil fraction contained by the berries seems to be richer in bioactive compounds in the peel in comparison to pulp and seeds, with slight compositional variations [12]. Furthermore, toxicity studies confirmed that the berry oil is safe to use in food or supplements [13]. The established "exceptional" antioxidant potential of the sea buckthorn berries arises from the presence of the two different types of antioxidant species, specifically hydrophilic (represented by phenolic species) and lipophilic compounds (through carotenoids and tocopherols) [14]. The nutritional quality of sea buckthorn berries is augmented in value by the composition in vitamins (especially ascorbic acid, A, and B vitamins), sugars, fibers, minerals, as well as organic and amino acids [15,16]. Lastly, the high composition in bioactive species allows the development of products enriched with sea buckthorn extracts or by-products, with potential nutritional and therapeutical applications [17–19].

Ultrasound assisted extraction (UAE) acts as a modern technique that can be used for the enrichment of extracts, with the possibility to obtain higher yields in comparison to conventional techniques [20]. For sea buckthorn, UAE has been successfully applied for the oil fraction, especially for the seed or berries oil [20–22]. Enzymatic-assisted extraction (EAE) is another example of "green" extraction technique, and it has been applied to a variety of plant matrices, with applicability in the development of new food products and nutraceuticals [23]. In this context, sea buckthorn pomace and seeds have recently been fractionated using an association of high pressure extraction and EAE [24]. Other methods aiming to improve the enzymatic hydrolysis have been applied for this species, for example a mix of steam explosion and anaerobic alkalization [25].

Considering the great potential of the sea buckthorn berries, this research aimed to refine an optimized extraction method for phenolic derivatives, employing a mix between EAE and UAE (E-UAE) and using only water as solvent, as a modern and non-polluting technique. One of the objectives was to ascertain the impact of extraction parameters extract quality using controls, which aimed to examine the influence of phosphate buffer on the assessed parameters. Furthermore, the extracts were additionally assessed with regards to their content in phenolic species and *in vitro* bioactive potential. This evaluation was beneficial for the study of the correlation between the protocol of extraction, phenolic profile, and bioactive potential of these preparations. To sum up, the objective was to ascertain the viability of water extraction and the utilization of a phosphate buffer, with the prospect of facilitating large-scale production and exploitation of sonochemical principles of extraction.

2. Experimental setup

2.1. Plant material

Plant material (*H. rhamnoides* fresh fruits) was collected in November 2021 near Vânători-Neamț (Neamț County, Romania, geographical coordinates: 47.21°N, 26.30°E), from a natural culture, and kept in the freezer until further processing. The collected fruits were subjected to an optimized drying process based on a protocol that was previously described by Moldovan *et al.*, in which hot air at 70 °C was used for a total of 24.35 h [26]. After reaching a stable dry weight, the plant material was stored in the freezer until extraction, which was accomplished the following day.

2.2. Extraction

To initiate the extraction, the dried berries were powdered with a laboratory mill (Grindomix® GM 200, Retsch Gmbh., Germany), for 4 min at 10,000 rpm, ensuring a homogenized and pulverized material. Recent studies highlighted the importance of environmentally friendly extractive techniques for *H. rhamnoides*, such as subcritical water, hydroethanolic mix, and pressurized water [27–29]. Therefore, in the present study, distilled water was selected for extraction. A SFX 150 Sonifier (Branson Ultrasonics Corporation) with tapered microtip (3.2 mm diameter) was used for the UAE procedure, in which three factors have been altered: the amplitude of the ultrasounds (10, 30, and 50 %), the extraction time (10, 30, and 50 min), and respectively the ratio between plant material and water (1:10, 1:15, and 1:20). According to every ratio, 30 g of exactly measured mixture was subjected to extraction. The uniformity of extraction was assured using a magnetic stirrer (VELP Scientific, New York, USA), and the extraction flask was submerged inside an ice bath to cancel the thermal effect induced by the presence of ultrasounds.

After UAE, the extraction mixture was centrifuged, and then the supernatant was collected (without the lipophilic phase) and consequently filtered (using cotton and paper filters until proper clarification). Furthermore, to eliminate the solid unextracted particles and to obtain a clear water extract, the obtained liquids were subjected to another filtration through 0.2 μm MV-20/25 cellulose membrane filters (Chromafil Xtra, Macherey-Nagel). The determined optimal extraction parameters have been used to obtain an optimized extract (**HRO**). To determine the influence of phosphate buffer solution (PBS), a control of HRO using PBS instead of water has also been obtained (**HROB**). Finally, the aqueous extracts were subjected to freeze-drying and then stored in a desiccator at room temperature.

Correspondingly, an analogous UAE procedure has been applied after EAE as pre-treatment, obtaining a mixed extract (**HRE**). In a similar manner to HRO, to determine the influence of the PBS, a control of HRE using PBS instead of water has been obtained (**HREB**). EAE has been accomplished with an enzymatic mix, which consisted of pectinase (0.6 U/mL), cellulase (0.3 U/mL), and hemicellulase (0.3 U/mL), based on the protocol developed by Nicolescu et al. [30]. PBS was involved in the extraction procedure to assure a constant pH of 5.6 for maximal enzymatic activity. Essentially, the same quantity of powder as for HRO has been mixed with the enzymatic blend in PBS, in a 50 mL Falcon, at 50 °C and with continuous shaking at 500 rpm using a MultiTherm Shaker Incubator (H5000-H-E, Benchmark Scientific Inc., Edison, NJ, USA), for a total of 60 min. After incubation with enzyme mixture, UAE was applied, and the same filtration and freeze-drying procedures were used, obtaining the powdered extracts.

The determination of the assayed activities for the four types of extracts has been realized by eliminating the mass of impurities introduced in the extraction procedure (in the form of enzyme and potassium phosphate salts), thus allowing an accurate comparison between the extracts. This has been accomplished by using the following formula, which was previously used in recent studies regarding EAE applications to plant matrices (Eq. (1)) [31]:

$$\%\,\text{yield} = \frac{\text{amount of extract} - \text{amount of impurities}}{\text{weight of dried powder used for extraction}} \times 100 \quad (1)$$

Where "amount of extract" refers to the mass after freeze-drying and "amount of impurities" refers to the sum of enzyme and solid buffer residue (determined to be 0.002 mg enzyme/mL and 0.012 mg sodium phosphates/mL).

2.3. Experimental design

MODDE 13.0 software (Sartorius AG, Sweden) was employed as development platform for the design of the experiment (DoE) [32]. With an aim to maximize the total phenolic content, a D-optimal type of DoE was selected, consisting of 18 experimental runs (of which 3 represent replicates needed for statistical analysis of the model). In comparison to the classical approach of the response surface methodology, the D-

optimal designs have the advantage of establishing the most suitable combination of experimental runs. In mathematical terms, a D-optimal design is developed by the minimization of the determinant of the $(X'X)^{-1}$ matrix [33]. Therefore, given certain factors and responses, the software will generate the largest possible volume for the experimental region [34]. The D-optimality criterion is among the most popular ones applied in experimental design [35]. Data analysis has been accomplished using the software, and it consisted of the evaluation of raw data, a summary of model fit, and regression analysis, followed by the application of the MODDE optimizer function [32,34].

2.4. Total phenolic content (TPC)

Total phenolic content (TPC) was assessed using a modification of the Folin–Ciocalteu (FC) assay, adapted to a microplate reader, based on the method by Nicolescu et al. [30]. Concisely, a volume of 20 µL of sample (of 5 mg/mL concentration) was homogenized with 100 µL of FC reagent solution (10 %, *v/v*). After 3 min of incubation, another 80 µL of 1 % Na_2CO_3 solution was added. TPC was determined photometrically after 30 min at 760 nm and was expressed as milligrams of gallic acid equivalents per gram of dried plant material (mg GAE/g, for the determinations corresponding to the experimental design) or per 100 g of dried extract (mg GAE/100 g dw, for the freeze-dried extracts obtained after optimization).

