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Which extraction technique is the best for LC × LC analysis of bioactive compounds from European medicinal plants: Conventional or sustainable extraction techniques?

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ABSTRACT

The growing interest in medicinal plants calls for further information about the extraction and chemical composition of potential bioactive compounds. Extraction is the most essential part of generating highly bioactive herbal remedies by isolating and preconcentrating bioactive compounds while, nowadays, the greenness of the method needs to be considered and improved. In this approach, different plant parts such as flowers, berries, and barks from *Sambucus nigra* L., a plant that has been studied for two decades for its promising hepatoprotective activity were investigated. Conventional and sustainable extraction techniques (infusion, magnetic-assisted extraction, ultrasound assisted extraction, and microwave-assisted extraction) were optimized for the recovery of bioactive compounds from these plant parts considering the greenness of the method as well as the total phenolic content, the antioxidant activity and the extraction yield. The parameters solvent-to-plant ratio, time, temperature, water-ethanol ratio as solvent and microwave power were optimized by an experimental design according to the extraction technique. The profile of the bioactive compounds of each plant part and extraction method was evaluated by LC × LC and hyphenated to tandem mass spectrometry for identification purposes. Compared to previously reported green extraction techniques, all four optimized methods were able to obtain similar results by being less time consuming, yielding good greenness values and were suited for high-throughput analysis. MAE proved to be the best of the investigated methods in terms of greenness and efficient extraction of bioactive compounds and will therefore play a significant role in future research applications.

1. Introduction

The growth of antibiotic-resistant bacteria due to prescription drug abuse is undoubtedly. Moreover, the existing drugs also show strong side or patient-dependent effects in many other diseases. In this sense, the ancient knowledge of monastic medicine has been used as an alternative to the present day. For example, the treatment of liver diseases with European medicinal plants has a centuries-old tradition in monastic medicine based on the therapeutic properties of these natural remedies [1–5]. Although there is a large knowledge about the therapeutic effects of these traditional plants, there is a lack of understanding about how these effects are achieved and which bioactive compounds are responsible for such beneficial effects. Out of multiple options of solvents that can cover the extraction of many compounds, nowadays the importance of complying with the green chemistry principles leads to the use of environmentally friendly solvents, with low toxicity, harmless to the environment and economically affordable [1,2]. Ethanol comprises all these criteria, since it has a high extraction capacity of water- and fat-soluble substances and is, likewise water, recognized as safe (GRAS) by the Food and Drug Administration (FDA) [3]. Lately, special attention is paid to extraction techniques that enable short extraction times, miniaturized setups, and low energy consumption. Conventional and emerging extraction techniques like infusion, solid-liquid extraction, Soxhlet extraction, magnetic, ultrasound (UAE), and microwave

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assisted extractions (MAE) and pressurized fluid extractions, are widely applied to herbal remedies [4–7]. Extraction methods must be optimized considering the plant species as well as the different plant parts from which the bioactive compounds are derived [8–11].

The objective of this work was the study of the medicinal plant *Sambucus nigra* L. which has shown promising results in their antioxidant, neuroprotective and hepatoprotective activity related to the fruits (known as elderberries), and the observed effects have been correlated to the secondary metabolite composition, phenolic acids, flavonoids, and polyunsaturated fatty acids [8,12–16]. Former investigations about the metabolite extraction from *Sambucus nigra* have evaluated the effect of different extraction techniques such as solid-liquid extraction of the elderberries or UAE, MAE and maceration of the elderflowers [15,16], however, reported green extraction methods can still be time- and solvent consuming [17–20]. Therefore, in this work, conventional and sustainable extraction techniques (infusion, magnetic assisted extraction, UAE and MAE) have been optimized for the extraction of berries, flowers and barks from *S. nigra* via a design of experiment (DoE) approach with the aim of obtaining highly bioactive extracts and improving the greenness of the process. To evaluate the optimization, the total phenol content and antioxidant activity were determined and a comprehensive two-dimensional liquid chromatography (LC × LC) method coupled to high resolution mass spectrometry (HRMS) for the separation of the optimized extracts was performed in order to investigate the analytes being extracted using different plant parts and extraction techniques. Moreover, the methods normally used for scavenging activity of phenolic compounds with 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were miniaturized. After optimization, the greenness of the extraction techniques was assessed according to the AGREEprep metrics [21,22].

2. Material and methods

2.1. Materials

Dried flowers, berries, and barks from *Sambucus nigra* L. harvested in the years 2020 and 2021, were obtained in a drug store (Herbathek, Berlin, Germany). The plant material was ground to a homogeneous powder after foreign bodies were removed. Ethanol (> 99.7 % (*v/v*)) was purchased from VWR (Darmstadt, Germany). Acetonitrile (100 %) was obtained from VWR (Rosny-sous-Bois-cedex, France), and methanol (≥ 99.9 % (*v/v*)) from VWR (Leuven, Belgium), both in HPLC-MS grade. Formic acid (≥ 99 %) was supplied from Fisher Scientific (Schwerte, Germany). Sodium carbonate (Na_2CO_3, ≥ 99.5 %) was purchased from AppliChem (Darmstadt, Germany). Potassium persulfate (≥ 99.0 %), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97 %) and gallic acid (97.5 %) were bought from Sigma Aldrich (Taufkirchen, Germany). Potassium dihydrogen phosphate (99.5 %), di-sodium hydrogen phosphate and Folin-Ciocalteau reagent were supplied from Merck (Darmstadt, Germany). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was bought from neoFroxx (Einhausen, Germany). Cellulose filters type 15A with a 110 nm diameter were obtained from Carl Roth (Karlsruhe, Germany) and PTFE filters with 0.20 µm pore size and a diameter of 13 mm from Macherey-Nagel (Düren, Germany). Ultrapure water (resistivity 18.2 M Ω cm^{-1}) was daily obtained from an Ultrapure Water System (Sartorius, Goettingen, Germany).

2.2. Optimization of extraction techniques

2.2.1. Design of experiment

For the experimental design and evaluation, a DoE approach via MODDE (13.0.2.34314, Sartorius, Goettingen, Germany) was used. The DoE consisted of a three-level two-factor full factorial design with 11 trials if not noted otherwise. Middle points were performed as triplicates for repeatability measurements. For the infusion extraction process, the plant material was extracted with boiling water for 5, 10 and 15 min and with solvent-to-plant ratios of 62.5, 125 and 187.5 mL g^{-1}. The optimization of magnetic-assisted extraction was done as Box Behnken design. The solvent-to-plant ratio was varied for 10, 25 and 40 mL g^{-1}, the ethanol content in the extraction was 0, 50 and 100 % aqueous ethanol (*v/v*) and the extraction time was 5, 15 and 25 min. After evaluation, for the following optimizations with the solvent composition was kept constant at 20 % aqueous ethanol for *S. nigra* berries and 60 % for flowers and bark and the solvent-to-plant ratio was set at 40 mL g^{-1}. For UAE (USR 30 H, Merck Eurolab, Belgium), the tested temperatures were 30, 55 and 80 °C with times of 5, 10 and 15 min at a frequency of 35 kHz. MAE with a microwave system (Mars NP-1185, Matthews, USA) was optimized using 5, 10, and 15 min with a microwave power of 200, 400 and 600 W at 55 °C. The extracts were filtered and lyophilizated for colorimetric assays carried out in a 96 well microplate reader Epoch BioTek (Agilent, Santa Clara, USA) using the Agilent BioTek Gen5 software). The remaining volume of the extracts were stored after filtration at −80 °C. The optimized extraction methods were evaluated regarding their greenness according to AGREEprep metrics [21,22]. For comparability, intake values for the evaluation were normalized to 1 mL extract.

