Sustainable Chemistry and Pharmacy 41 (2024) 101722

Contents lists available at ScienceDirect

Sustainable Chemistry and Pharmacy

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

Improving phenolic compound extraction from *Arnica montana* flowers through multivariate optimization of heat and ultrasound-assisted methods

Paula Garcia-Oliveira, Franklin Chamorro, Jesus Simal-Gandara, Miguel A. Prieto, Lucía Cassani *

Universidade de Vigo, Nutrition and Bromatology Group, Department of Analytical Chemistry and Food Science, Instituto de Agroecoloxía e Alimentación (IAA) – CITEXVI, 36310, Vigo, Spain

ARTICLE INFO

Handling Editor: Vania Zuin

Keywords:
Bioactive compounds
Chemometric analysis
Optimization
Efficient processes

ABSTRACT

This study aimed to optimize phenolic compound extraction from *Arnica montana* (AM) L. flowers, comparing heat- and ultrasound-assisted extraction (HAE and UAE) through a multivariate approach. Critical parameters, including time, temperature or ultrasonic power, and ethanol concentration, were evaluated through a circumscribed central composite design. Unsupervised multivariate analysis of LC-MS/MS data identified key extraction conditions influencing the phenolic profile. Response surface methodology (RSM) determined optimal levels of enhancing yield and total phenolic content. Among the 24 identified phenolic compounds, dicaffeoylquinic acid was the most abundant. Ethanol concentration proved crucial in extracting specific phenolic compounds, supported by multivariate and RSM analyses. Optimal HAE conditions outperformed UAE, resulting in a 26% increase in phenolic compounds. Utilizing extraction cycles under these conditions, especially two cycles for HAE and three for UAE, surpassed traditional Soxhlet extraction, indicating potential industrial applications for AM flower extracts with improved efficiency and resource utilization compared to conventional methods.

Abbreviations

AM	*Arnica montana*
ANOVA	Analysis of variance
CCC	Circumscribed central composite
ESI	Electrospray ion
GRAS	Generally recognized as safe
HAE	Heat-assisted extraction
HCA	Hierarchical clustering analysis
HPLC	High-performance liquid chromatography
LC-MS/MS	Liquid chromatography coupled to mass spectrometry
PC	Principal component

(*continued on next page*)

* Corresponding author.
E-mail address: luciavictoria.cassani@uvigo.es (L. Cassani).

https://doi.org/10.1016/j.scp.2024.101722
Received 7 February 2024; Received in revised form 8 July 2024; Accepted 24 July 2024
Available online 1 August 2024

(*continued*)

PCA	Principal component analysis
RSM	Response surface methodology
SRM	Selected reaction monitoring
TPC	Total phenolic content
UAE	Ultrasound-assisted extraction

1. Introduction

Arnica montana (AM) L., is a perennial herbaceous plant belonging to the Asteraceae family and originates from the central mountains of Europe (Clauser et al., 2014). In the traditional medicine of this region, AM flowers have been used to produce tinctures and ointments for treating a variety of ailments, primarily those associated with inflammation, such as skin inflammation, bruises, sprains, or rheumatic pain (Garcia-Oliveira et al., 2021; Jaiswal and Kuhnert, 2011). Several studies have reported that different parts of the plant, mainly the flowers, possess anti-inflammatory, antioxidant, and antibacterial properties (Garcia-Oliveira et al., 2021). AM contains a wide range of bioactive compounds, including essential oils, terpenoids and polysaccharides, carotenoids, alkaloids, sesquiterpene lactones and phenolic compounds (Flórez-Fernández et al., 2021). Between these compounds, the most widely studied are the sesquiterpene lactones, in particular helenalin, known for its anti-inflammatory effects. Additionally, phenolic acids such as dicaffeoylquinic, chlorogenic and caffeic acids, along with flavonoids like quercetin and kaempferol derivatives are recognized for their antioxidant and antimicrobial activities (Gaspar et al., 2014; Oliveira et al., 2017; Röhrl et al., 2023). For example, the antioxidant activity of tinctures derived from some parts of AM (flowers, stem, and rhizome) was studied. Results revealed a strong correlation between the phenolic content of each part and *in vitro* antioxidant activity (Gawlik-Dziki et al., 2011). Likewise, when comparing AM extracts rich in phenolic compounds and polysaccharides, the former showed significantly higher *in vitro* antioxidant activity and effectively reduced the oxidative stress induced by H_2O_2 in mouse fibroblast cells (Gaspar et al., 2014). The antioxidant and anti-inflammatory properties of AM were further confirmed *in vivo*. For instance, in a collagen-induced arthritis rat model, oral administration of a methanolic extract significantly alleviated oxidative stress and inflammation in the animals, attributed to the high content of phenolic acids and flavonoids in the extract (Sharma et al., 2016). Nowadays AM is primarily distributed in a dehydrated state for infusion and integration into topical products. Considering its bioactive potential, the value of this species could be enhanced by extracting valuable phenolic compounds, which could find applications in the fields of feed, food, cosmetics, and pharmaceuticals (Garcia-Oliveira et al., 2021).

In the food industry sector, phenolic-rich extracts find diverse applications as food ingredients, additives and preservatives (Albuquerque et al., 2021). The efficient extraction of these compounds from the matrix is the critical initial step for their development. Several factors impact phenolic compounds extraction, including sample pre-treatment, solid/liquid ratio, solvent, time and extraction technique (Gil-Martín et al., 2022). Evaluating these factors is crucial to determining the optimal conditions for compound recovery (Domínguez et al., 2020; Leichtweis et al., 2023). Polar solvents like alcohols, organic solvents, or water-alcohol mixtures are commonly used for phenolic compound extraction (Gil-Martín et al., 2022). Methanol is usually replaced by water-ethanol mixtures in eco-friendly applications (Backes et al., 2018; Jovanović et al., 2017; Leichtweis et al., 2023). Traditional methods (such as maceration, percolation or Soxhlet) are being replaced by more eco-friendly, energy-efficient techniques like ultrasound-assisted extraction (UAE) (Gajic et al., 2019). This technology employs ultrasound waves to facilitate cell content release, requiring less time and solvent (Benarfa et al., 2020; Shehata et al., 2021). However, there is a need for further exploration into the scalability and economic viability of UAE, a crucial prerequisite for its potential integration with conventional industrial extraction methods, such as heat-assisted extraction (HAE). HAE employs increased temperatures to facilitate the migration of bioactive compounds into the solvent. This method is straightforward to implement, cost-effective, and readily adaptable for industrial use (Backes et al., 2018). Nevertheless, the drawback is that the application of high temperatures can result in the degradation of the desired compounds and encourage the extraction of unwanted substances. The optimization of HAE conditions for phenolic component extraction from AM was carried out in this study, owing to its simple adaptability for industrial applications. However, given the growing interest in scaling up the environmentally friendly UAE approach, optimization of UAE conditions was also done to compare with HAE results, thereby contributing to the advancement of environmentally responsible technology.