2.5. Total flavone and flavonol content (TFC)

Total flavone and flavonol content (TFC) was determined using the simple $AlCl_3$ method, as previously described by Nicolescu et al. [36] Briefly, 100 µL of sample (of 5 mg/mL concentration) was homogenized with 100 µL of 2 % $AlCl_3$ solution, and after 10 min the absorbance was measured at 420 nm alongside a sample blank. Results were presented as milligrams of quercetin equivalents per 100 g of dried extract (mg QE/100 g dw) for the freeze-dried extracts obtained after optimization.

2.6. LC-DAD-ESI-MS analysis

The phenolic profiling of the samples described above has been accomplished using a HPLC-DAD-ESI/MS^n tool (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, United States), based on a previously described method [36,37]. The sample preparation was accomplished by re-solubilization in HPLC vials at a 10 mg/mL concentration, using a mix of ethanol and water (20:80, *v/v*), followed by double online detection with DAD detector at 280, 330, 370 and 520 nm. The column used was Waters Spherisorb S3 ODS-2 C_{18} (3 µm, 4.6 x 150 mm, Waters, Milford, MA, USA) adapted to a gradient elution method, consisting in two phases: formic acid (0.1 %) in water and pure acetonitrile. The mass spectrometry detection was carried out using negative mode, with a Linear Ion Trap MS with ESI (LTQ XL, Thermo Finnigan, San Jose, CA, United States). The following parameters have been applied: 50 psi (nitrogen gas), 325 °C (source temperature), −20 V (capillary voltage), 5 kV (spray voltage), and − 66 V (tube lens offset). Mass acquisition was done between *m/z* 100 and 1500, and the software used for interpretation was Xcalibur® (Thermo Finnigan, San Jose, CA, USA) [37].

2.7. In vitro antioxidant potential

In vitro antioxidant potential was determined by means of five different assays, aiming to assess the activity through different mechanisms: radical scavenging potential (using TEAC or ABTS and DPPH methods), the ferric reducing power (using FRAP method), the oxidative hemolysis inhibition (OxHLIA), and lipid peroxidation inhibition (TBARS). The results for the first three assays were expressed as equivalents of Trolox per gram of dried extract (mg TE/g dw), and for the next two as IC_{50} values (µg/mL).

For TEAC, an $ABTS^+$ radical solution was needed. This solution was prepared by mixing the ABTS reagent (7 mM) with potassium persulfate solution (2.45 mM), and subsequently incubated (room temperature, 14 h, dark). The radical solution was further diluted with water, and 200 µL of the solution was mixed with 20 µL of diluted sample, and after 30 min the absorbances were read at 734 nm [30]. For DPPH, 270 µL of 0.004 % DPPH methanolic solution was mixed with 30 µL of diluted sample, and subsequently incubated for 30 min. The absorbance was recorded at 517 nm [36]. The FRAP reagent has been prepared using a mixture of 10 mM TPTZ, 20 mM ferric chloride in 40 mM HCl and acetate buffer (0.3 M, pH 3.6) in a 1:1:10 ratio (*v/v/v*). 175 µL of reagent was added to 25 µL of diluted sample, and the absorbance was read after 30 min at 593 nm [30].

For TBARS, the procedure described by Pinela et al. was used [38]. In short, 0.1 mL of a porcine brain homogenate solution (1:2, *w/v*) prepared in ice-cold Tris-HCl buffer was mixed with 0.2 mL of sample extract (9.8–5000 µg/mL), and then 0.1 mL of $FeSO_4$ (0.01 mM) and 0.1 mL of ascorbic acid (0.1 mM) were added. Following one-hour incubation at 37 °C, 0.5 mL of trichloroacetic acid (28 %, *w/v*) was supplemented to the solution. To assess the degree of oxidation, 0.38 mL of thiobarbituric acid solution (2 % w/v) was introduced, followed by heating the mixture at 80 °C for 20 min. The pink coloration of the thiobarbituric acid–malondialdehyde complex was determined after centrifugation, at 532 nm.

For OxHLIA, the assay was performed based on the protocol of Lockowandt et al. [39]. A solution of erythrocytes (2.8 %, *v/v*; 200 µL) in phosphate-buffered saline (PBS, pH 7.4) was combined with 400 µL of one of the following: extract solution (62.5–2000 µg/mL), Trolox (3.91–4000 µg/mL) serving as a positive control, PBS solution as a negative control, or ultrapure water (baseline). Following a 10 min incubation at 37 °C with continuous stirring, 200 µL of 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH; 160 mM) was introduced, and the optical density was continuously measured at 690 nm in the microplate reader until complete hemolysis occurred.

2.8. In vitro enzyme inhibitory potential

The extracts were assessed for in vitro enzyme-inhibitory activity against α-glucosidase, acetylcholinesterase, and tyrosinase. Acarbose, galantamine, and kojic acid served as positive control. The percentages of inhibition (I, as %) were derived from the formula:

$$I\,(\%) = 100^{*}\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \quad (2)$$

The results were expressed as IC_{50} values (µg/mL), using the normalized logarithmic curve for the determined I (%), which was graphed using GraphPad Prism®. IC_{50} values were expressed considering the microplate dilutions, and not the original resolubilized samples concentration, in a similar manner to previous studies [30].

The α-glucosidase inhibition method consisted in mixing 50 µL of different sample dilutions with the same quantity of both enzyme (0.75 U/mL in PBS, pH 6.8) and substrate (PNPG, 0.5 mM in PBS). After incubation for 5 min at 37 °C, the absorbance was measured at 405 nm. The tyrosinase inhibition experiment involved mixing 40 µL of sample with an equal quantity of tyrosinase (125 U/mL) and 80 µL of PBS (pH of 6.5). After 10 min, 40 µL of the L-DOPA (10 mM in PBS) was added. After incubation for 20 min, the absorbance values were measured at 475 nm [30]. In the acetylcholinesterase (AChE) inhibitory activity assay, 25 µL of samples were combined with 50 µL of Tris-HCl buffer (50 mM, pH 8.0), 125 µL of DTNB solution (0.9 mM) and 25 µL of AChE solution (0.078 U/mL). After incubation for 15 min, 25 µL of ATCI solution (4.5 mM) were added, and followed by an additional incubation for 10 min, with reading at 405 nm [30,40].

2.9. Statistics and correlation analysis

Every determination of biological activity was realized in triplicate, expressing the values as average ± standard deviation. The determination of the statistical difference between determinations has been assessed using one-way ANOVA. The statistical evaluation regarding the determinations in DoE was analyzed in MODDE by analyzing the summary of model fitting, residuals, and replicates [34]. Principal component analysis (PCA) was implemented using the FactoMiner factoextra package [41]. Heatmap and dendrograms were performed with the default clustering method (Euclidean distance) with a complete linkage algorithm to highlight the similarities and discrepancies between the bioactive components and various extraction techniques (i.e., UAE and EAE) of *H. rhamnoides,* by the use of Cluster R [42] and ggplot packages from R (version 4.3.2) [43].

3. Results and discussion

3.1. Experimental design and optimization process

The evaluation of the selected D-optimal design has been accomplished using the *condition number* and the *G-efficiency* (G_{eff}) as statistical parameters. The condition number is relevant as indicator of the symmetry and sphericity of a design. The performance of the design before implementation is indicated by orthogonality, which is shown by a value close to 1. For efficient optimization designs, this value can be up to about 8. G_{eff} is used to indicate the performance of the design in comparison to the classical type of fractional factorial design. The design that was developed for the current study was characterized by a condition number of 4.97 and a G_{eff} value of 65.91 %, which shows high quality and reliability.