2.2.2. Determination of bioactivity

Three tests were used to determine the antioxidant activity of different plant parts. The total phenolic content (TPC) was determined according to the Folin-Ciocalteu test [23]. The scavenging activity of phenolic compounds was determined by an ABTS and DPPH assay. For ABTS a traditional method from *Re et al.* [24] was used as model and its miniaturization was tuned in this work (see supplementary material SM1). Briefly, the extracts were diluted to a concentration of 0.25 mg mL^{-1} and gallic acid was used as a control at 7.5 µg mL^{-1}. 12 µL of sample, control and calibration standard reacted directly in wells with 290 µL of diluted ABTS (0.1 mM) solution and the absorbance was measured at 734 nm after 7 min incubation in the dark. The antioxidant activity of the samples was calculated using an external calibration with five concentrations of Trolox at 0.0625, 0.125, 0.25, 0.375 and 0.5 mM and given as Trolox equivalents (TE) in mg TE g^{-1} extract. A similar miniaturization was tuned for the DPPH assay according to Brand-Williams *et al.* [25] where a Trolox calibration with six concentrations (0.0625, 0.125, 0.25, 0.4, 0.5 and 0.6 mM) was used (SM2). The samples and α-tocopherol as control were diluted to 0.625 mg mL^{-1}. 15 µL of sample, control or calibration standard reacted with 285 µL DPPH solution (0.6 mM) for 30 min in the dark and were measured at 516 nm. Samples were further diluted if necessary. Samples, standards, and the control were measured as triplicates. The precision was assessed as intraday and interday repeatability by calculating the inhibition for each radical reagent for the lowest and highest standard concentration and for the control within one day and over all measurements on three days, respectively. The same was applied to a sample within two days.

2.3. LC × LC-HRMS/MS analysis

A 1290 Infinity II LC × LC-DAD-HRMS system consisted of a multisampler module (G7167B), two high speed pumps (G7120A), a MCT oven compartment (G7116B), and an automated controlled 2 positions/4-ports dual valve (G1170A). The LC system was operated and controlled using OpenLab CDS Edition (Agilent, Santa Clara, USA). 2 µL of the obtained plant extracts were injected. A Kinetex® pentafluoro phenylpropyl (PFP) (150 × 2.1 mm, 1.7 µm, Phenomenex, Torrance, USA) was used in the first dimension (^{1}D) and a Kinetex® polar C18 that has a polar modified surface (50 × 4.6 mm, 2.6 µm, Phenomenex, Torrance, USA) in the second dimension (^{2}D). The ^{1}D flow rate was 60 µL min^{-1} with a gradient of 0 min, 0 % B; 10 min, 5 % B; 16 min, 26 % B; 25 min, 28 % B; 30 min, 30 % B; 36 min, 35 % B; 45 min, 45 % B; 50 min,

60 % B and 58 min, 95 % B until 70 min using water, 0.1 % formic acid (A), and methanol, 0.1 % formic acid (B) as mobile phases. The ^{2}D flow rate was 2.0 mL min^{-1} using water, 0.1 % formic acid (A), and acetonitrile, 0.1 % formic acid (B) as mobile phases with a segmented gradient of 0–15 min, 0–2 % B; 15.5–23 min, 5–17 % B; 24–35 min, 10–23 % B; 36–49 min, 15–30 % B; 50–70 min, 40–95 % B. From 50 to 70 min a hold time at 95 % B from 0.6 min to 0.9 min during the modulation was applied. The modulation time was 1 min and two identical 80 μL loops were installed in the switching valve for the injection of the ^{1}D fractions onto the ^{2}D in countercurrent filling direction. The ^{2}D flow rate was split in a ratio of 1:5 before entering the HRMS. For the contour plots, the LC × LC method was hyphenated to an Exactive Orbitrap mass spectrometer (ThermoFisher Scientific, California, USA) equipped with a heated electrospray (HESI) ionization source for a reduced background signal. The Orbitrap was operated with a scan range of 80–1500 m/z, positive polarity, resolution of 10,000 at 10.8 Hz, AGC target reached at 10^{6}, and a maximum inject time of 10 ms. The HESI source parameters were spray voltage at 3.5 kV, capillary voltage of 37.5 V, tube lens voltage of 125 V and skimmer voltage of 26 V. Capillary and heater temperature were at 320 °C and 350 °C, respectively, sheath gas flow of 25 L min^{-1}, auxiliary gas flow of 10 L min^{-1}, and sweep gas flow of 1 L min^{-1}. The orthogonality was manually estimated for the *S. nigra* flower MAE extract by the bin-counting method suggested by Leonhardt *et al.* [26] with a bin size of 10 as it proved to be a more realistic tool to determine the orthogonality by considering unused space between analytes.

To confirm the findings of the Orbitrap, and to generate MS/HRMS data, a 6546 Q-TOF mass spectrometer (Agilent, Santa Clara, USA) was configured to operate in positive mode in a full scan data-dependent MS/MS acquisition mode, with a mass range of m/z 100 - 1700. The ion source parameters were set as follows: gas temperature was maintained at 320 °C, with a drying gas flow rate of 8 L/min, nebulizer pressure held at 35 psi, sheath gas temperature set to 350 °C, and sheath gas flow rate maintained at 11 L/min. The capillary voltage was set to 3500 kV and fragmentor voltage to 175V. Instrument control was performed using Mass Hunter LC/MS Acquisition (version 10.1), while data processing and analysis were conducted using MS-Dial. Parameters for data collection and peak detection were set as follows: MS1 tolerance was set to 0.01 Da, MS2 tolerance was set to 0.025 Da, minimum peak height for detection of a feature was set to 3000 in amplitude, while the peaks were smoothed with a linear moving average level 3. For compound identification and structural validation, acquired MS1 data were compared against spectral databases such as the MassBank of North America (MoNA). The features were positive matches when reaching at least 85 % similarity in centroid alignment between the acquired MS/HRMS spectra and reference spectra from the database. These features were then annotated as tentative candidates (level 3) according to the Schymanski scale [27].

3. Results and discussion

3.1. Optimization of conventional and sustainable extraction techniques

The optimization of the extraction techniques was based on three responses, i.e., total phenolic content (TPC) measured by Folin-Ciocalteu test, antioxidant activity by ABTS and DPPH assays for each plant part and extraction technique. Typically, the methods reported for ABTS and DPPH antioxidant activity measurements are carried out in 1 mL total reaction volume prepared in individual reaction tubes while only 300 μL are transferred to the well plate before analysis. Moreover, these assays measured different sample concentrations which led to three samples per well plate [24,28]. These facts imply the use of unnecessary solvents and the waste of a lot of plastic material and time. To improve this, it was aimed to increase the number of samples that can be analyzed per well plate, to decrease waste by reducing the number of steps and by miniaturizing the assay in general. Thereby, a concentration that lied within the calibration curve of each sample (flowers, berries, and barks) was selected and measured in triplicate and the antioxidant activity was determined using an external calibration. The samples and standard reacted directly in the wells with the reagent to avoid waste. By this, the sample throughput was increased by a factor of 6 and the volume of reagent used was halved. In this way, the procedure was less time-consuming due to a reduced number of pipetting steps leading at the same time to less waste generated during preparation. All measurements were done in triplicate for the samples, the standards and controls (gallic acid for ABTS assay and α-tocopherol for DPPH assay), ensuring that the quality of the results was maintained. Generally, DPPH values varied more than ABTS values due to the higher volatility of methanol used as solvent (Tables 1, 2). In general, the deviations occurring for sample measurements were lower than the ones measured for the lowest calibration standard and did not exceed a relative standard deviation of 9 %, demonstrating the reliability of these miniaturized procedures.