To the best of our knowledge, there are few studies optimizing the extraction of bioactive compounds from AM (Žitek et al., 2022). To address this gap, the objectives of our research were as follows: 1) characterize the phenolic composition of dried flowers from AM using liquid chromatography coupled to mass spectrometry (LC-MS/MS); 2) employ a multivariate strategy that combines unsupervised analysis and the phenolic profile obtained through LC-MS/MS to unravel the impact of critical extraction parameters for both HAE and UAE (including time, temperature/power, and solvent concentration) on the extraction of phenolic compounds from AM; 3) use response surface methodology (RSM) to further study possible interactions between the critical extraction parameters and to optimize the extraction conditions for HAE and UAE maximizing both yield and total phenolic content (TPC) simultaneously; 4) evaluate the effect of including additional extraction cycles under optimal conditions to enhance yield and TPC and 5) compare the results obtained under the optimal conditions for HAE and UAE with those obtained using the Soxhlet method as a reference. This proposed protocol is expected to significantly increase the value of an underutilized plant source. Offering two optimized and efficient extraction methods creates opportunities for potential industrial use.

2. Material and methods

2.1. Reagents

Ethanol, hydrochloric acid, formic acid, and high-performance liquid chromatography (HPLC) grade acetonitrile were acquired from Carlo Erba Reagents (Sabadell, Spain). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). Standards (ferulic acid, gallic acid, luteolin, quercetin and kaempferol) were purchased from Merck (Madrid, Spain).

2.2. Plant material

Dried flowers of arnica (*Arnica montana* L., AM) containing a 7% moisture content, were obtained from "Pinisan" (Madrid, Spain; www.pinisan.com) in November 2022. Flowers were finely grounded to obtain a homogeneous matrix and were then vacuum sealed for preservation at −80 °C for subsequent extraction assays.

2.3. Determination of phenolic profile of A. montana using LC-MS/MS

First, an initial literature search was conducted to determine phenolic compounds previously found in AM flowers (Barral-Martinez et al., 2021; de Athayde et al., 2021; Flórez-Fernández et al., 2021), which enabled the creation of a compound database. This database was then used to identify the compounds present in an AM extract obtained through a conventional extraction procedure, which involved mixing 2 g of sample with 40 mL of 80:20 (v/v) ethanol:water solution. This mixture was continuously stirred (LBX instruments, Barcelona, Spain) for 1 h at room temperature. Then, the extract was centrifuged (Labprocess, Barcelona, Spain) at 6000 rpm for 15 min, and the resulting liquid fraction was filtered through 0.22 μm syringe filters (Waters, Camden, USA) before being transferred to sample vials for further analysis. LC-MS/MS analysis of the phenolic profile was carried out in a Dionex Ultimate 3000 UPLC+ (Thermo Scientific, USA) system coupled to a triple quadrupole mass spectrometer (TSQ Quantis, Thermo Scientific, USA). Analytical separation was carried out with a Waters Spherisorb S3 ODS-2C18 (3 μm, 4.6 mm × 150 mm, Waters, USA) column kept at 35 °C. Mobile phases were (A) mili-Q water acidified with 0.1% of formic acid and (B) acetonitrile. The elution gradient employed was 15% B (5 min), 15–20% B (10 min), 20–25% B (10 min), 25–35% B (10 min), 35–50% B (10 min), and re-equilibration of the column, with a constant flow rate of 0.5 mL/min. The injection volume was 10 μL. Mass detection was performed using a TSQ Quantis, equipped with an electrospray ion (ESI) source, working in negative mode. The phenolic profile of the AM flower extract was analyzed in selected reaction monitoring (SRM) scan mode. This scanning mode aids in identifying and quantifying compounds in the sample by monitoring the fragmentation of selected precursor ions into product ions. The following parameters were used as universal conditions: sheath gas 30 Arb; auxiliary gas 10 Arb, ion transfer tube temperature 325 °C and vaporizer temperature: 350 °C. Retention time, precursor/product ion combination, collision energy and RF lens voltage were optimized for each identified compound. Data acquisition and LC-MS/MS analysis interpretation were conducted using FreeStyle software (1.8 SP1, ThermoFinnigan, San Jose, CA, USA). After characterization, the compounds present in the AM extract were systematically categorized into phenolic classes and subclasses.

2.4. Extraction techniques

2.4.1. Heat-assisted extraction (HAE)

AM flowers (2 g) were placed into amber glass bottles and combined with 40 mL of acidified solvent (pH ~2.3), resulting in a solid/liquid ratio of 50 g/L. Subsequently, these bottles, equipped with a magnetic stirrer, were placed within a thermostatic water bath (Raypa, Barcelona, Spain) set to provide orbital agitation (1400 rpm). Detailed conditions for critical HAE parameters (namely, time, temperature, and solvent concentration) in the 28 experimental runs obtained by the circumscribed central composite (CCC) design are shown in Table S1. The chosen ranges were time (t, 5–60 min), temperature (T, 30–90 °C) and solvent concentration (S, 0–100% ethanol). After extraction, the resulting mixtures were centrifugated (6000 rpm, 15 min), and the supernatants were collected and subjected to filtration through nylon syringe filters (0.22 μm). The filtrated samples were stored at −80 °C until further LC-MS/MS analysis.

2.4.2. Ultrasound-assisted extraction (UAE)

UAE was carried out in an ultrasonic system (Optic Ivymen System sonicator, model CY-500, Spain) operating at 20 kHz and equipped with a titanium probe. In this case, AM flowers (2 g) were placed in a graduated cylindrical tube with 40 mL of acidified solvent. The temperature sensor was also placed in the reaction tube. This system was then immersed in a cold-water bath to avoid overheating and keep this parameter constant at 20 °C. Experimental runs were carried out according to the CCC design matrix (Table S1), which combined different levels of time (t, 5–45 min), power (P, 150–400 W), and solvent concentration (S, 0–100% ethanol). Once extracted, the resulting mixtures were centrifugated, filtrated, and stored, following the same procedure as previously described for HAE samples.

2.4.3. Quantification of phenolic compounds from AM extracts

The phenolic compounds present in the AM extracts obtained from HAE and UAE were quantified using calibration curves established from pure analytical standards of a representative compound. The quantified results were expressed as equivalent amounts of the representative compound per gram of dry sample weight (mg/g dw). Specifically, hydroxycinnamic acids content was expressed as ferulic acid equivalents; hydroxybenzoic acids content was expressed as gallic acid equivalents; flavone and flavanones content

were expressed as luteolin equivalents; flavonol content was expressed as quercetin equivalents, except for kaempferol derivatives, which were expressed as kaempferol equivalents.

2.5. Multivariate data analysis

In a preliminary study, unsupervised multivariate analysis, comprising Hierarchical Clustering Analysis (HCA) and Principal Component Analysis (PCA), was conducted to investigate the similarities and differences among the phenolic compounds extracted from AM using HAE and UAE across the 28 experimental runs established within the CCC design (section 2.4). The chemometric analyses were performed on data obtained in subsection 2.4.3 using SPSS Software (IBM, SPSS Statistics for Windows, Version 25.0. Armonk, New York, USA). Ward's clustering method and squared Euclidean distance was employed for the creation of dendrograms.

2.6. Optimization of HAE and UAE procedures through RSM

2.6.1. Experimental design

A second study using RSM with a CCC design was performed to analyze the combined effect of the most critical extraction conditions of HAE and UAE on the extraction yield, and TPC of AM flowers. CCC design consisted of a 5-levels–3-factors with six replicates at the central point, in which each variable was tested at five different coded levels, resulting in 28 experiments. Table S1 shows the experimental design matrix in the coded and actual levels of the independent variables for both HAE and UAE techniques.

2.6.2. Response variables

Three dependent variables were chosen in this study.