The software generated a D-optimal design containing three quantitative factors, as independent variables: amplitude of ultrasounds (10, 30, and 50 %), extraction time (10, 30, and 50 min), and the ratio between plant material and solvent (1:10, 1:15, and 1:20). The initial design included 18 experimental runs, from which 3 were replicates; however, the statistical analysis led to the removal of two statistically insignificant determinations. The results for the determinations, where TPC (as mg GAE/g dw) has been chosen as response factor, are presented in Table 1. Further, data analysis has been accomplished using multiple linear regression (MLR), and the statistical evaluation of the model has been determined using relevant parameters, such as: goodness of fit (R^2 = 0.992; R^2 Adj. = 0.980), goodness of prediction (Q^2 = 0.964), relative standard deviation (RSD = 0.066), validity (0.926), and reproducibility (0.970). These values indicate the fact that the model that was developed is statistically viable and valid, with a ($R^2 - Q^2$) difference of 0.028 [34]. Moreover, the statistical evaluation shows similarities with a previous optimization which our team has successfully developed [30]. The overall statistical evaluation can be noticed in Fig. 1(A–D).

Table 1
Experimental values for TPC (mg GAE/g of dried berries) according to each experimental run, as well as the experimental design.

No.	ID	Independent variables (factors)			TPC (mg GAE/g)
		Amplitude (%) X_1	Time (min) X_2	S-L ratio (1:n) X_3	
1	N1	10 (−1)	10 (−1)	10 (−1)	4.156
2	N2	50 (+1)	10 (−1)	10 (−1)	4.644
3	N3	10 (−1)	50 (+1)	10 (−1)	5.095
4	N4	50 (+1)	50 (+1)	10 (−1)	5.321
5	N5	30 (0)	30 (0)	10 (−1)	5.536
6	N6	10 (−1)	10 (−1)	20 (+1)	4.173
7	N7	50 (+1)	10 (−1)	20 (+1)	4.880
8	N9	10 (−1)	50 (+1)	20 (+1)	4.788
9	N10	50 (+1)	50 (+1)	20 (+1)	5.273
10	N11	50 (+1)	30 (0)	20 (+1)	5.404
11	N12	50 (+1)	10 (−1)	15 (0)	4.073
12	N13	50 (+1)	50 (+1)	15 (0)	4.723
13	N15	10 (−1)	30 (0)	15 (0)	4.312
14	N16	30 (0)	30 (0)	15 (0)	5.088
15	N17	30 (0)	30 (0)	15 (0)	4.938
16	N18	30 (0)	30 (0)	15 (0)	5.064

Note: S-L (solid-to-liquid); 1:10 relates to 1 g of material and 9 g of water. The average power generated according to every amplitude was: 1.5 W (for 10 %), 6.26 W (for 30 %) and 15.8 W (for 50 %). Runs N8 and N14 have been excluded from the model due to statistical insignificance.

3.1.1. Effects of process variables on TPC

A quadratic equation describing the process has been generated according to the regression coefficients determined, expressed as scaled and centered values (**Eq. (2)**):

$$Y = 5.00 + 0.23X_1 + 0.33X_2 - 0.05X_1X_2 + 0.06X_1X_3 - 0.07X_2X_3 - 0.47X_1^2 - 0.34X_2^2 + 0.58X_3^2 \quad (2)$$

Where Y is the response (TPC); 5.00 is the constant of the model; 0.23 and 0.33 represent linear coefficients; −0.05, 0.06, and −0.07 are interaction coefficients, and −0.47, −0.34, and 0.58 are quadratic coefficients. The multilevel factors are represented by X1, X2, and X3 and correspond to ultrasound amplitude (%), extraction time (min) and sample-liquid ratio (SLR).

The coefficient plot (represented in Fig. 1**C**), translated quantitatively in the quadratic equation of the process, is an indicator of the relative influence of considered parameters on the TPC value, which is the dependent parameter of the process. Consequently, in this model the amplitude and the ratio had significant linear coefficients, and all the quadratic parameters (Amp^2, $Time^2$, $Ratio^2$, $p < 0.05$) were significant. Despite the fact that ratio alone did not show a notable influence, the quadratic interaction of this parameter was the most significant of the model (with a relative value of 0.58). Ultimately, the interaction between the two parameters (Amp × SLR and Time × SLR) were statistically significant ($p < 0.05$); nevertheless, SLR and Amp × Time interaction coefficients were less significant ($p = 0.81$ and 0.054, respectively).

The applicability of these observations has been correlated with the representation of the three-dimensional response surface plots, as it can be observed in Fig. 2. Firstly, the graphs suggest that the highest TPC value should be theoretically obtained for a 1:20 ratio, however with limited differences in comparison to the other ratios. For this reason, the comparative representation of the plots has been realized in the three cases of ratios, being able to map the other more relevant interactions.

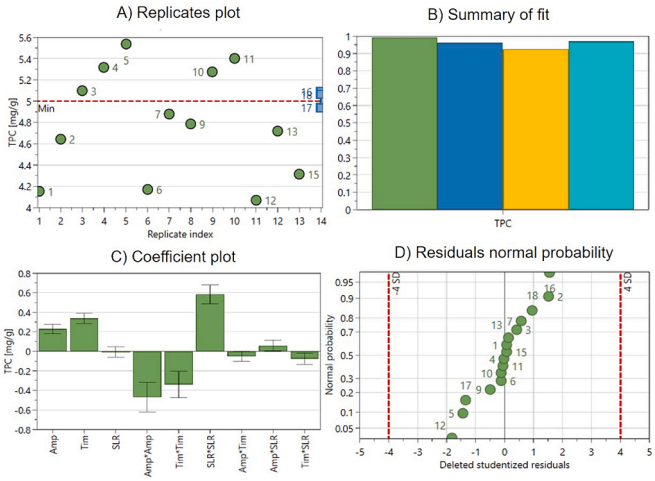

Fig. 1. Graphical overview of statistical analysis for the experimental model developed for HR optimization process: A) replicate analysis, B) summary of fit, C) coefficient plot (scaled and centered), and D) residuals.

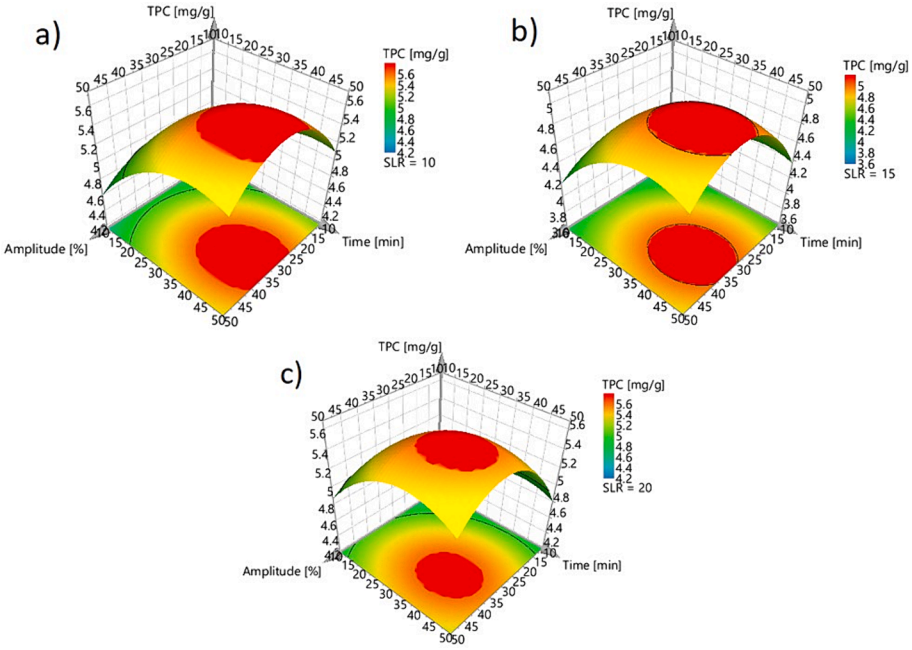

Fig. 2. Response surface plots (RSP) representing the quadratic relationship between TPC (mg/g), amplitude (%), and time (min), in three cases of SLR (ratio): a) 1:10; b) 1:15; and c) 1:20.