Once the colorimetric assays were validated, they were applied to all the experiments described by the DoE. The optimization of the extraction techniques was based on three responses, i.e., total phenolic content (TPC) measured by Folin-Ciocalteu test, antioxidant activity by ABTS and DPPH assays. The extraction yield was considered at the beginning as a fourth response. The extraction yield results were found to be within 35–47 % for flowers, 32–42 % for berries and 9–16 % for barks for all extraction techniques. Since the main aim of the extraction technique optimization was to obtain highly bioactive extracts and to highlight the greenness of the methods, the extraction yield was not considered a priority. Therefore, the optimal extraction conditions were determined by the maximization of the TPC, ABTS and DPPH responses for each plant part and extraction technique. For the investigation of statistical significance of the parameters, effect plots were evaluated where the bar charts indicated the 95 % confidence interval. As mentioned above, each extraction parameter has a different impact on the targeted compounds depending not only on the extraction method but also on the matrix to be extracted. Fig. 1 summarizes the impact of each extraction parameter on the flower extraction (TPC, ABTS and DPPH) for each extraction technique. As can be seen in this Figure, some extraction parameters did not have a strong influence on some responses, for instance, the TPC was unaffected by any extraction parameter in all the evaluated extraction methods. However, some parameters had a significant effect to other responses, like the solvent composition that had a significant effect over the ABTS and DPPH assays in the infusion and magnetic stirring extractions methods, or the effect of the solvent-to-plant ratio on the DPPH values obtained during the optimization of the infusion method. Moreover, the time was shown to have a significant impact on the DPPH values of the infusion extraction of flowers. Considering the berry samples, the solvent-to-plant ratio had a significant influence on the DPPH of the extracts obtained with the infusion method. On the other hand, the solvent strongly affected all the responses of the extracts obtained with magnetic-assisted extraction, and the time was a statistically significant variable for the DPPH of the berry extracts obtained with infusion (Fig. S1). Finally, for barks, the solvent was the only parameter with statistical relevance, in particular, it was relevant for the TPC and DPPH values obtained for the bark extracts obtained with magnetic-assisted extraction (Fig. S2).

Moreover, for *S. nigra* elderflower extracts, the surface-response curves displayed as contour plots for each response are shown in Fig. 2. The effect and surface-response plots of berries and barks are presented in Figs. S3 and S4. Interestingly, the behavior and trend of TPC, ABTS and DPPH of the extraction of flowers and berries was remarkably similar when using infusion, UAE, and MAE. However, magnetic-assisted extraction provided opposite results for these two plant parts. On the other hand, barks showed completely different extraction conditions for each extraction technique for the recovery of bioactive compounds. These results could show that berries and flowers can present more similar secondary metabolites while the bioactive

Table 1
Repeatability of the ABTS and DPPH assay expressed as mean, standard deviation and relative standard deviation (RSD) of the measured inhibition in triplicate. Gallic acid was used as control at 7.5 μg mL^{-1} for ABTS and α-tocopherol at 0.0625 mg mL^{-1} for the DPPH assay. The sample is picked out of 20 measured samples during assay revision due to the highest standard deviation.

			Trolox (0.0625 mM)	Trolox (0.5 mM)	Control	Sample
ABTS inhibition [%]	Intraday	Mean±SD	8.6 ± 0.2	70 ± 0.6	30 ± 0.2	52 ± 0.2
		RSD [%]	2.7	1.0	0.9	0.4
	Interday	Mean±SD	7.9 ± 0.8	70 ± 1	30 ± 0.4	49 ± 3
		RSD [%]	8.7	1.7	1.4	6.8
DPPH inhibition [%]	Intraday	Mean±SD	11 ± 1	82 ± 5	36 ± 0.3	25 ± 1
		RSD [%]	11	6.4	0.88	4.3
	Interday	Mean±SD	11 ± 2	84 ± 5	35 ± 3	27 ± 2
		RSD [%]	18	5.6	8.6	8.7

Table 2
Optimized extraction parameters for each plant part and technique (- indicates that this parameter was constant for the technique; * indicates that the parameter was previously optimized using another technique).

Plant part	Technique	Solvent-to-plant ratio [mL g^{-1}]	Ethanol content [%]	Time [min]	Temperature [°C]	Microwave power [W]
Flowers	Infusion	187.5	–	5	–	–
	Magnetic	40	60	5	–	–
	UAE	40*	60*	11	55	–
	MAE	40*	60*	5	55*	400
Berries	Infusion	187.5	–	5	–	–
	Magnetic	40	20	5	–	–
	UAE	40*	20*	11	55	–
	MAE	40*	20*	5	55*	400
Bark	Infusion	187.5	–	15	–	–
	Magnetic	40	60	15	–	–
	UAE	40*	60*	11	55	–
	MAE	40*	60*	15	55*	200

compounds present in barks could belong to different metabolite families. Overall, the ethanol ratio in the solvent was the most important parameter to be optimized as in the effect plots for magnetic-assisted extraction (Figs. 1, S1, and S2), the whisker ends within the box indicating a significant influence on the response. This was observed for flowers for the antioxidant activity measured by ABTS and DPPH, for berries it affected every outcome and influenced other extraction parameters as well as seen due to the multiplicative abbreviation, and for barks TPC and DPPH was influenced by the ethanol ratio. The optimal ethanol ratio of the different plant parts varied as observed in the surface-response plots (Figs. 2, S3 and S4) as for berries lower percentages were better while for flowers and barks, it varied for each response and, therefore, this parameter has to be chosen carefully. In the case of flowers and berries, a higher solvent-to-plant ratio may be more favorable as well as a longer extraction time for barks, but both would not be desirable for assessing the greenness and were therefore not further investigated.

Optimal conditions were estimated as desirability based on all three responses and compared for each plant part manually to state the optimal conditions for each technique. The optimal extraction conditions for infusion, UAE and MAE were in compliance with sustainability while for magnetic-assisted extraction of flowers and barks optimal parameters were obtained that resulted in higher extraction time, organic solvent and energy consumption. As the greenness of the methods was of foremost importance in this work, these optimal conditions were replaced by desired ones by reducing the extraction time, temperature, or percentage of ethanol in the solvent. As example, for magnetic-assisted extraction the optimal extraction conditions would be a solvent-to-plant ratio of 40 mL g^{-1}, 80 % aqueous ethanol and 25 min for flowers from *S. nigra*. The theoretical values achieved with these conditions resulted in a TPC value of 135 mg GAE g^{-1} extract, 22.8 mg TE g^{-1} extract for ABTS and 1.45 mg TE g^{-1} extract for DPPH, while at more sustainable conditions with 60 % aqueous ethanol and 5 min, the estimated values for TPC, ABTS and DPPH were 130 mg GAE g^{-1} extract, 21.8 mg TE g^{-1} extract and 1.43 mg TE g^{-1} extract, respectively. Therefore, the change in optimal parameters did not lead to a reduction loss greater than 5 %. A similar behavior was observed for the magnetic-assisted extraction of barks with a solvent-to-plant ratio of 40 mL g^{-1}, 90 % aqueous ethanol and 22 min resulting in predicted values of 29.3 mg GAE g^{-1} extract for TPC, 14.8 mg TE g^{-1} extract for ABTS, and 0.19 mg TE g^{-1} extract for DPPH. With more sustainable parameters of 60 % aqueous ethanol and 15 min, the value for TPC would be improved by 32 % to 38.7 mg GAE g^{-1} extract and lowered by 28 % for ABTS with 10.6 mg TE g^{-1} extract and 21 % lower with 0.15 mg TE g^{-1} extract for DPPH. Even though the prediction changed significantly compared to the optimal parameters, the more sustainable parameters were chosen since the TPC would rise while losing antioxidant activity. The optimized extraction conditions can be found in Table 2.