- Extraction yield (referred to as Y_1), which was gravimetrically determined according to the methodology explained by Cassani et al. (2022) and represented as g extract/100 g of dry weight,
- TPC, which was calculated as the cumulative sum of the individual quantified phenolic compounds, as detailed in subsection 2.4.3. TPC was expressed in two formats: Y_2 (mg/g dry weight) and Y_3 (mg/g dry extract), which represented the overall concentration of phenolic compounds in the dried flowers of AM and in the resulting dry extract, respectively.

2.6.3. Mathematical modelling

The measured response variables were fitted to second- and/or third-order mathematical models (**Eq. (1)**) according to the least-squares regression method.

$$Y_n = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=2}^{n} \beta_{iij} X_i^2 X_j + \sum_{i=1}^{n} \beta_{iii} X_i^3 \quad \text{Eq. 1}$$

where Y_n is the predicted response variable, X_i is the dimensionless coded value of the independent variable, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ij} is the coefficient for the interaction effect, β_{ii} the quadratic coefficient, β_{iii} the cubic coefficient, and n is the number of variables considered in the analysis.

2.6.4. Simultaneous optimization and validation

Considering that the optimization procedure comprises three response variables, finding a global solution can be difficult, because improving one response may have an undesirable effect on others. As a result, finding a compromise solution involving an optimal region that provides a certain amount of concordance with the goal of the individual response variables is critical. The "desirability function" was used in this case since it is one of the most extensively used ways for optimizing several responses at the same time (Cassani et al., 2018). Regarding the Desirability criteria, all factors were optimized with a relative importance of three. TPC (expressed in two formats) was maximized with the highest relative importance of five. On the other hand, yield was maximized with a relative importance of three, given that yield represents the dry extract weight from each extraction, which may include other compounds apart from phenolic compounds.

Following that, a new series of experiments was carried out under optimal extraction conditions to assess the reliability of the simultaneous optimization. In this regard, AM samples were extracted under optimal operational parameters determined for HAE and UAE, and response variables were assessed to compare predicted and experimental results.

2.7. Enhancing extraction efficiency through multiple cycles under optimal conditions

Once the optimal extraction conditions for HAE and UAE were determined, a subsequent series of experiments was conducted to evaluate the influence of three sequential extraction cycles on the enhancement of phenolic compound extraction from AM flowers. To achieve this objective, a new batch of dried AM flowers was subjected to the HAE and UAE procedures under the optimal operational parameters, following the methodology stated in subsections 2.4.1 and 2.4.2, respectively. The supernatants obtained from this initial extraction were named "cycle 1".

Simultaneously, another batch of AM flowers was subjected to the same HAE and UAE procedures under optimal conditions, and the residue obtained after centrifugation was then mixed with 40 mL of solvent. This mixture was then extracted using the same methods as previously stated. The resulting supernatants from this second extraction step were mixed with those obtained in cycle 1, referred to as "cycle 2". In addition, a third batch of AM flowers was processed using the same approach, and the remaining solid ma-

terial after centrifugation was combined with 40 mL of solvent. The same extraction process as in previous cycles was applied to this mixture. The resulting supernatants were mixed with those from cycles 1 and 2, and this mixture was termed "cycle 3". Extraction yield and TPC were assessed in all samples using the methodology described in subsections 2.6.2. All experiments were performed in triplicate in three independent experimental runs.

2.8. Soxhlet extraction as the reference method

Soxhlet extraction technique was chosen as the reference method to evaluate the extraction system's efficiency in contrast to the performance observed with the optimized HAE and UAE procedures. In brief, 3 g of dried AM flowers were extracted with 120 mL of ethanol at 78.4 °C for 3.5 h. This extraction was carried out with an automatic Soxhlet Büchi Extraction System B-811 (Büchi Labortechnik AG, Flawil, Switzerland) in its standard configuration. Extraction yield and TPC were assessed in the AM extracts obtained through Soxhlet using the methodology described in subsection 2.6.2.

2.9. Statistical analysis

Stat-Ease Design-Expert 11.0 software (Stat-Ease, Inc., Minneapolis, USA) was employed to perform RSM analysis with CCC design, simultaneous optimization using Desirability function, and the creation of figures. The coefficients of the developed mathematical models were calculated using backward multiple regression. To statistically analyze the regression models, an analysis of variance (ANOVA) test was conducted, considering a significance level of p-value <0.05 and an insignificant lack of fit ($p > 0.05$). Only those factors exhibiting a p-value <0.05 were retained in the models, as these demonstrated a significant impact on the responses. The adjusted coefficient of determination (R^2_{adj}) was computed for each model, representing the proportion of variance explained by the respective models. Subsequently, these models were validated through ANOVA tests ($p < 0.05$).

Data from analysis of multiple extraction cycles under optimal conditions were analyzed using R, software version 2.12 (R Development Core Team, 2011). ANOVA was performed and the Tukey-Kramer comparison test was used to estimate significant differences between cycles ($p < 0.05$). Experiments were carried out in triplicate.

3. Results and discussion

3.1. Phenolic profile of AM extracts

3.1.1. Characterization of AM phenolic compounds using conventional extraction method

Table 1 provides the list of the compounds tentatively identified in the AM extract obtained through conventional extraction, while Fig. S1 shows the corresponding SRM chromatogram. Twenty-four phenolic compounds were identified in the AM flower extract, predominantly belonging to the flavonol and hydroxycinnamic acid families. Within hydroxycinnamic acids, eight compounds were found, mostly compounds derived from quinic acid. Peak 1 was recognized as caffeic acid (C1), due to its mass (*m/z* 179) and characteristic fragments at *m/z* 161 and 135. Peak 2 was designated as quinic acid (C2), based on the [M − H] ion at *m/z* 192 and the product ion at *m/z* 86 (Clifford et al., 2003, 2005). This compound is one of the most distinctive hydroxycinnamic acids identified in the extract. It has been described that this bioactive presents diverse *in vitro* and *in vivo* biological activities, including antioxidant, cytotoxic, and antimicrobial, among others (Benali et al., 2022). Peak 4 was attributed to a caffeoylquinic acid (C4, *m/z* 353), with two notable product ions: the most intense at *m/z* 191, corresponding to the [quinic acid-H] ion, and the second at *m/z* 179 (Clifford et al., 2003, 2005). Previous studies have confirmed the presence of diverse caffeoylquinic acid isomers in AM samples, 5-caffeoylquinic acid (chlorogenic acid) being the most commonly reported (de Athayde et al., 2021; Jaiswal and Kuhnert, 2011; L. Z. Lin and Harnly, 2008). Caffeoylquinic acids, and particularly chlorogenic acid, have been widely investigated from *in vitro* to clinical studies due to their bioactive properties. These compounds stand out by their antioxidant activity, and they also exhibit other effects such as anti-inflammatory, anti-inflammatory, antitumoral, antimicrobial, cardioprotective or anti-diabetic (Alcázar Magaña et al., 2021; W. Liu et al., 2020). Peak 7 was ascribed to feruloylquinic acid (C7), due to its mass *m/z* 367, which produced product ions at *m/z* 191 and 134 (C. Liu et al., 2022). Previous studies have also identified 5-feruloylquinic acid in AM flowers (Jaiswal and Kuhnert, 2011; L. Z. Lin and Harnly, 2008). Peak 8 displayed an [M − H] ion at *m/z* 163, accompanied by a prominent product ion at *m/z* 119, which can be attributed to the loss of CO_2. Thus, this compound was recognized as *p*-coumaric acid (C8) (de Athayde et al., 2021). Peak 12, displayed a precursor with a mass of *m/z* 515, releasing a characteristic ion at *m/z* 353 due to the loss of caffeoyl moieties. As a result, it was suggested to be a dicaffeoylquinic acid (C13) (C. Liu et al., 2022). Notably, this peak stands out as the most distinctive feature in the SRM chromatogram (Fig. S1). Numerous studies have reported the beneficial effects of dicaffeoylquinic acid isomers, especially their antioxidant, anti-inflammatory and antimicrobial activities, both *in vitro* and *in vivo* (Chen et al., 2016; Deng et al., 2013; Zhou et al., 2020). Peak 14 was confirmed as ferulic acid (C16) by comparing its retention time and production ions with those observed for a commercial standard. Lastly, peak 20 was identified as sinapoylquinic acid (C23) based on its chemical formula, the [M − H] ion at *m/z* 397, and the product ion at *m/z* 191, which is indicative of quinic acid derivates (L. Z. Lin and Harnly, 2008).