Secondly, a maximal TPC value is obtained for an intermediate ultrasound amplitude (the range 30–40 % correlates to a power range of 6.26–10.15 W). In a previous study accomplished by our team, a similar tendency has been obtained for the water UAE applied to *Rosa canina* pseudo-fruits, with an optimal amplitude range between 40 and 50 % [30]. This phenomenon is acknowledged in the case of UAE applied to plant matrices, and essentially an intermediate ultrasound power induces maximal yields. Higher amplitudes induce a decline in the extractive power as a result of the collapse of violent cavitation bubbles; ultimately, the cavitation effect decreases at high concentrations of bubble volume [44,45]. As it can be observed, the factor interactions were less significant for the current experimental model, and the most important one was the interaction between time and SLR; this indicates a synergistic effect between the two factors. Lastly, all three quadratic interactions proved to be significant, with the SLR being the most relevant. This suggests that all the independent variables that have been chosen can indeed induce a non-linear influence on the model.

3.1.2. Process optimization

The optimization of UAE has been accomplished using an optimizer function from MODDE software [32], aiming to determine a suitable combination of factors that can act as maximal yield of TPC (acting as dependent variable). Considering the maximal and minimal determined during the experimental runs (ranging from 4.073 to 5.536 mg GAE/g), the condition was chosen to maximize the yield, considering TPC as required response. The value for the predicted maximal value of TPC was 5.734 mg/g. Finally, the optimal extraction parameters for this model were 34 % amplitude (average ultrasound power of 7.85 W), 39.33 min of extraction, and 1:10 ratio, having a probability of failure of 0.052 %. These optimal parameters have been used for the development of the four types of extracts using UAE or E-UAE, as described earlier. Consequently, the theoretically predicted value corresponds to HRO extract, and the obtained TPC value was 5.74 ± 0.20 mg/g, showing a recovery of 100.10 %. These results comply with the range of ± 5 % in comparison with the predicted value (5.04–6.02 mg/g). Furthermore, for the preliminary assessment of the difference between the liquid extracts developed, we have obtained values of 5.62 ± 0.13 mg/g (HRE), 6.07 ± 0.11 mg/g (HROB), and 5.49 ± 0.30 mg/g (HREB). As it can be observed, the fresh liquid samples did not show statistically significant differences among the TPC values, with the exception of HROB.

Considering the optimized extraction of phytocompounds, using the principles of response surface methodology (RSM) presents the possibility of assessing not only the effect of variables, but also their interaction pattern. This concept has been recently applied for the development of unexpensive but enriched extracts, using water and UAE, with promising applicability in the field of phytotherapy [46–48]. Consequently, the optimization accomplished in the present study managed to determine relevant factors influencing modern extraction of sea buckthorn berries using a non-toxic and low-temperature environment, highlighting a safe and low-cost process that can be applied industrially.

3.2. Extractability and yield

Using water as the only solvent appears to be an effective technique for the selective recovery of secondary metabolites with polar characteristics, the main class being represented by poly-hydroxylated flavonoid derivatives. The highest extraction yield for flavonoids seems to be obtained using a mixture of ethanol and water. However, due to high polarity, water extraction has a negative influence on the carotenoid species in plant matrices [49]. For example, Um et al. determined that for the fruits of *Rosa rugosa*, the highest antioxidant activity was obtained for a mix of 50 % ethanol and water during UAE, which was also the most effective concentration for the yield of TPC and TFC [50].

In addition to the extraction optimization, the amount of residual extract after freeze-drying has been used for the determination of the extraction yields (average of 6.71 ± 0.72 %), using the formula described in **Eq. (1)**. By studying the extraction using UAE (6.39 and 5.91 %) in comparison to EAE and UAE (7.60 and 6.95 %), the application of buffer demonstrated a considerable decrease in extractive yields ($p < 0.05$). Moreover, the yield reduction is correlated for both extraction methods, which suggests that phosphate buffer has an analogous reduction effect in both cases. Salts from buffers can act not only through assuring a pH, but also through the ionic strength; this parameter can increase or decrease the extraction yields depending on the nature of the compounds and the pH of the medium [51]. Recent

findings suggest the fact that ionic strength has a significant impact in the case of polyphenol – fiber interactions; specifically, rising ionic strength induces molecular association, creating hydrophobic interactions that aim to reduce the contact with the ions present in the medium [52]. Sea buckthorn berries have been extensively investigated for their complex chemical composition, and their high amounts of polyphenolic species, as well as dietary fiber (especially in seeds), are established [53]. From this point of view, the obtained extractive yields suggest that phosphate buffer might decrease the extractability of polyphenols as a result of inducing an interaction between polyphenols and fibers, which could in turn decrease their solubility in the water phase. These results underscore the significance of studying the EAE parameters, and specifically the use of saline buffers which can therefore affect the extractability.

Using E-UAE showed a higher recovery of extractible chemical compounds in comparison to only using UAE. Pham et al. showed that the combination of EAE and UAE in this order was able to extract efficiently the highest amount of antioxidant and antimicrobial compounds from Pomelo (*Citrus maxima*) peels, using water as solvent [54]. Similarly, Gao et al. have successfully applied a green ultrasound-assisted and enzymatic extraction on *Empetrum nigrum* air parts [55]. This aligns with our findings, which suggest that an enzymatic mix can rise extractability from sea buckthorn berries. The observation is relevant only in the context of specific metabolites of interest, and for some classes (e.g., anthocyanins) the difference between UAE and EAE could be insignificant [56]. The extractive yields are similar to others determined for analogous techniques; for example, Dienaitė et al. found that sea buckthorn pomace defatted by supercritical CO_2 yielded 4.8 and 11.9 % after pressurization with water and ethanol [29].

3.3. Total phenolic content (TPC)

TPC method was chosen as a rapid screening technique for the experimental runs in DoE, as well as for the assessment of final optimized extracts. However, no colorimetric method can be applied to complex matrices with perfect analytical performances, and there are disadvantages that can alter the determinations, such as the presence of ascorbic acid as main interferent [57]. The choice of selective extraction of phenolic species has been confirmed by the phenolic profiling, described in detail in section 3.5, where a high number of isorhamnetin derivatives has been identified, certifying a high recovery of flavonol derivatives.

The obtained values show that E-UAE association led to an increase in total phenolic species (118.26 ± 2.77 and 104.47 ± 1.27 mg GAE/100 g dw) in comparison to only UAE (103.25 ± 2.50 and 90.49 ± 1.00 mg GAE/100 g dw), with the buffer having a positive effect on the extraction in both cases ($p < 0.05$). For *H. rhamnoides* pomace, it was previously shown that 100 % methanolic and 70 % aqua-methanolic extraction show better phenolic recovery than water [58,59]. However, among additional objectives employed in the current study, we also aimed to investigate the relevance of water as non-polluting solvent, with applicability in the development of extracts readily accessible for oral administration. Moreover, water UAE methods might result in less degradation of the phytocompounds [60].

3.4. Total flavone and flavonol content (TFC)

Although the aluminum chloride method is generally depicted as a determination of "total flavonoid content", critical literature suggests that its specificity is limited (mainly) to flavones and flavonols with free hydroxyl moieties, capable of chelating the Al^{3+} ions in the solution [61,62]. Regarding the TFC, the values were in the range of 3.00 to 18.93 mg QE/100 g dw. EAE had a significant positive effect on the value of the TFC, while the usage of the phosphate buffer leads to a significant decrease in TFC (in both HROB and HREB, in comparison to non-buffered extracts). A similar relative range of TFC values (1.98–8.96 mg RE/g) was identified for different varieties of berries from China, for which ultrasonic hydro-ethanolic extraction has been applied [63]. Even though we have identified various flavonol derivatives (mainly of isorhamnetin and quercetin) as the main phenolic constituents of the extracts, the TFC values proved to be greatly reduced in comparison to the TPC values. However, this could be explained on one of the disadvantages of the aluminum chloride method, namely the reduced affinity of the Al^{3+} ions towards species with absent free hydroxyl groups, as well as methoxylated species [61,64], which is the case of the isorhamnetin-*O*-glycosides identified in our samples.

Recent studies reported a rich composition of the sea buckthorn berries, with significant quantities in phenolic species and flavonoids. For example, Guo et al. found that berries from four different subspecies present TFC values in the range of 34.9 ± 1.2 to 51.5 ± 0.9 mg catechin equivalents/g dw. Nonetheless, these values have been determined for extracts obtained with 80 % acetone, different in comparison to our aqueous-only extracts [65]. The determinations suggest that the most abundant type of flavonoids in this species is represented by flavonols, and their glycosides are especially important for the sensory quality [14].