Regarding the prediction of responses for TPC, ABTS and DPPH compared to the observed values, high variance can be found (Table 3). For TPC, the deviation between observed and predicted laid between 62 and 162 % with MAE often overperforming compared to the predicted values. For ABTS, the range was 65 to 100 % indicating that the predicted values were not often met and the closest values to the predicted ones were obtained for UAE. DPPH proved to be an unstable response with deviations varying from 18 to 450 % where the prediction did not prove to be reliable. On the other hand, it was observed that magnetic-assisted extraction yielded overall higher values improving the predicted values by at least doubling the response.

After the extraction of each plant part under optimal conditions, it could be seen that for berries and barks, MAE was the best extraction technique for the phenolic compound recovery providing an extract with TPC of 97 and 68 mg GAE g^{-1} extract, respectively (Table 3). Interestingly, the extraction technique that provided the highest TPC from flowers was the magnetic-assisted extraction (150 mg GAE g^{-1} extract), while infusion, UAE and MAE obtained similar TPC values (130 mg GAE g^{-1} extract). Flowers were the part with the highest TPC value (150 mg GAE g^{-1} extract) followed by berries (97 mg GAE g^{-1} extract) and barks (68 mg GAE g^{-1} extract). Regarding the ABTS and DPPH antioxidant activities, according to TPC for flowers, magnetic-assisted

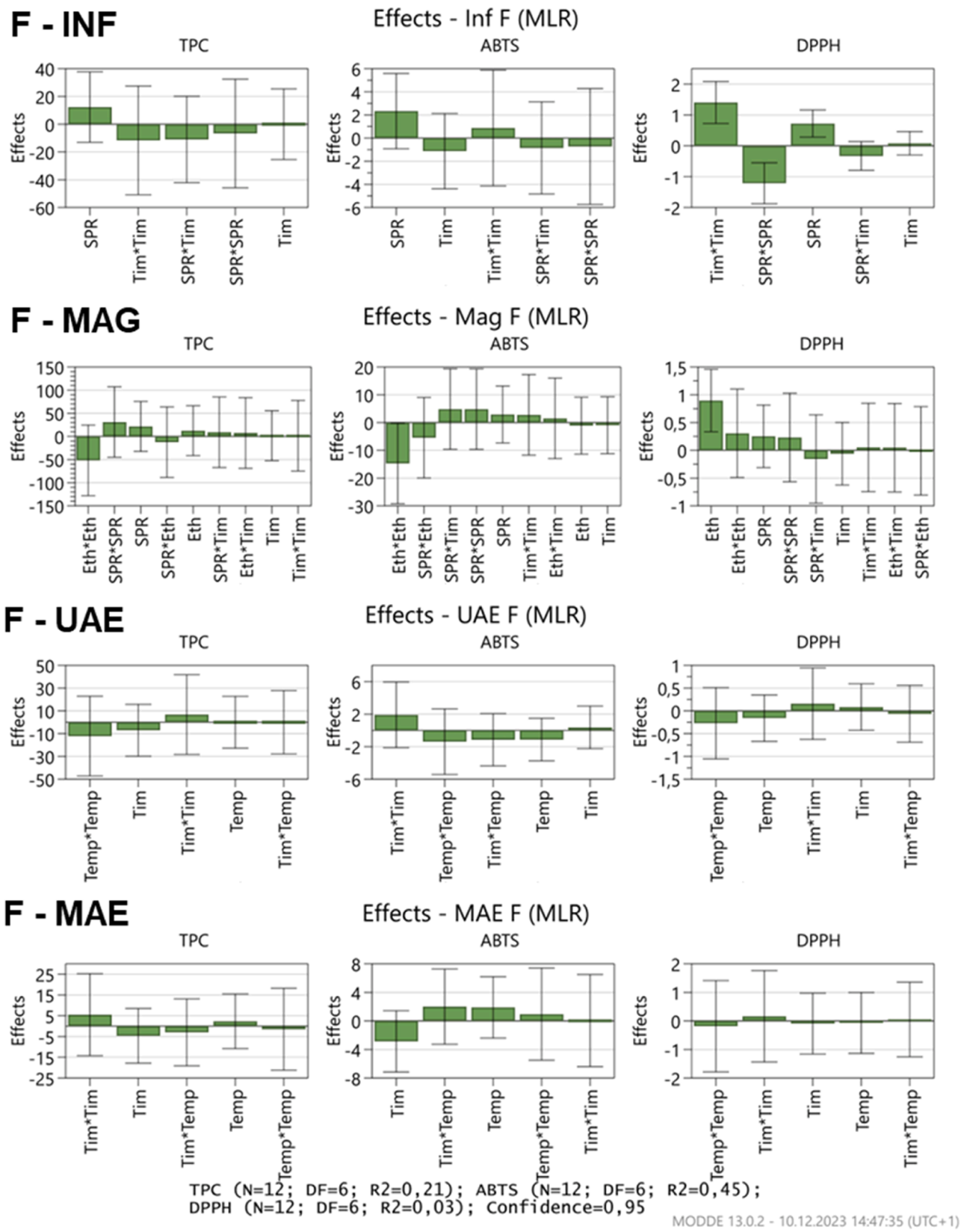

Fig. 1. Effect plots of the responses TPC, ABTS and DPPH for the extraction techniques infusion (INF), magnetic-assisted (MAG), ultrasound-assisted (UAE) and micowave-assisted extraction (MAE) for flower (F) extracts from *Sambucus nigra* L. The effects of the parameters solvent-to-plant ratio (SPR), time (Tim), ethanol ratio (Eth), and temperature (Temp) are indicated as individual or multiplicative effects. Individual means the effect itself has an influence on the outcome while multiplied parameters indicate that the parameter(s) do not only influence the outcome but also change the influence of other parameters to the outcome.