Regarding flavonols, there was a remarkable diversity, with the detection of eleven distinct compounds. Peak 5 exhibited a mass of *m/z* 317 along with two prominent product ions at *m/z* 151 and 179, leading to the identification of myricetin (C5) (Y. Lin et al., 2012). Peak 9 featured two compounds with [M − H] ions at *m/z* 609 and 610, both exhibiting a similar fragmentation pattern with product ions at *m/z* 300, attributed to the quercetin aglycone, and 271, suggesting the tentative identification of quercetin-*O*-rutinoside (C9) and quercetin-(*p*-coumaroyl)-glucopyranoside (C10), respectively (Gulsunoglu et al., 2020). Peak 10, with a mass of *m/z* 477, showed an intense signal at *m/z* 301, which was attributed to quercetin-*O*-glucuronide (C11). This compound has not been

Table 1
Tentative identification of phenolic compounds of *A. montana,* chromatographic and mass spectroscopy data.

	Compound	Group	Chemical formula	RT (min)	**M-H (m/z)**	Transition[1] (Collision energy[2])	RF Lens (V)
C1	Caffeic acid	Hydroxycinnamic acids	$C_9 H_8 O_4$	3.3	179.034	161 (6), 58 (16)	61
C2	Quinic acid	Hydroxycinnamic acids	$C_7 H_{12} O_6$	3.7	191.055	110 (12), 86 (16)	76
C3[a]	Gallic acid	Hydroxybenzoic acids	$C_7 H_6 O_5$	5	169.013	125 (15), 79 (24)	121
C4	Caffeoylquinic acid	Hydroxycinnamic acids	$C_{16} H_{18} O_9$	6	353.087	191 (16), 178 (17)	108
C5[a]	Myricetin	Flavonols	$C_{15} H_{10} O_8$	13.8	317.029	179 (19), 151 (24)	161
C6[a]	Eriodictyol-*O*-glucuronide	Flavanones	$C_{21} H_{20} O_{12}$	14.7	463.087	300 (27), 287 (44)	198
C7	Feruloylquinic acid	Hydroxycinnamic acids	$C_{17} H_{20} O_9$	15	367.102	191 (15), 134 (33)	107
C8	*p*-Coumaric acid	Hydroxycinnamic acids	$C_9 H_8 O_3$	17.4	163.039	119 (14), 93 (31)	77
C9	Quercetin-*O*-rutinoside	Flavonols	$C_{27} H_{30} O_{16}$	17.8	609.145	300 (37), 271 (59)	260
C10	Quercetin (*p*-coumaroyl) glucopyranoside	Flavonols	$C_{30} H_{27} O_{14}$	17.8	610.132	300 (37), 271 (57)	260
C11	Quercetin-*O*-glucuronide	Flavonols	$C_{21} H_{18} O_{13}$	18.4	477.066	301 (20), 150 (37)	153
C12	Quercetin-*O*-glucoside	Flavonols	$C_{21} H_{20} O_{12}$	18.5	4623.079	300 (27), 271 (43)	119
C13	Dicaffeoylquinic acid	Hydroxycinnamic acids	$C_{25} H_{24} O_{12}$	20	515.118	353 (15), 191 (30)	150
C14[a]	Quercetin-*O*-rhamnoside	Flavonols	$C_{21} H_{20} O_{11}$	20.4	447.092	255 (39), 227 (45)	133
C15	Kaempferol-*O*-glucoside	Flavonols	$C_{21} H_{20} O_{11}$	20.4	447.092	284 (27), 255 (39)	132
C16[a]	Ferulic acid	Hydroxycinnamic acids	$C_{10} H_{10} O_4$	22	193.052	178 (13), 134 (16)	83
C17[a]	Kaempferol-*O*-glucuronide	Flavonols	$C_{21} H_{18} O_{12}$	24.5	461.072	285 (23), 195 (12)	110
C18	Quercetin	Flavonols	$C_{15} H_{10} O_7$	26.5	301.034	178 (19), 151 (22)	190
C19	Luteolin	Flavones	$C_{15} H_{10} O_6$	26.6	285.039	151 (26), 133 (34)	173
C20[a]	Kaempferol	Flavonols	$C_{15} H_{10} O_6$	26.6	285.039	239 (27), 187 (29)	162
C21	Isorhametin	Flavonols	$C_{16} H_{12} O_7$	27.5	315.051	300 (19), 271 (30)	132
C22[a]	Apigenin	Flavones	$C_{15} H_{10} O_5$	30.7	269.045	151 (25), 117 (35)	135
C23	Sinapoylquinic acid	Hydroxycinnamic acids	$C_{18} H_{22} O_{10}$	33	397.113	300 (25), 271 (41)	142
C24	Hispidulin	Flavones	$C_{16} H_{12} O_6$	35.4	299.055	284 (20), 178 (27)	173

[a] The indicated compounds were identified but not quantified in any of the samples, so they are considered traces. The sum of these compounds does not exceed 1% of the total compounds. [1]m/z, [2]Voltage.

as investigated as quercetin and other of its derivatives, but there is scientific evidence that demonstrates its antioxidant, anti-inflammatory and neuroprotective properties (Lesjak et al., 2018; Nishikawa et al., 2022; Suganthy et al., 2016). Peak 11 with a mass of *m/z* 463 was assigned as quercetin-*O*-glucoside (C12), based on the most intense product ions *m/z* 300, and 271 (Gulsunoglu et al., 2020). Both of these compounds have been previously reported as two of the most prevalent flavonols in AM samples (Flórez-Fernández et al., 2021; Ganzera et al., 2008; Kimel et al., 2019). In peak 13, two compounds with similar masses (*m/z* 447) co-eluted, but they exhibited different fragmentation patterns. The first one produced fragments at *m/z* 271 and 255, leading to its identification as quercetin-*O*-rhamnoside (C14) (Gulsunoglu et al., 2020), while the second one was identified as kaempferol-*O*-glucoside (C15), another relevant flavonol identified in AM samples (Pljevljakušić et al., 2014). This identification was based on the strong product ion at *m/z* 285 corresponding to the kaempferol aglycone (Clauser et al., 2014; Enomoto, 2020). Peak 15 was attributed to kaempferol-*O*-glucuronide (C17, *m/z* 461), with a significant product ion at *m/z* 285 due to the loss of a hexose moiety (162 Da) (Clauser et al., 2014; Enomoto, 2020). Peaks 16 and 17 were identified as quercetin (C18), and kaempferol (C20), based on the results obtained from commercial standards. Lastly, peak 18, with a mass of *m/z* 315, was designated as isorhamnetin (C21), considering the characteristic product ions *m/z* 300 and 271 (Li et al., 2016; Paşayeva et al., 2021). This compound has been previously reported in AM tincture (Kimel et al., 2019). Isorhamnetin has been reported to exert a miscellaneous of bioactivities, investigated both *in vitro* and *in vivo*, including antioxidant, anti-proliferative and anti-inflammation (Jaramillo et al., 2010; Lesjak et al., 2018; Tsai et al., 2019). Other reported properties include cardioprotective, neuroprotective, anti-obesity and immunomodulation (Gong et al., 2020).