3.5. Phenolic profiling of the extracts

The phenolic profiling using HPLC-MS^n is presented in Table 2. All four extracts presented a rich composition in *O*-glycosylated flavonoids, especially ten identified isorhamnetin (IRh) derivatives, which are classified as 3′-methoxylated-flavonols (see also Fig. 3 for the general chemical structure). Moreover, one luteolin-*O*-glycosyl and two quercetin-*O*-glycosyl derivatives have been identified, belonging to the flavone and flavonol class, respectively. Only two simple aglycones have been identified, namely the flavan-3-ols epigallocatechin and (+)-catechin, based on their parent ions with $[M-H]^-$ at *m/z* 305 [66] and respectively 289 [67]. The compounds have been putatively determined based on retention times, the profiles of UV spectra, and MS^n fragmentation, in comparison to other analogous chromatographic determinations for *H. rhamnoides* [26,68,69], as well as to data available in the scientific literature.

Compound **7**, with $[M-H]^-$ at *m/z* 785, had a peak at ***m/z* 315**, that correlates to deprotonated IRh. The base peak was registered at *m/z* 623, showing a loss of hexosyl moiety (162 u) and a loss of hexosyl-deoxyhexosyl (308 u). Thus, it was tentatively assigned as IRh-*O*-deoxyhexoside-dihexoside and served as model for identification. Further, the peak *m/z* 315 corresponding to deprotonated IRh has been found for other flavonoids, specifically in compounds **4, 6, 7, 9 – 15**. In a similar manner, the loss of hexosyl and/or deoxyhexosyl moieties has been analyzed, concluding a total of nine IRh-glycosyl and polyglycosyl derivatives, as visualized in Table 2 and Fig. 3**.** For instance, compound **4** was identified as IRh-*O*-deoxyhexosyl-dihexoside (due to the loss of hexosyl moiety from *m/z* 785 to *m/z* 623, followed by a 308 u loss to *m/z* 315, that corresponds to a hexosyl-deoxyhexosyl or rutinosyl moiety). The compounds **4, 6, 7** and **9, 10, 11** have shown a pseudomolecular ion $[M-H]^-$ at *m/z* 785 and 769, with a distinctive fragment at *m/z* 315 in MS^3, which corresponds to IRh aglycone, as well as hexosyl or deoxyhexosyl fragments (324 u corresponds to dihexosyl and 308 corresponds to rutinosyl). Compounds **12, 13, 14, 15** had a pseudomolecular ion $[M-H]^-$ at *m/z* 623, with fragments at *m/z* 423 and 315, preliminary classified as IRh-*O*-deoxyhexosyl-hexoside [26]. The *m/z* 605 remained unidentified. Compounds **3** and **8** presented a pseudomolecular ion $[M-H]^-$ at *m/z* 771 and 755, with distinctive fragments at *m/z* 301, which correspond to quercetin aglycone. A supplementary fragment at *m/z* 625 suggested a loss of 146 u (deoxyhexosyl) and another one of 324 u (dihexosyl), hence **3** has been tentatively identified as quercetin-*O*-deoxyhexosyl-dihexoside. In a similar manner, due to the loss of 454 u (equivalent to two deoxyhexosyl units and one hexosyl unit), **8** was identified as quercetin-*O*-dideoxyhexosyl-hexoside. Finally, compound **5**, luteolin-*O*-dihexosyl-deoxyhexoside, was identified based on the

Table 2
The tentatively identified flavonoid derivatives from *H. rhamnoides* L. extracts, presented in terms of retention time (Rt), maximum absorption wavelength (λmax), base peaks and fragmentation until MS^3 (*m/z*), as well as quantification (as mg/g of extract).

Peak	Rt (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	MS^3 (*m/z*)	Tentative identification	Quantification (mg/g dw)			
							HRO	HROB	HRE	HREB
	4.18	255	305	–	–	Epigallocatechin	0.53 ± 0.06	0.52 ± 0.02	0.94 ± 0.03	1.68 ± 0.06
	6.71	261	289	–	–	(+)Catechin	1.48 ± 0.03	1.56 ± 0.05	1.55 ± 0.04	1.33 ± 0.02
	9.69	350	771	625(100)	301(93)	Quercetin-*O*-deoxyhexosyl-dihexoside	0.59 ± 0.00	0.65 ± 0.00	0.51 ± 0.00	0.76 ± 0.00
	11.36	350	785	623(100)	315 (100)	Isorhamnetin-*O*-deoxyhexosyl-dihexoside	0.59 ± 0.00	0.65 ± 0.00	0.52 ± 0.00	0.73 ± 0.00
	12.49	349	755	609(100)	285(98)	Luteolin-*O*-dihexosyl-deoxyhexoside	0.63 ± 0.00	0.72 ± 0.00	0.57 ± 0.00	0.83 ± 0.00
	12.92	349	785	639(100)	315 (100)	Isorhamnetin-*O*-deoxyhexosyl-dihexoside	0.55 ± 0	0.59 ± 0	0.48 ± 0.00	0.67 ± 0.00
	13.31	353	785	639(100)	315 (100)	Isorhamnetin-*O*-deoxyhexosyl-dihexoside	0.67 ± 0.00	0.76 ± 0.00	0.6 ± 0.00	0.88 ± 0.00
	14.48	350	755	591(50), 301 (100)	–	Quercetin-*O*-dideoxyhexosyl-hexoside	0.56 ± 0.00	0.61 ± 0.00	0.48 ± 0.00	0.69 ± 0.00
	16.91	353	769	605(100), 315(70)	–	Isorhamnetin-*O*-dideoxyhexosyl-hexoside	0.6 ± 0.00	0.66 ± 0.00	0.54 ± 0.00	0.78 ± 0.00
	17.30	350	769	605(69), 315 (100)	–	Isorhamnetin-*O*-dideoxyhexosyl-hexoside	0.54 ± 0.00	0.58 ± 0.00	0.46 ± 0.00	0.67 ± 0.00
	18.04	353	769	623(100)	315 (100)	Isorhamnetin-*O*-deoxyhexosyl-*O*-deoxyhexosyl-hexoside	0.56 ± 0.00	0.6 ± 0.00	0.49 ± 0.00	0.71 ± 0.00
	18.43	352	623	477(100), 315(21)	315(20)	Isorhamnetin-*O*-deoxyhexosyl-hexoside	0.79 ± 0.00	0.84 ± 0.00	0.75 ± 0.00	1.12 ± 0.00
	18.71	353	623	477(100), 315(30)	315(74)	Isorhamnetin-*O*-deoxyhexosyl-hexoside	0.62 ± 0.00	0.64 ± 0.00	0.55 ± 0.00	0.78 ± 0.00
	19.13		623	477(89), 315 (10)	315(15)	Isorhamnetin-*O*-deoxyhexosyl-hexoside	0.53 ± 0.00	0.58 ± 0.00	0.47 ± 0.00	0.67 ± 0.00
	21.97	354	623	477, 315 (100)	–	Isorhamnetin-*O*-deoxyhexosyl-hexoside	0.7 ± 0.00	0.67 ± 0.00	0.71 ± 0.00	1.27 ± 0.00
Total flavonoid species							**9.94 ± 0.02**	**10.63 ± 0.01**	**9.63 ± 0.06**	**13.57 ± 0.09**

pseudomolecular ion $[M-H]^-$ at *m/z* 755, with fragments at *m/z* 609 (loss of deoxihexosyl) and 285 (luteolin aglycone, loss of dihexosyl).

Regarding the semi-quantitative analysis of the abovementioned compounds, it can be observed that the HREB extracts showed the highest cumulative sum of phenolic species (13.57 ± 0.09 mg/g dw), similar to HROB (10.63 ± 0.01 mg/g dw). Consequently, the values for the non-buffered extracts showed a lower content, specifically 9.94 ± 0.02 mg/g for HRE and 9.63 ± 0.06 mg/g for HRO.