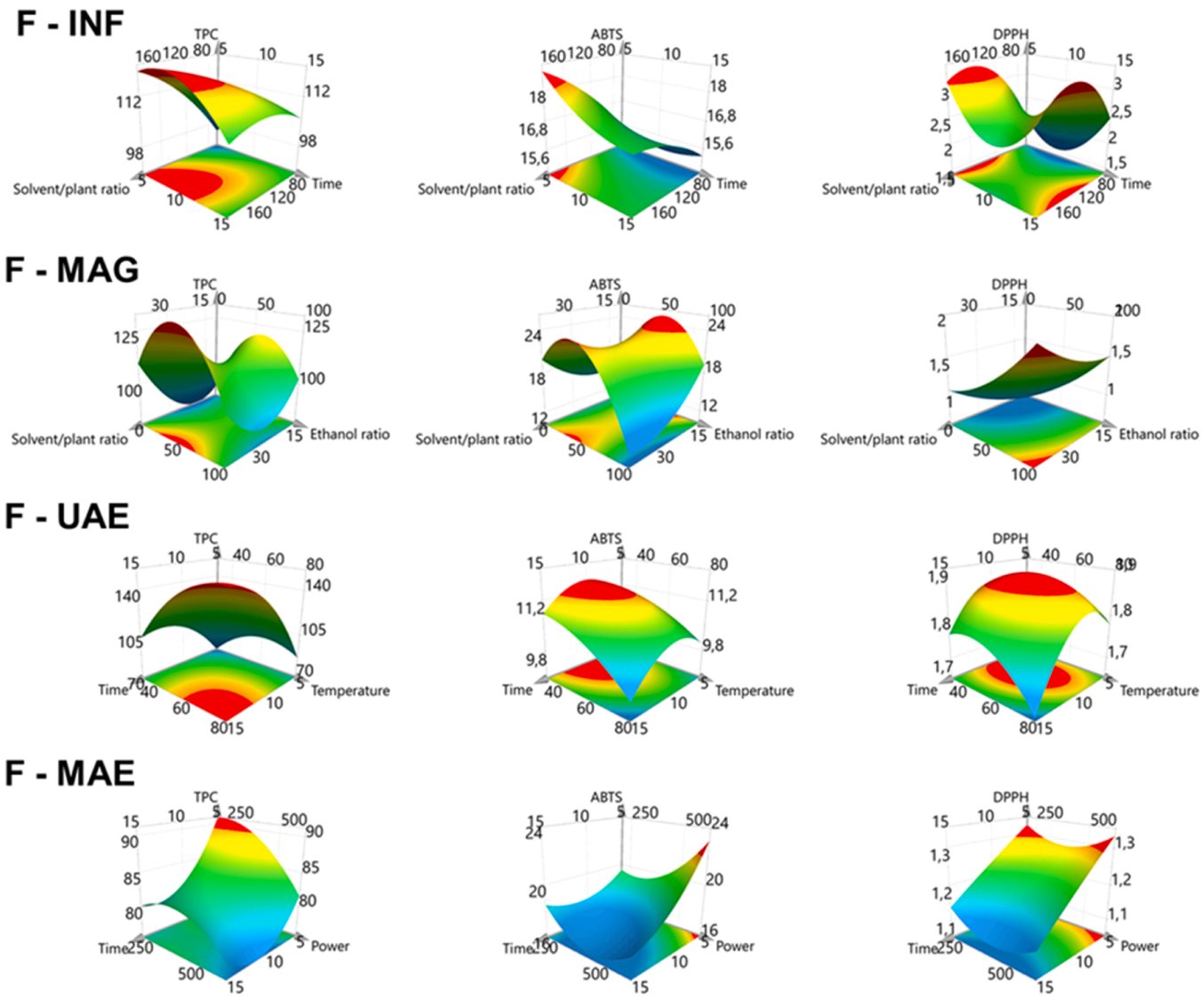

Fig. 2. Surface plots of the responses TPC, ABTS and DPPH for the extraction techniques infusion (INF), magnetic-assisted (MAG), ultrasound-assisted (UAE) and micowave-assisted extraction (MAE) for flower (F) extracts from *Sambucus nigra*.

Table 3
TPC, ABTS, DPPH, and extraction yield of the different plant parts extracted by optimized extraction techniques, measured in triplicates. Obs. indicates the through experiments observed values and Pred. indicates the predicted values from the software where the percentual deviation to the observed ones is given in brackets.

Plant part	Technique	TPC [mg GAE g^{-1} extract]		ABTS [mg TE g^{-1} extract]		DPPH [mg TE g^{-1} extract]		Extraction yield [%]
		Obs.	Pred. (Dev. [%])	Obs.	Pred. (Dev. [%])	Obs.	Pred. (Dev. [%])	Obs.
Berries	Infusion	85 ± 5	78 (109)	11 ± 1	13 (85)	0.58 ± 0.04	0.58 (100)	42 ± 1
	Magnetic	63 ± 1	102 (62)	13 ± 0.7	14 (93)	1.0 ± 0.005	0.22 (450)	37 ± 0.3
	UAE	88 ± 2	132 (67)	15 ± 2	11 (136)	0.35 ± 0.05	1.9 (18)	47 ± 2
	MAE	97 ± 0.5	90 (108)	13 ± 2	20 (65)	1.1 ± 0.05	1.3 (85)	43 ± 0.6
Flowers	Infusion	130 ± 13	120 (108)	17 ± 2	19 (89)	1.3 ± 0.2	1.1 (118)	36 ± 2
	Magnetic	150 ± 12	130 (115)	20 ± 0.3	22 (91)	3.0 ± 0.3	1.4 (214)	33 ± 0.6
	UAE	130 ± 0.9	133 (98)	17 ± 0.5	17 (100)	2.1 ± 0.3	3.7 (57)	37 ± 3
	MAE	130 ± 0.9	131 (99)	19 ± 3	22 (86)	2.5 ± 0.4	3.6 (69)	41 ± 1
Bark	Infusion	46 ± 2	57 (81)	5.8 ± 0.2	7.0 (83)	0.36 ± 0.01	0.92 (39)	14 ± 1
	Magnetic	63 ± 1	39 (162)	8.3 ± 0.1	11 (75)	0.44 ± 0.03	0.15 (293)	10 ± 3
	UAE	52 ± 3	50 (104)	6.4 ± 0.5	7.4 (86)	0.69 ± 0.07	1.2 (58)	14 ± 0.3
	MAE	68 ± 3	62 (110)	11 ± 2	14 (79)	0.58 ± 0.06	1.0 (58)	14± 0.0

extraction was the extraction technique that provided better ABTS (20 mg TE g^{-1} extract) and DPPH (3.0 mg TE g^{-1} extract) results. For the barks, the ABTS results were also in agreement with the TPC, being MAE the best extraction technique for this antioxidant activity (11 mg TE g^{-1} extract), while MAE and UAE obtained the extract with higher DPPH value (0.58 and 0.69 mg TE g^{-1} extract, respectively). Finally, for berries, DPPH results were also in relation to the TPC values, being MAE the best extraction technique (1.1 mg TE g^{-1} extract). However, although the

antioxidant activity is commonly related to TPC, there are other phenolic compounds present in the plants that cannot be quantified by the Folin method and present antioxidant activity [29]. In this regard, UAE provided the best ABTS results for berries (15 mg Trolox g^{-1} extract). This result could be related to the availability of UAE to extract other phenolic compounds from the berries like anthocyanins, compounds that required other spectrophotometric assays for their better quantification [29].

3.2. Evaluation of the greenness and comparison to previously reported methods

According to the AGREEprep software, the criteria for the intake values are: to favor in situ sample preparation (1), usage of safe solvents and reagents (2), usage of sustainable, reusable or renewable materials (3), amount of waste (4), miniaturization of sample, chemical and material amounts (5), sample throughput (6), automation and number of steps (7), energy consumption (8), post-sample preparation (9), and safety of the operator (10) [21,22]. The investigated extraction techniques differed in the fields of waste (criterion 4), sample consumption and throughput (criterion 6), number of steps (criterion 7) and energy consumption (criterion 8) (Fig. 3). Weights for the criteria are suggested and increased for criterion 3 since the usage of renewable materials has a significant influence on the procedure, especially for high throughput analysis and should be considered of higher value. Opposite to that, the weight of criterion 9 was decreased due to the independence of post-extraction steps on post-preparation analysis and was, accordingly, negligible when evaluating sample preparation. For infusion, the preparation of aqueous ethanol can be omitted resulting in less waste and number of steps being one of the greenest methods due to its high sample throughput of 30 samples h^{-1}. The differentiation between the other techniques relies on the sample throughput and energy consumption only. Magnetic-assisted extraction is the least green technique due to the lowest sample throughput of 12 samples h^{-1} and highest energy consumption of 30.6 Wh per sample. With UAE, 18 samples h^{-1} can be carried out and it had the lowest energy consumption (10.3 Wh per sample). MAE and UAE yielded similar end values for their greenness, but MAE was considered greener due to a higher sample throughput of 30 samples h^{-1} despite the higher energy consumption (21.8 Wh per sample).