Concerning flavones, three compounds were detected. Luteolin (C19) was found to co-elute with kaempferol in peak 17. Peak 19 was tentatively identified as apigenin (C22) based on its mass (*m/z* 269) and the observed product ions at *m/z* 151 and 117 (Gai et al., 2021; Han et al., 2019). Peak 21, which had a mass of *m/z* 299, was considered to be hispidulin (C24) due to the presence of the product ion at *m/z* 284 (Clauser et al., 2014).

For the remaining compounds, gallic acid (C3) was confidently identified in peak 3, and this identification was verified by comparing its mass fragmentation and retention time with a commercially available standard. In the case of peak 6, an [M − H] ion at *m/z* 463 and a strong peak at *m/z* 287 were observed, tentatively suggesting it as eriodictyol-*O*-glucuronide (C6).

3.1.2. Quantification of phenolic compounds in AM extracts: A comparison of HAE and UAE methods

After characterizing AM using conventional extraction, the database of twenty-four identified phenolic compounds was used to analyze their composition in 28 experimental runs conducted with both HAE and UAE methods. In the HAE experiments, 16 out of the 24 compounds were quantified across these runs, encompassing flavones, flavonols, and phenolic acids, as detailed in Table S2. Major compounds were phenolic acids, ranging from 18.38 to 73.86 mg/g dw, followed by flavonols, varying from 0.32 to 3.87 mg/g dw (Table S2). These results are consistent with findings from previous authors, reporting that total phenolic acids ranged between 12.1 and 22.4 mg/g dw, while total flavonoids vary between 11.7 and 15.2 mg/g dw (Clauser et al., 2014; Ganzera et al., 2008). Among phenolic acids, di-caffeoylquinic acid, quinic acid, caffeoylquinic acid, and ferulic acid stood out. Particularly di-caffeoylquinic acid had levels ranging from 8.75 to 50.51 mg/g dw (Table S2). Regarding the flavone composition, isorhamnetin and quercetin-*O*-glucuronide were the most prominent.

In the case of UAE, 12 out of the 24 compounds were quantified in the 28 experimental runs, and their respective quantities are displayed in Table S3. These phenolics extracted belong to phenolic acids, with levels ranging from 8.68 to 49.29 mg/g dw, and flavonols, with quantities varying from 0.31 to 11.06 mg/g dw. Similarly, the major compounds were phenolic acids, with di-caffeoylquinic acid being again the most prominent. The content of this compound varied between 6.01 and 32.13 mg/g dw (Table S3). Previous studies have shown that di-caffeoylquinic acid ranged from 5.1 to 11.3 mg/g dw in methanolic-water extracts subjected to ultrasonic waves at room temperature (Table S3) (Clauser et al., 2014; Ganzera et al., 2008). The observed differences in these values could be due to the inherent variability of the plant matrices and variations in the extraction conditions, such as the choice of the solvent, time, temperature and other factors. When comparing the UAE experiments carried out in short times, the values are similar to those previously reported.

The variation in the quantified compounds between HAE and UAE techniques can be attributed to the use of different energy sources. Specifically, HAE relies on temperature, while UAE utilizes acoustic cavitation controlled at room temperature. Although both techniques have proven to promote the extraction of phenolic compounds, in this particular case, acoustic cavitation alone may not be as effective as temperature. In fact, it is common to apply ultrasound in combination with moderate temperatures, because temperature reduces the surface tension and viscosity of the solvent. This reduction enhances diffusion and, as a result, increases the rate of mass transfer and extraction efficiency (Jahromi, 2019; Osorio-Tobón, 2020; Thilakarathna et al., 2023). Additionally, previous studies have noted differences in the phenolic profile of extracts obtained through UAE when compared to other extraction techniques (Leichtweis et al., 2023; Rocchetti et al., 2019).

Overall, AM extracts are a valuable source of phenolic compounds whose content is affected by the extraction conditions established in the CCC design for both HAE and UAE techniques. Due to the extensive analytical data collected, it was proposed to utilize multivariate analysis to identify key extraction parameters in discriminating among compounds.

3.2. Unsupervised analysis of phenolic compounds in AM extracts obtained through HAE and UAE

A preliminary study using unsupervised analysis was conducted to reveal patterns that facilitate the distinction of phenolic compounds within the experimental setups (time, temperature/power, ethanol concentration) established by the 28 experiments. The input data used in HCA and PCA is presented in Table S2 and Table S3 for HAE and UAE, respectively. Initially, HCA was employed to identify patterns within the LC-MS/MS data, resulting in the formation of compound clusters that facilitate the assessment of similarities and differences among compounds. Then, to delve deeper into the relationship between individual compounds and extraction conditions, an unsupervised PCA was performed. The results of multivariate analysis are illustrated in Fig. 1.

Regarding the HAE dendrogram (Fig. 1A), the sixteen quantified compounds displayed a separation into two major clusters, labeled I and II. Notably, compounds C21, C23, C19, C24, C18, C10, C15, and C12 were grouped separately from the remaining compounds. Moreover, each of these clusters also exhibited subgroups. Compounds within the same subgroup share common characteristics, but these characteristics differ from those of compounds grouped in other subgroups. For instance, compounds C21, C23, C19, and C24, which belong to cluster I, exhibited similar features. In contrast, their characteristics were distinct from those of C9 and C13, which were grouped in cluster II. Interestingly, the compounds' clustering did not align with their phenolic categories. For instance, C21, C23, C19, and C24 do not share the same category as indicated in Table 1. Thus, it is interesting to find the factors that drove the grouping of these compounds.

When examining the UAE dendrogram, a pattern similar to that observed in HAE emerged, with two major clusters, denoted as I and II. In this instance, the compounds found in cluster I are primarily hydroxycinnamic acids, except C9, while the compounds in cluster II are categorized as flavonols. Therefore, unlike the HAE dendrogram results, the primary classification of compounds in the UAE case was based on their categories. HCA enabled the identification of two additional subgroups in which these compounds were further grouped. Thus, it is also interesting to find the factors that drove the last grouping of compounds. This highlights the intricate relationships between the extraction variables, which play a role in the recovery of these compounds.

Regarding PCA analysis, three PC were selected for HAE, based on the Kaiser criterion (Granato et al., 2018) (eigenvalues higher than 1), which collectively explained 84.83% of the total variance. Likewise, for UAE, three PC were considered which together accounted for 84.45% of the total variance (Table S4).