IRh and its derivatives are known for being one of the major flavonoid classes represented in *H. rhamnoides* [70,71], and it has been suggested that IRh is the principal bioactive compound of this species, accounting for the antioxidant and anti-inflammatory effects. For example, some IRh-3-*O*-glycosides showed radical-scavenging activity and α-glucosidase inhibitory effects. Regarding cytotoxicity, it has been shown that IRH had antitumor and growth inhibitory effects on human hepatocellular carcinoma cells [72,73]. Moreover, the compound has been investigated for the effect of cardiovascular and cerebrovascular protection [74]. Other studies have noticed the capacity of water to extract IRh-*O*-glycosides, such as from the water extract of *Sophorae flos* [75]. The phytochemical analysis confirmed the presence of IRh glycosides in the *H. rhamnoides* extracts; this is probably due to the higher extractability of the sugar portions with higher affinity for polar solvents, including IRh and kaempferol or luteolin, which are aglycones with lower polarity [76]. At the same time, the extensive amount of *O*-glycosylation of IRh in the extracts can cause a reduction of bioactivity, since usually the aglycone accounts for the beneficial effects [77]. This means that further studies regarding UAE and E-UAE water extracts should also take into consideration the potential bioactivity, bioaccessibility, and bioavailability of glycosylated derivatives, in comparison to their separate aglycones [26].

3.6. In vitro antioxidant capacity

HRO and HRE extracts were tested for their antioxidant potential through three *in vitro* assays (ABTS or TEAC, DPPH, and FRAP), as well as other two assays, namely inhibitions of oxidative hemolysis (OxHLIA), and lipid peroxidation (TBARS). The results are shown in Table 3. The comparative graphical representation of the results is shown in Fig. 4. As a general trend, the tests based on radical and ferric reducing mechanisms indicated that the E-UAE method obtained the extracts with the highest antioxidant potential. Moreover, for these extracts the activity was even higher in association with the phosphate buffer, although in the case of UAE extracts there was no correlation. The highest values of the antioxidant activity have been observed in the FRAP assay, ranging from 123.46 to 205.59 mg TE/100 g dw. Regarding lipid peroxidation and oxidative hemolysis inhibition, the extracts showed a less significant activity, approximatively ten times lower in comparison to the Trolox standard for TBARS (IC_{50} = 5.42 ± 0.33 μg/mL) and 10–20 times lower for OxHLIA (IC_{50} = 21.47 ± 0.18 at Δt = 60 min and IC_{50} = 43.49 ± 0.25 at Δt = 120 min). As it can be observed, HREB demonstrated an increased activity in the TBARS assay, and HRO the highest activities in OxHLIA, in both time frames.

3.7. In vitro enzyme inhibitory activity

HR extracts were tested for their inhibitory activity against α-glucosidase (α-Glu), tyrosinase (Tyr), and acetylcholinesterase (AChE). None of the extracts were able to inhibit the enzymes, since the determined IC_{50} values (results not shown) have been at least ten times higher than the values of the control standards (acarbose, kojic acid and galantamine). The most potent extract was HREB, with IC_{50} values of 1.854 mg/mL (AChE), 1.875 mg/mL (Tyr), and 2.42 mg/mL (α-Glu), followed by HROB. These results show a similar trend with the

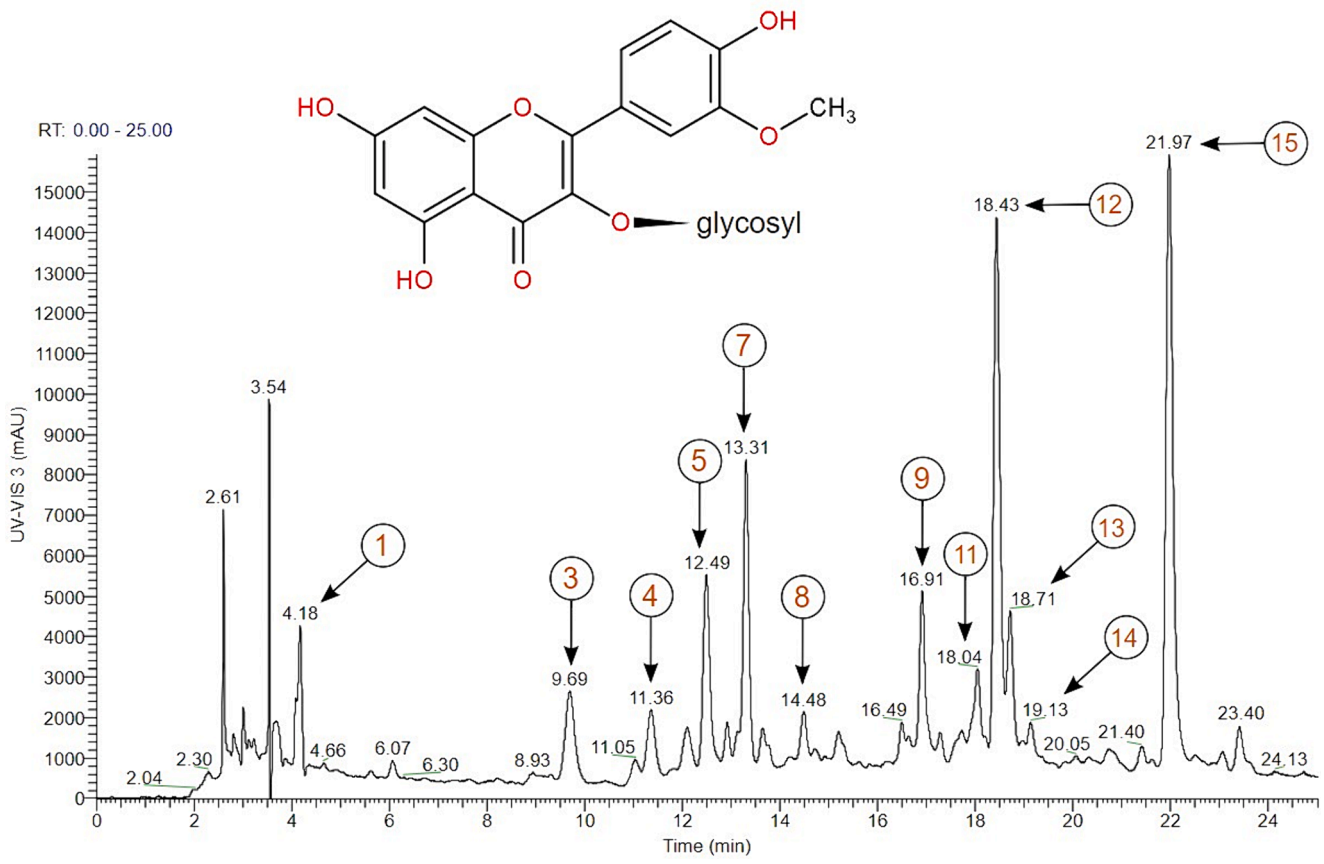

Fig. 3. Liquid chromatogram for UV–VIS identification (represented for λ = 370 nm) for the sample HREB (which showed the highest cumulative total flavonoid content). The chemical structure represented corresponds to a general isorhamnetin-3-*O*-glycoside, which is the major class of phenolic species identified in the HR extracts (the glycosylation can also occur at other free –OH positions). The tentatively identified **glycosyl** groups for isorhamnetin derivatives are as following: **4,6,7** – deoxyhexosyl-dihexoside; **9,10** – dideoxyhexosyl-hexoside; **11** – deoxyhexosyl-deoxyhexosyl-hexoside; **12–15** – deoxyhexosyl-hexoside.

Table 3
Extractive yields, TPC, TFC, *in vitro* antioxidant capacity and anti-glucosidase activity values for the four *H. rhamnoides* L. extracts.