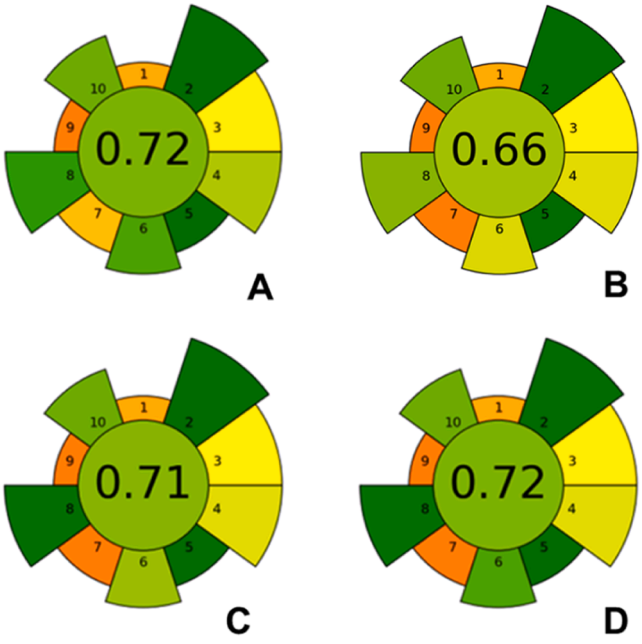

Fig. 3. AGREEprep metrics for the optimized extraction techniques infusion (A), magnetic-assisted extraction (B), UAE (C), and MAE (D).

Several papers about the extraction of *S. nigra* were reported issuing different extraction techniques, sustainability and the bioactivity of the plant determined by TPC, ABTS or DPPH [17–20,30,31]. As reported by Uzlasir et al., the best TPC, ABTS and DPPH values were reached for aqueous infusion extracts of *S. nigra* flowers after 30 min yielding 1.36 g L^{-1} for TPC [18]. During the optimization in this work, the highest TPC value of the infusion extracts of 1.42 g L^{-1} was obtained for a 5 min extraction with 50 % aqueous ethanol and a solvent-to-plant ratio of 10 ml g^{-1}, while the TPC value at the optimum considering the ABTS and DPPH reaction was 1.23 g L^{-1} for the infusion. Whereas for UAE and MAE, the optimal extraction conditions resulted in 1.25 and 1.35 g L-1, respectively, showing that MAE is capable of reaching similar TPC values within 5 min. As Pascariu et al. found, UAE (30 mins at room temperature and 40 kHz) and MAE (10 mins at 600 W) of alcoholic extracts gave better results than conventional stirring for 30 mins or water as a solvent, demonstrating the ability of sustainable extraction techniques to recover bioactive compounds [17]. The highest TPC value of 44.27 mg GAE g^{-1} dry matter for *S. nigra* flowers were yielded for alcoholic extracts of MAE followed by UAE and conventional extraction [17]. Compared to our MAE method, we reached a TPC value about twice as high for all extraction techniques with a time reduction of 50 % for MAE by optimizing the extraction parameters. For *S. nigra* berries, MAE using 50 % aqueous ethanol was also preferred over other techniques such as UAE and maceration or water as solvent [30,31]. MAE therefore proves to be the better choice over conventional extraction techniques or UAE and, as described here, is also the more sustainable technique. Specifically, for *Sambucus nigra* L., deep-eutectic solvents were used for the green extraction of flowers yielding similar TPC values of approximately 130 mg GAE g^{-1} plant extract while the method was more time-consuming with 150 min and involved more steps during extraction [19]. For elderberries, UAE with natural deep-eutectic solvents and additional stirring for 30 min were used to extract the phenolic compounds, followed by a second step prior to LC-MS/MS analysis, which consisted of a 5-h stirring extraction with 50 mL of 50 % aqueous methanol [15]. Considering reported literature about other medicinal plants, sustainable extraction methods had greenness values of 0.53 to 0.63 evaluated by AGREE [21] and extraction times between 3 and 120 min [32]. For UAE and MAE, reported extraction times were 20–150 min and 12 min, respectively, with more steps involved. Thus, various methods have been developed with the main aim of providing green solutions for the extraction of phenolic compounds with good results in terms of phenolic extraction. However, very long extraction times are often required to obtain the final enriched extract. In contrast, the higher greenness value of the methods developed in this work was a result of the optimization process focusing on the greenness of the technique, resulting in more time-saving methods (5–15 mins) while maintaining the bioactivity of the extracts. Although in most of these references ABTS and DPPH were used for the determination of antioxidant activity, the assays cannot be readily compared as they were often reported in terms of inhibition values, which were dependent on the concentration of the reagent and the sample. It is advisable to use a more general procedure for the ABTS and DPPH assays, taking into account the concentration of both substances, to facilitate comparison between the literature, as is possible with the TPC values. The miniaturized assays described here proved to be more sustainable, time efficient and cost effective, repeatable and offer a comparable measure for future reference.

3.3. LC × LC-HRMS analysis of the optimized extracts and identification

The chemical profile of plants is characterized as being a complex mixture of metabolites with a wide variety of families of compounds and therefore presenting analytes with very different physicochemical properties and a wide range of concentrations. This fact becomes a great analytical challenge, and conventional analytical techniques like one-dimensional liquid chromatography are not able to provide enough

separation power to obtain the relevant information about the complete chemical composition of the medicinal plant. To solve this problem, LC × LC appears as a powerful analytical tool able to increase the peak capacity and therefore, the separation power of very complex samples. In LC × LC a sample is analyze by two different separation mechanisms in one run which enhanced separation of all the compounds present in the sample [33]. Among a PFP column, C18, HILIC, and cyano phases have been tested for the ^{1}D and HILIC, C8, C18, and polar C18 for the ^{2}D after selecting PFP in the ^{1}D. The best separation was achieved by PFP × polar C18 due to the π-π interactions between aromatic compounds of the extracts and the stationary phase while polar C18 exhibited a good orthogonality with the possibility to use gradients below 5 % organic solvent. The orthogonality of the MAE extract of *S. nigra* flowers reached an orthogonality of 63 % which is comparable to reported RP × RP methods in literature although the orthogonality was determined using different metrics [34,35]. The colorimetric phenolic content and antioxidant activity measures did not provide a powerful enough insight into the real composition of the extracts obtained by the optimized extraction methods. Thus, LC × LC was employed to complete the information achieved with the spectrophotometric assays. As expected, the results achieved by the measurement of TPC were correlated with the data observed in the LC × LC plots. The extraction by infusion provided the lower TPC values, and in agreement with this data, the LC × LC analysis of these extracts showed the least number of separated compounds for each plant part (Fig. 4). Only in the last 20 mins of the analysis was it possible to observe a group of compounds that were also detected in the other extraction methods. However, in the case of the infusion extracts, fewer compounds with lower intensities were observed in this area compared to the other extraction methods. The elution time at which these peaks occur also provides information about the physico-chemical properties of these compounds. Since a reversed phase PFP column was used in the ^{1}D, compounds that elute at the end of the analysis are related to lower polarity. It could be expected that the infusion extraction using water as a solvent would increase the extraction of polar compounds, however, these chromatograms were characterized by the absence of polar compounds and the elution of medium- and low-polar compounds. This could be explained by the fact that phenolic compounds can present several sugar moieties attached to the aglycone structure, and the polarity of these compounds increases with the number of attached sugars, however, it has been reported that boiling water produces hydrolysis of the bonds between sugars and aglycones [36,37], giving rise to free aglycones which typical elute at the end of reversed phase chromatographic separation. Therefore, the high temperature used under boiling infusion extraction could produce the hydrolysis of the glycoside phenolic compounds. The chemical fingerprint of magnetic-assisted extraction was similar to the ones obtained by UAE and MAE, presumably because the extraction solvent was the same (mixtures of ethanol and water) as well as a medium extraction temperature (55 °C) which led to more compounds being extracted. Among these three methods (magnetic-assisted, UAE, and MAE), the intensity of the extracted compounds varied slightly based on the efficiency of the method. Due to measured reference standards, it can be stated that in the first area (5 to 25 min) compounds such as amino acids and monosaccharides were eluted and after 15 min phenolic compounds were observed with mainly phenolic acids between 25 and 50 min and polyphenols such as flavonoids between 50 and 70 min. Upon these three areas within the HRMS contour plots, the presence, absence or intensity of the compounds varied for each plant part leading to unique fingerprints. Compounds like chlorogenic acids, quercetin or its glycoside rutin were found in the flower extracts but not in berries or barks. Flowers contained overall more compounds or at least higher intensities than berries and barks which was in correlation to their total phenolic content and antioxidant activities. As expected from the evaluation of the in-vitro activity, the plant parts contained different secondary metabolites as seen in the variety of compounds displayed in the contour plots.