Based on the analysis of factor loadings in HAE technique, PC1 accounted for most of the variability, explaining over 49% of the total variation. PC1 effectively separated compounds with negative PC1 values (C2 and C4) from the other compounds that had high positive values on this component (C2, C15, C19, C21, C23 and C24), as shown in Table S4 and Fig. 1B. From the PCA results (Fig. 1B), it could be observed that compounds C19, C21, C23, and C24 consistently cluster together in both the HCA dendrogram and PCA score plot. Regarding loading plot, these compounds were located near experiments 22, 14, and 18. Notably, these experiments all share a common feature: the use of 100% ethanol as extraction solvent (Table S1). This suggests that this specific solvent condition

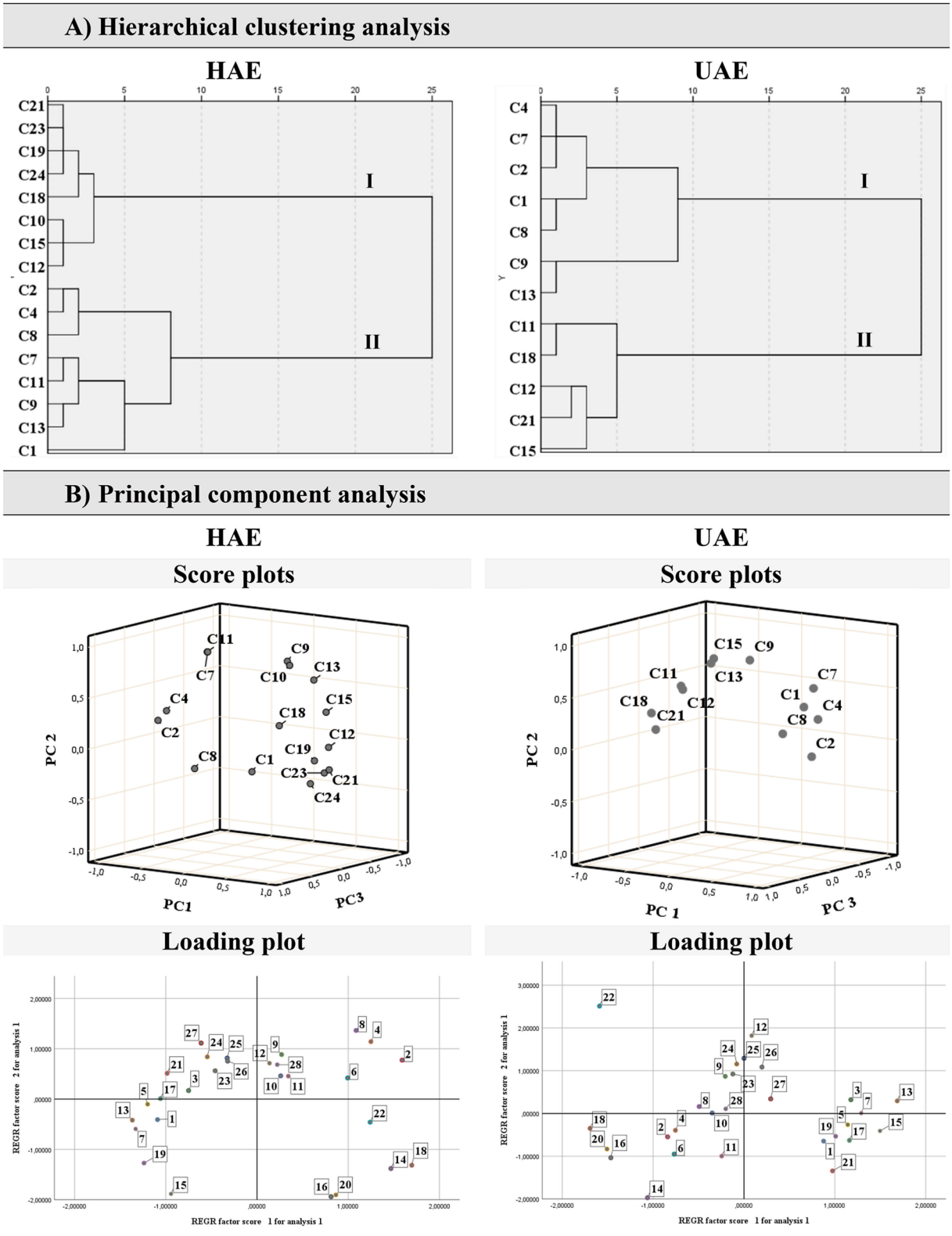


Fig. 1. Unsupervised multivariate analysis of phenolic compound profiles in AM extracts obtained using HAE and UAE: (A) HCA dendrogram using Ward's method and squared Euclidean distance, and (B) PCA score and loading plots.

was favorable for the extraction of these compounds. Conversely, compounds C2 and C4 were located near runs 21 and 17, characterized by the use of 0% ethanol (Fig. 1B). In the loading plots of PCA, the distribution of the 28 experimental points is shown in the space defined by PC1 and PC2. This allows for the grouping to be associated with specific extraction conditions and also links these conditions with the extraction of individual compounds. Overall, these results indicated that a solvent-based pattern may be crucial in distinguishing between different phenolic compounds found in AM flowers. PC2 explained another 24% of data variability and it positioned compounds with loading factors exceeding 0.7 (specifically, C7, C9, C10, and C11) near experiments 24, 25, 26, and 28, which were associated with intermediate levels of time, temperature, and solvent. In contrast, PC3 explained a minimal amount of variability and had little to no distinguishing impact on the compounds.

Regarding UAE, PC1 explained 45% of the data's variability and separated compounds with PC1 values greater than 0.7 (namely, C1, C2, C4, C7, and C8) from those with negative PC1 values (specifically, C12 and C21), as shown in Table S4 and Fig. 1B. These findings were consistent with what was previously observed in the HCA dendrogram, highlighting the effective separation of compounds by PCA based on their respective categories. According to the loading plot for UAE (Fig. 1B), the extraction of C1, C2, C4, C7, and C8 was more favorable when runs 3, 5, and 7 were employed, demonstrating a preference for lower ethanol levels in the extraction of hydroxycinnamic acids (Table S1). C12 and C21, both flavonols, on the other hand, were found towards run 8, which is related to increased ethanol levels.

While PCA successfully indicated that ethanol concentration plays a crucial role in extracting specific phenolic compounds, it is necessary to conduct a more detailed investigation into how different extraction conditions interact and impact the extraction of bioactive compounds. To address this gap, we applied RSM, and the results are detailed in the following section.

3.3. Optimizing the HAE and UAE conditions by RSM

3.3.1. Model fitting

Table 2 displays the average values for the response variables under investigation, including extraction yield (Y_1) and TPC expressed in two different units (Y_2 in mg/g of dry weight and Y_3 in mg/g of dry extract). These values correspond to each of the 28

Table 2
Results obtained for Y_1 (yield in g extract/100 g dw), Y_2 (total polyphenolic content in mg TP/g dw), and Y_3 (total polyphenolic content in mg TP/g extract) using both heat-assisted extraction (HAE) and ultrasound-assisted extraction (UAE) techniques. The experiments were conducted under 28 experimental conditions defined in the circumscribed central composite design using coded values.