Type of extraction applied		Extraction by UAE		Extraction by EAE and UAE	
		HRO Non-buffered	HROB Buffered	HRE Non-buffered	HREB Buffered
Extractive yield		6.39 %	5.91 %	7.60 %	6.95 %
TPC (mg GAE/100 g dw)		90.49 ± 1.00^{c}	103.25 ± 2.50^{b}	104.47 ± 1.27^{b}	118.27 ± 2.77^{a}
TFC – $AlCl_3$ (mg QE/100 g dw)		8.87 ± 0.07^{c}	3.00 ± 0.10^{d}	18.93 ± 0.66^{a}	17.52 ± 0.34^{b}
TEAC (mg TE/100 g dw)		69.31 ± 2.95^{d}	78.45 ± 1.27^{c}	85.81 ± 1.48^{b}	99.92 ± 4.03^{a}
FRAP (mg TE/100 g dw)		127.16 ± 1.69^{c}	123.46 ± 0.45^{c}	149.91 ± 1.58^{b}	209.59 ± 5.84^{a}
DPPH (mg TE/100 g dw)		79.39 ± 2.15^{c}	67.03 ± 2.24^{d}	92.08 ± 2.00^{b}	122.48 ± 1.65^{a}
TBARS (IC_{50}, μg/mL)		65.72 ± 0.96^{c}	66.18 ± 0.86^{c}	61.91 ± 0.89^{b}	43.80 ± 0.55^{a}
OxHLIA (IC_{50}, μg/mL)	Δt = 60 min	253.49 ± 13.82^{a}	412.12 ± 8.42^{b}	253.96 ± 15.41^{a}	413.24 ± 13.14^{b}
	Δt = 120 min	598.65 ± 13.45^{a}	687.88 ± 9.45^{b}	688.91 ± 11.81^{b}	843.71 ± 9.73^{c}
α-glu. inhibition (IC_{50}, mg/mL)		2.801 ± 0.174^{b}	3.039 ± 0.251^{b}	3.163 ± 0.292^{c}	2.425 ± 0.131^{a}

Note: The results are illustrated as average ± standard deviation (n = 3). The analysis of statistical variances was assessed using one-way ANOVA, where every superscript letter indicates significant statistical differences ($p < 0.05$) compared to different HR extracts.

antioxidant activity, with higher potential for the extracts obtained through U-EAE. Tkacz et al. have found an important effect of freshly berries on α-amylase, α-glucosidase, lipase, and lipoxygenase, although they have applied specific extraction methods for the recovery of phytochemicals, and not water as in the present study [78]. Moreover, Khan et al. have identified α-glucosidase, β-glucuronidase, and tyrosinase inhibitory potential, in the case of 70 % methanol extracts [79].

3.8. Statistical correlations

Several statistical methods were used to evaluate the correlations between the association of optimized extracts with UAE and EAE parameters, their evaluated phenolic profile, along with *in vitro* antioxidant and enzyme inhibitory activities. Heatmapping and hierarchical clustering (HCA) were employed to highlight the similarities and discrepancies between phenolic compounds, and extraction procedures (i.e., UAE, E-UAE) of dried *H. rhamnoides* L fruits. The HCA revealed that the samples were grouped according to their extraction procedures. Unambiguous discrimination between the extraction types (i.e., UAE, E-UAE), buffered and non-buffered solutions are distinguished by the different cluster positions of UAE and E-UAE extraction types. As it can be visualized in Fig. 5, the different extraction methods by E-UAE were

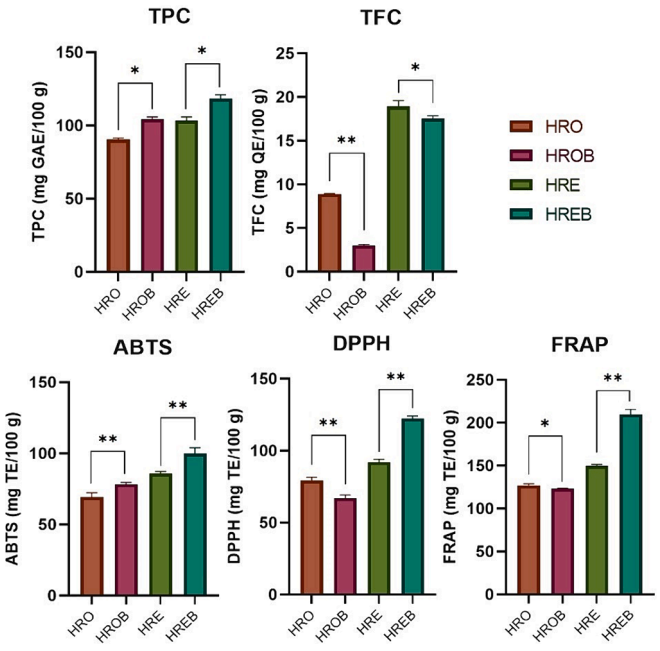

Fig. 4. Graphical representation of preliminary phytochemical testing (through TPC and TFC) and *in vitro* antioxidant activity (through ABTS, DPPH, and FRAP assays) for the four HR extracts. The results are illustrated as average ± standard deviation (n = 3). The analysis of statistical variances (between buffered and non-buffered extracts) was assessed using one-way ANOVA: * = $p < 0.05$; ** = $p < 0.01$.

discriminated by UAE by the different positions in the cluster. Following the importance score, the 1st cluster highlights the highest extractive yields which were identified for non-buffered extracts and the E-UAE combination obtained extracts with the highest overall *in vitro* antioxidant capacity. EAE had a significant positive effect on the value of the TFC (in both buffered and non-buffered extracts), compared with the extraction by UAE which led to a significant decrease in TFC. Regarding lipid peroxidation and oxidative hemolysis inhibition, the HREB exhibited a particularly significant activity in the TBARS assay compared to the other extracts, as seen by the negative correlation of the importance score. The 2nd cluster underlines the non-buffered extract HRE and the U-EAE combination extraction method which gave the highest extraction yield, as seen by the positive score. A comparable tendency to that of the HREB extract has been observed in the case of TFC; however, a strong inhibitory activity against α-glucosidase has been observed as compared with HREB, as depicted by the positive score. The following sub-cluster emphasizes the grouping of HRO and HROB as evidenced by the similar extractive yields and significantly low TFC in both extraction methods, particularly in HRO. As a general trend, the tests based on radical and ferric reducing mechanisms indicated that using the UAE method leads to a lower antioxidant potential in both buffered and non-buffered extracts.

The PCA results revealed that the first four components, with eigenvalues ranging from 1.78 to 7.14, were significant in explaining the variation between the optimized extracts, accounting for 94 % of the total variance (Fig. 6). The 1st PC accounted for 71.5 % variation and highlighted the accumulation of major phenolic compounds in the HREB extract with the highest levels in epigallocatechin and isorhamnetin-*O*-deoxyhexosyl-hexoside. Furthermore, the significantly higher TPC and *in vitro* antioxidant activity was revealed as compared to the other extracts. The following quadrant emphasizes the HRE extract with the highest extractive yield, which exemplifies the increased accumulation of TFC and the strong inhibitory activity against *α*-glucosidase. The 2nd PC accounted for 22.7 % variation and revealed the similarities between the extraction method by UAE, particularly of HRO and HROB regarding the accumulation of cathechin and inhibition of lipid peroxidation. Conversely, an increased accumulation of phenolic compounds in the