Of the individual compounds found in all extracts, 24 compounds were preliminarily identified and selected for comparison between the extraction methods and the plant parts as they were analyzed for their bioactive properties such as antioxidant capacity (Fig. 5). As expected, the compounds present in the extracts obtained by infusion presented the lowest intensities for all the plan parts except for 3-indoleacetic acid in *S. nigra* berries. For flowers, magnetic-assisted, UAE and MAE yielded similar intensities overall not showing a clear trend which extraction method would be favorable. Naringenin, ferulic acid, phenylacetyl aldehyde, and salsolinol were the main compounds observed in flowers. In the case of berries, infusion surprisingly resulted in comparable intensities for the compounds phenylacetyl aldehyde, nicotinamide, nicotinic acid, ferulic acid, naringenin and rutin, whereas for all other compounds the intensities were lower than the ones observed for the other investigated techniques. As already discussed for the flowers, the berries extracted with magnet-assisted, UAE and MAE extraction also show similar intensities, with the exception of kaempferol and hesperitin, for which magnet-assisted extraction was best. Among the barks extracts, infusion showed lower intensities than the other extraction techniques as expected, while the other techniques resulted often in comparable intensities. Interestingly, some compounds showed high intensities with specific extraction methods, such as bergapten, which was highly present in the UAE extract, indicating some selectivity of this technique for this compound. Magnetic-assisted extraction showed also some selectivity for other compounds such as quercetin and quercetin-ribonobioside. This selectivity could be explained by the temperature used during magnetic-assisted extraction since this was the only extraction technique operated at room temperature. The correlation analysis between the antioxidant assays (TPC, ABTS, and DPPH) revealed strong positive relationships. The Pearson correlation coefficients for TPC-ABTS (0.936), TPC-DPPH (0.863), and ABTS-DPPH (0.819) indicated a high degree of association. The statistical significance of these correlations was confirmed by Pearson correlation and low p-values ($p < 0.01$), suggesting that the observed relationships were not due to random variations but rather reflect meaningful biological interactions. For the identified compounds, some molecules exhibited statistically significant correlations with antioxidant parameters like indole, 4-hydroxybenzaldehyde, benzoic acid and colchicine (Table S1). The observed correlations suggested that these compounds contributed to the antioxidant potential measured in the different assays. Notably, benzoic acid and 4-hydroxybenzaldehyde showed the strongest correlation across all assays, particularly with DPPH ($r = 0.928$, $p < 0.0001$ and $r = 0.815$, $p = 0.0012$, respectively). Interestingly, indole exhibited moderate correlations with TPC ($r = 0.716$) and ABTS ($r = 0.719$), but its correlation with DPPH was weaker ($r = 0.448$, $p = 0.144$), suggesting that its antioxidant activity may be influenced by the different chemical environments of assays. Colchicine, on the other hand, demonstrated consistent moderate correlations across all assays. The variability in correlation strengths implies that antioxidant activity is likely influenced by a complex interaction of multiple compounds rather than individual constituents alone. As seen in the last 20 min of the LC × LC analysis, all extraction techniques showed similar compounds that were identified as quercetin, hesperitin, colchicine, rutin and the quercetin glycoside but with lower intensities for the infusion extracted plant parts. Accordingly, the metabolome profiles obtained by magnetic-assisted extraction, UAE and MAE showed a high diversity of phenolic compounds with different chemical properties, while the extracts from the infusion contained a lower diversity of phenolic compounds and thus fewer compounds known for their health-promoting effects, as observed for antioxidant activity in vitro.

4. Conclusion

The study of conventional and sustainable extraction methods is essential to propose greener alternatives. In this work, infusion, magnetic assisted extraction, UAE and MAE using green solvents were

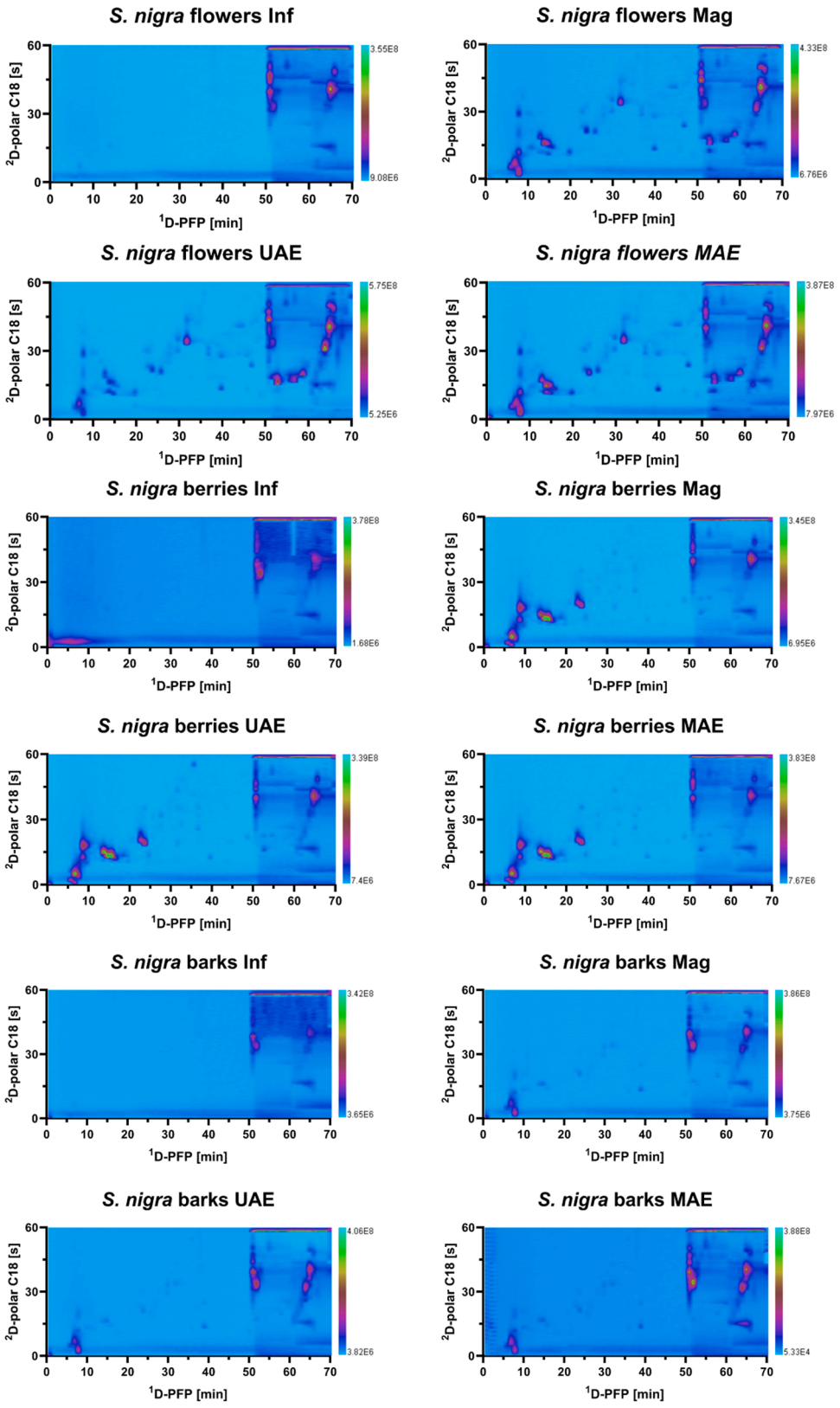

Fig. 4. PFP x polarC18-HRMS contour plots of flowers, berries, and barks of *S. nigra* extracted under optimized methods for infusion, magnetic-assisted, UAE, and MAE.