EXPERIMENTAL RESPONSES									
	FACTORS			HEAT-ASSISTED EXTRACTION			ULTRASOUND-ASSISTED EXTRACTION		
Run	X_1: Time	X_2: Temperature/Power	X_3: Ethanol concentration	Y_1 (g extract/100 g dw)	Y_2 (mg TP/g dw)	Y_3 (mg TP/g extract)	Y_1 (g extract/100 g dw)	Y_2 (mg TP/g dw)	Y_3 (mg TP/g extract)
1	−1	−1	−1	18.39	44.34	241.09	10.18	17.896	175.79
2	−1	−1	1	23.36	63.99	273.92	6.27	35.022	558.80
3	−1	1	−1	18.69	52.90	283.10	16.94	37.907	223.82
4	−1	1	1	22.67	63.57	280.42	9.24	37.202	402.80
5	1	−1	−1	17.09	45.89	268.49	15.43	24.662	159.78
6	1	−1	1	16.70	74.54	446.34	7.19	27.804	386.56
7	1	1	−1	20.76	23.02	110.91	18.58	24.369	131.13
8	1	1	1	29.64	79.20	267.27	13.86	43.409	313.16
9	1.68	0	0	23.13	74.89	323.80	12.70	43.001	338.59
10	−1.68	0	0	20.47	66.91	326.91	10.04	38.294	381.22
11	0	−1.68	0	20.31	67.10	330.32	8.68	10.623	122.41
12	0	1.68	0	23.93	67.62	282.52	19.51	52.892	271.09
13	0	0	−1.68	16.35	40.46	247.54	15.79	29.516	186.89
14	0	0	1.68	9.82	54.37	553.44	3.30	23.744	719.42
15	−1.68	−1.68	−1.68	13.97	19.42	138.99	13.78	17.451	126.62
16	−1.68	−1.68	1.68	5.92	42.86	723.80	4.56	35.022	768.69
17	−1.68	1.68	−1.68	17.03	45.57	267.60	14.82	20.099	135.60
18	−1.68	1.68	1.68	10.23	61.60	601.91	6.23	38.798	622.97
19	1.68	−1.68	−1.68	15.27	23.98	157.02	14.23	22.570	158.56
20	1.68	−1.68	1.68	6.73	44.85	666.68	3.69	30.224	818.08
21	1.68	1.68	−1.68	19.62	59.08	301.08	16.38	9.093	55.52
22	1.68	1.68	1.68	17.75	62.37	351.37	10.04	53.379	531.80
23	0	0	0	26.76	59.46	222.23	16.32	46.329	283.85
24	0	0	0	28.19	64.46	228.64	17.02	46.337	272.30
25	0	0	0	25.04	69.11	276.02	16.27	46.434	285.47
26	0	0	0	25.04	68.08	271.88	18.72	45.620	243.74
27	0	0	0	27.55	60.64	220.11	16.96	38.589	227.60
28	0	0	0	22.53	70.60	313.31	15.54	40.011	257.44

TP: Total polyphenolic content was determined by summing all quantified polyphenolic compounds present in *A. montana* extracts using LC-MS/MS analysis. Runs 7 and 18 of Y_2 and Y_3 for HAE, respectively, were excluded from the RSM analysis.

combinations of conditions used in the RSM according to the CCC design. The experimental data were subjected to polynomial modeling, as described in **Eq. (1)**. Table 3A presents the results of the statistical analysis for fitting and the estimated regression coefficients.

The predictive models developed for the three response variables studied in HAE and UAE techniques displayed a high statistical significance. In all cases, the models were highly significant, as indicated by a p-value less than 0.0001, except for Y_2^{UAE}, which still exhibited a substantial significance level with a p-value below 0.001. These models exhibited high adjusted determination coefficients (R^2_{adj}), ranging from 0.737 to 0.818 for HAE and from 0.699 to 0.970 for UAE. Additionally, they demonstrated no significant lack of fit ($p > 0.05$). These findings confirm the suitability of the regression models in effectively fitting the experimental data for all response variables, enabling their application in predicting the impact of HAE and UAE conditions on extraction yield and TPC from AM flowers.

3.3.2. *Effect of HAE conditions on response variables*

Fig. 2 presents three-dimensional surface plots generated through the RSM analysis for HAE, illustrating the combined impact of two independent variables while keeping the third one at its central value.

In terms of extraction yield (Y_1), the solvent concentration had the most significant influence, affecting it quadratically, as shown in Table 3A. This finding suggests that the yield increases as the solvent concentration rises to a certain point. However, beyond this

Table 3

A. The regression coefficients obtained for each model using different techniques, and their associated statistical parameters expressed in terms of coded factors. B. Optimal levels for HAE and UAE conditions through simultaneous optimization via the Desirability function for each response, with comparison of predicted and experimental values in these conditions. C. Evaluation of extraction cycles on response variables for each technique.

A. RSM MODELS						
Regression coefficients	HAE			UAE		
	Y_1	Y_2	Y_3	Y_1	Y_2	Y_3
Intercept (β_0)	24.94 ± 0.90	67.41 ± 1.77	264.44 ± 17.11	14.42 ± 0.68	38.71 ± 1.97	263.64 ± 10.89
Linear						
β_1 (t)	NS	3.94 ± 1.78^{a}	NS	NS	0.31 ± 1.86^{NS}	-113.14 ± 33.72^{b}
β_2 (T or P)	2.80 ± 0.91^{b}	8.97 ± 1.78^{d}	-50.03 ± 18.22^{a}	2.62 ± 0.69^{c}	17.49 ± 3.77^{c}	-32.3 ± 33.86^{NS}
β_3 (S)	-1.67 ± 0.91^{NS}	9.58 ± 1.78^{d}	135.71 ± 18.22^{d}	-4.81 ± 0.69^{d}	0.51 ± 3.77^{NS}	162.51 ± 33.72^{c}
Quadratic						
β_{11}	NS	NS	NS	NS	1.26 ± 3.72^{NS}	69.38 ± 21.67^{b}
β_{22}	NS	NS	NS	NS	NS	-93.43 ± 21.67^{c}
β_{33}	-11.62 ± 1.43^{d}	-21.62 ± 2.78^{d}	102.07 ± 28.39^{b}	-4.32 ± 1.09^{c}	-12.73 ± 3.72^{b}	162.43 ± 21.67^{d}
Cubic				NS		
β_{111}	NS	NS	NS	NS	NS	99.68 ± 37.45^{a}
β_{222}	NS	NS	NS	NS	NS	106.34 ± 42.28^{a}
β_{333}	NS	NS	NS	NS	NS	116.72 ± 37.45^{b}
Interactive						
β_{12}	NS	NS	NS	NS	0.09 ± 2.22^{NS}	-27.77 ± 12.00^{a}
β_{13}	NS	NS	NS	NS	1.96 ± 2.22^{NS}	NS
β_{23}	NS	NS	-109.74 ± 22.23^{d}	NS	4.11 ± 2.22^{NS}	-47.22 ± 12.00^{b}
β_{123}	NS	NS	NS	NS	5.08 ± 2.3^{a}	NS
β_{112}	NS	NS	NS	NS	-15.94 ± 4.66^{b}	-139.65 ± 28.42^{c}
β_{113}	NS	NS	NS	NS	10.94 ± 4.66^{a}	NS
Statistical parameters						
Model significance (p-value)	<0.0001	<0.0001	<0.0001	<0.0001	0.0004	<0.0001
Lack of fit (p-value)	0.126	0.21	0.103	0.376	0.0565	0.112
R^2	0.766	0.846	0.837	0.767	0.821	0.983
R^2_{adj}	0.737	0.818	0.807	0.738	0.699	0.970
B. VALIDATION						
	PREDICTED RESPONSES			**EXPERIMENTAL VALUES**		
	Y_1	**Y_2**	**Y_3**	**Y_1**	**Y_2**	**Y_3**
HAE (60 min, 40 °C, 77% et.)	18.77 ± 2.13	64.20 ± 4.50	440.73 ± 41.32	21.47 ± 1.85	83.61 ± 4.15	389.98 ± 14.24
UAE (20 min, 400 W, 87% et.)	10.98 ± 2.01	50.82 ± 7.10	499.79 ± 42.87	12.42 ± 1.87	55.16 ± 1.50	447.94 ± 54.91
C. ADDITION OF EXTRACTION CYCLES						
Cycles	**HAE**			**UAE**		
	Y_1	**Y_2**	**Y_3**	**Y_1**	**Y_2**	**Y_3**
1	24.01 ± 0.54^{c}	65.95 ± 8.39^{b}	264.4 ± 14.6^{a}	13.93 ± 1.84^{c}	59.01 ± 8.77^{c}	427.83 ± 73.99^{c}
2	42.60 ± 0.00^{b}	108.75 ± 8.70^{a}	260.70 ± 12.27^{a}	25.00 ± 2.96^{b}	90.90 ± 2.09^{b}	484 ± 37.7^{a}
3	57.05 ± 0.78^{a}	135.05 ± 9.40^{a}	236.9 ± 19.7^{a}	35.40 ± 0.82^{a}	116.42 ± 1.20^{a}	541.92 ± 0.79^{a}