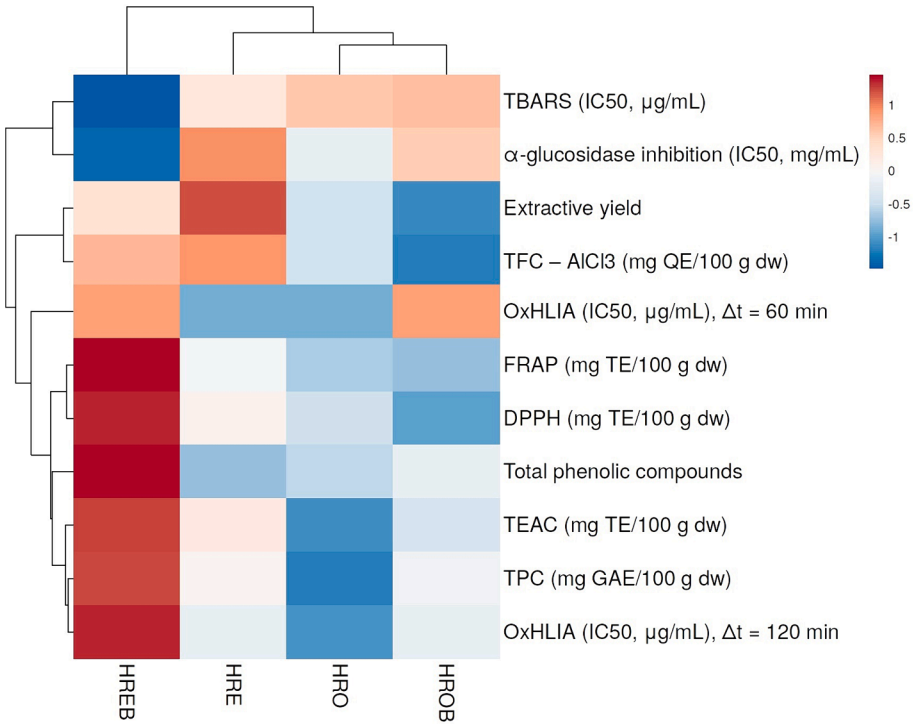

Fig. 5. HCA and heatmap representation of the bioactivities of *H. rhamnoides* based on the type of extraction applied. Columns indicate the different extraction methods and row the evaluated phenolic profile, *in vitro* antioxidant and enzyme inhibitory activities. Cells are highlighted according to the values of bioactivities and extraction types (i.e., UAE, E-UAE). In this context, red indicates a significant positive association, while blue indicates a significant negative association. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

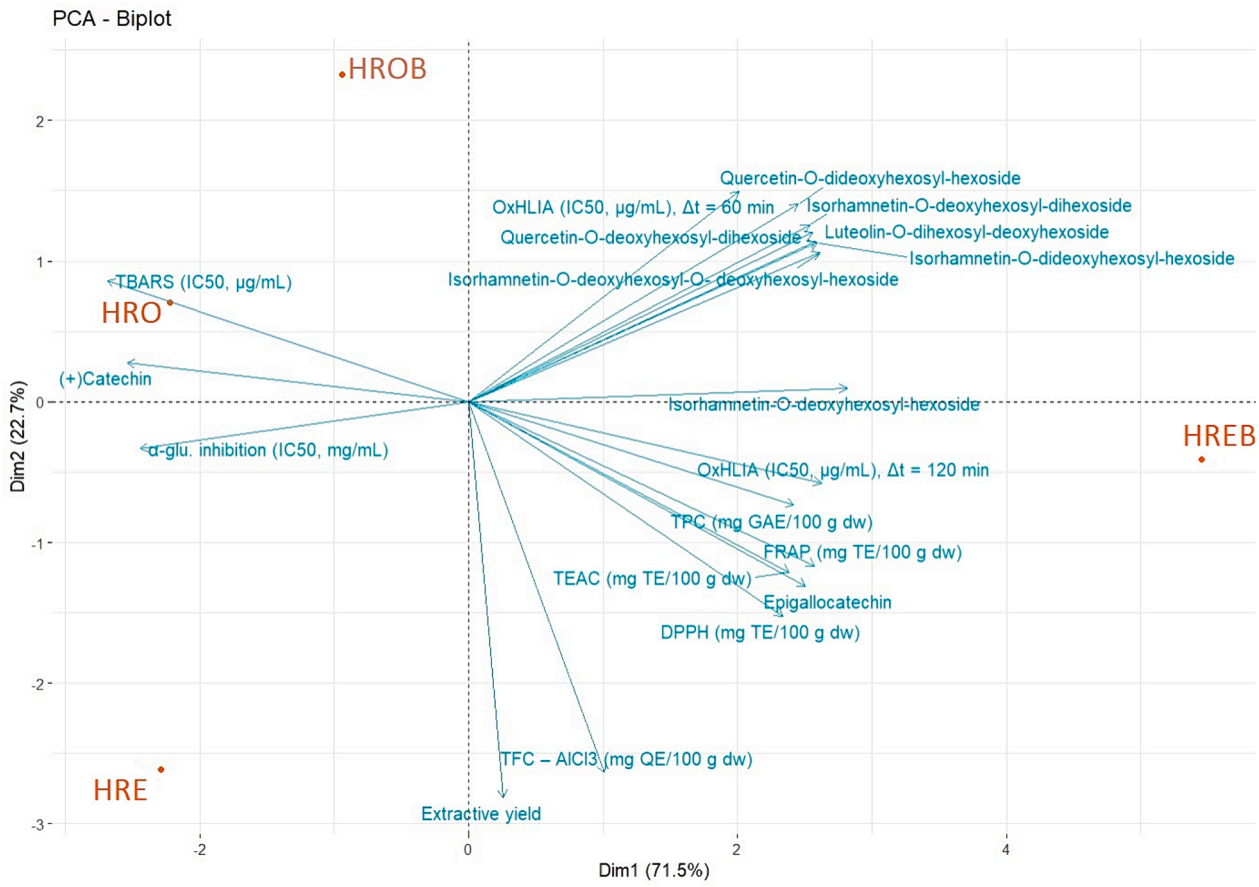

Fig. 6. PCA biplot of isolated phenolic compounds, phytochemical testing, and *in vitro* antioxidant activity for the four HR extracts. The main two factors explained 94% of the overall variance.

buffered extracts (i.e., HROB and HREB) has been observed particularly in the case of IRh derivatives, quercetin and luteolin glycosides. Therefore, the highest accumulation in phenolic compounds, *in vitro* antioxidant and enzyme inhibitory activities identified in the non-buffered extracts revealed the significant importance to select the most appropriate enzymatic extraction.

4. Conclusions

An optimization of aqueous ultrasound-assisted extraction of polyphenolic species from the dried *Hippophae rhamonides* L. fruits (sea buckthorn berries) has been developed. Furthermore, this method has been implemented with cellulolytic and pectinolytic enzymatic-assisted extraction, in the presence or absence of phosphate buffer. The optimization process considered three independent variables, namely ultrasound amplitude, exposure time and material-solvent ratio, with total phenolic content being the dependent variable. For this model, the optimal parameters obtained were 34 % ultrasound amplitude (average ultrasound power of 7.85 W), 39.33 min of extraction and 1:10 material: water ratio. The highest extractive yields have been identified for non-buffered extracts (7.60 and 6.95 %), suggesting the importance of rational choice of enzymatic extraction parameters. The HPLC-MS analysis revealed a rich isorhamnetin-*O*-glycosides content in all the tested extracts, confirming that flavonols represented the most abundant type of flavonoids in the aqueous extracts. In addition, the developed extracts showed a recovery of other specific flavonoids, such as catechin, epigallocatechin, as well as quercetin and luteolin glycosides. The *in vitro* antioxidant activity and the cumulative phenolic content was the highest for non-buffered E-UAE extracts, established through statistical analysis. This research holds significance because we have applied a previously developed hot air-drying process and studied the applicability of UAE ± EAE as pre-treatment, using the principles of green chemistry. Future perspectives include the bioaccessibility determination and the study of other possible factors that can alter polyphenolic extractability in water, including the optimization of EAE parameters.

CRediT authorship contribution statement

Alexandru Nicolescu: Writing – review & editing, Software, Methodology, Conceptualization. **Mihai Babotă:** Writing – review & editing, Investigation. **Eduardo Aranda Cañada:** Visualization, Investigation. **Maria Inês Dias:** Methodology, Investigation. **Mikel Añibarro-Ortega:** Investigation. **Mihaiela Cornea-Cipcigan:** Writing – review & editing, Software. **Corneliu Tanase:** Funding acquisition. **Cristian Radu Sisea:** Supervision. **Andrei Mocan:** Software, Methodology, Funding acquisition, Conceptualization. **Lillian Barros:** Software, Methodology. **Gianina Crișan:** Supervision.

Declaration of competing interest

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

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