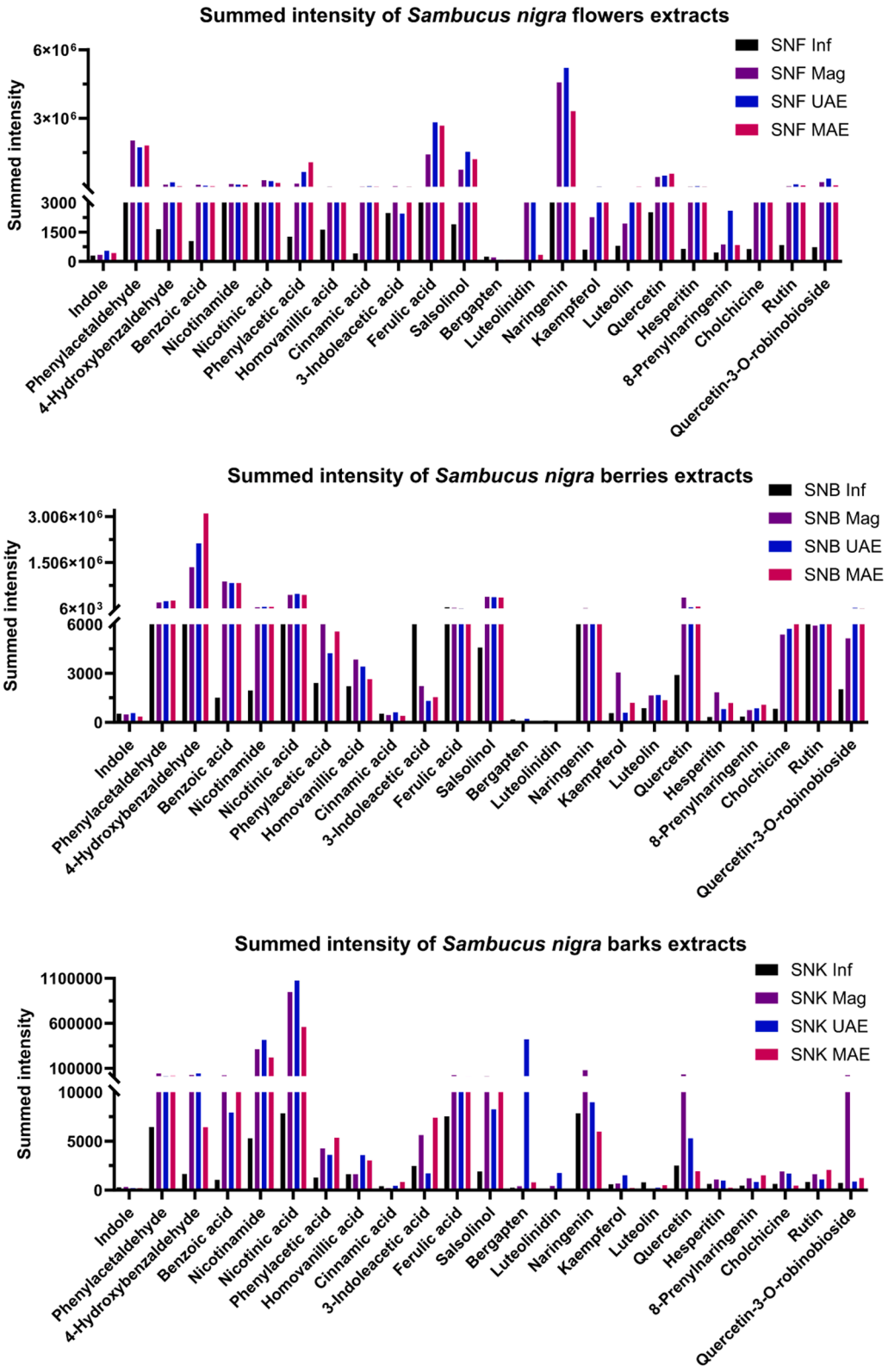

Fig. 5. Comparison of the intensities of 24 tentative candidates of flowers, berries, and barks of *S. nigra* extracted under optimized methods for infusion, magnetic-assisted, UAE, and MAE.

compared. The extraction solvent was one of the most important parameters during the optimization of the design of experiment and must be chosen for each plant part individually. The best extraction technique in terms of TPC, antioxidant activity and yield for berries and barks was MAE while for flowers magnetic-assisted extraction yielded higher but comparable results to other methods. Flowers were the part with the highest TPC value (150 mg GAE g^{-1} extract) followed by berries (97 mg GAE g^{-1} extract) and barks (68 mg GAE g^{-1} extract). In opposite to that, the antioxidant activity measured by miniaturized assays using ABTS and DPPH were the highest for berries reaching 20 and 3.0 mg TE g^{-1}

extract whereas 15 and 1.1 mg TE g^{-1} extract for flowers and 11 and 0.69 mg TE g^{-1} extract for barks were yielded for ABTS and DPPH respectively. Regarding the greenness of the methods, equivalent results of 0.71 or 0.72 were obtained for the tested methods except for magnetic-assisted extraction due to the least compatibility with higher sample throughputs. In terms of sample throughput and greenness, infusion and MAE are the methods of choice but for the total phenolic content and antioxidant activity, MAE extracts yielded higher values. Due to the extraction solvent, magnetic-assisted extraction, UAE, and MAE had similar chemical fingerprints obtained by LC × LC (PFP × polar C18) HRMS analysis. The use of LC × LC achieved greater separation power than conventional 1D-LC, improving the separation and identification of the compounds present in the sample and thus, enhancing the information about each sample. Regarding the recovery of compounds known for beneficial health effects, magnetic-assisted extraction, UAE and MAE could be used. To sum up, MAE proved to be the method of choice in terms of TPC, antioxidant activities, yield, greenness and based on the chemical fingerprint. Despite this, all four optimized methods outperformed previously reported ones in terms of time efficiency, sample and volume consumption and number of steps leading to more compatible methods for high throughput analysis while maintaining the high phenolic content and antioxidant activity of the plant extracts.

CRediT authorship contribution statement

Katharina Wetzel: Writing – original draft, Investigation. **Tatyana Tishakova:** Methodology, Investigation. **Marvin Häßler:** Investigation. **T. Niedenthal:** Conceptualization. **Juan F. Ayala-Cabrera:** Conceptualization, Writing – review & editing, Resources, Funding acquisition. **Lidia Montero:** Conceptualization, Writing – review & editing, Resources, Funding acquisition. **Oliver J. Schmitz:** Writing – review & editing, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

There is no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.greeac.2025.100233.

Data availability

Data will be made available on request.

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