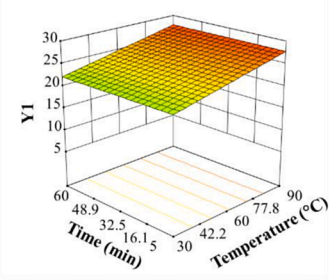
Y_1: yield in g extract/100 g dw, Y_2: total polyphenolic content in mg TP/g dw, and Y_3 (total polyphenolic content in mg TP/g extract). A. NS: non-significant ($p > 0.05$). Significant with: $^{a}p<0.05$; $^{b}p < 0.01$; $^{c}p < 0.001$; $^{d}p < 0.0001$. C. Results are expressed as mean values ± standard deviation. Values with different letter in the same column indicate significant differences ($p < 0.05$) between cycles for each response variable.

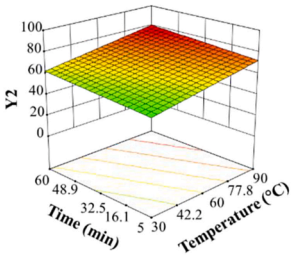
HEAT-ASSISTED EXTRACTION

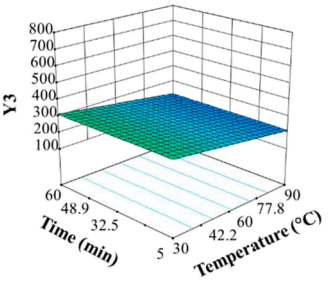
Y_1 (mg extract / 100 g dw)	Y_2 (mg / g dw)*	Y_3 (mg / g extract)**

A) Response surface plots

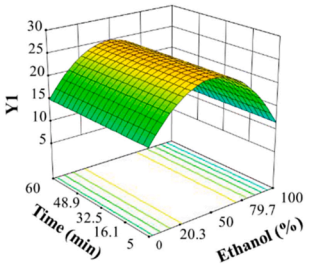
x_3= 50% ethanol

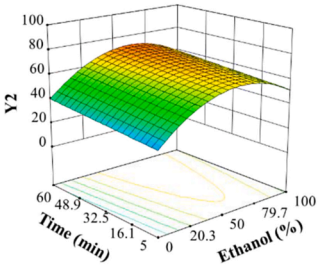


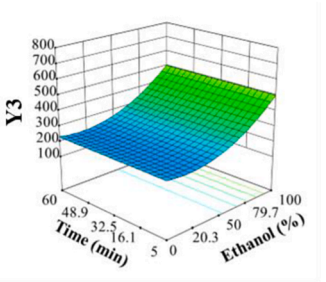




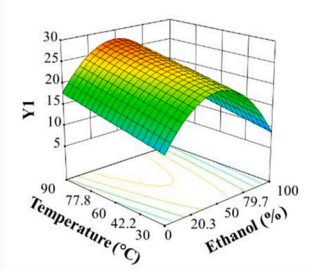
x_2= 60 °C

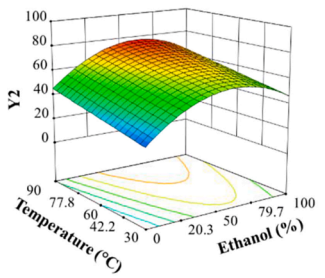


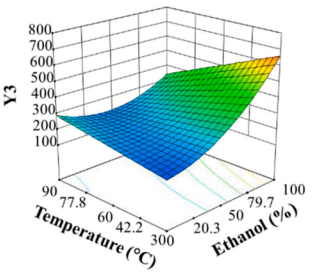




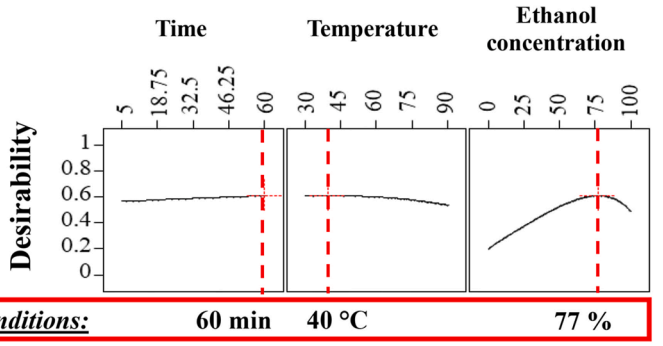
x_1= 32.5 min







B) Simultaneous optimization



Optimal conditions: **60 min** **40 °C** **77 %**

* TPC/ g dw was calculated by quantifying phenolic compounds in *A. montana* extracts using LC-MS.
** TPC given in milligrams of all phenolic compounds per gram of dry extract.

Fig. 2. Response surface methodology analysis in AM extracts using HAE. A) Response surface plots depicting the combined influence of two independent variables, with the third variable held constant at its midpoint. B) Predicted values of response variables under optimal conditions and Desirability function.

point, there was a noticeable decrease in yield, as depicted in Fig. 2A. Furthermore, temperature had a linear impact on extraction yield, with higher temperatures leading to higher extraction yields, regardless of the solvent concentration and time assayed (Fig. 2A). A higher extraction yield was observed with increased temperature when extracting other species in the Asteraceae family (Jurinjak Tušek et al., 2016).

Regarding TPC, Y_2 (mg TPC/g dw) displayed a similar pattern to what was observed in the extraction yield, with solvent concentration being the most influential factor affecting TPC extraction since their linear and quadratic terms were highly statistically significant ($p < 0.0001$) (Table 3A). Moreover, the solvent concentration was also the crucial factor influencing Y_3 (mg TPC/g extract), with a pronounced linear effect resulting in a greater quantity of TPC obtained when the ethanol content in the solvent increased. Temperature exhibited a notable and favorable linear influence with $p < 0.0001$, suggesting that higher Y_2 levels are achieved with increasing temperature values, irrespective of the tested solvent concentration (Fig. 2A). In addition, extraction time also yields a positive effect, as longer periods typically result in enhanced diffusion of phenolic compounds (Albuquerque et al., 2017; Jovanović et al., 2017). These results align with previous authors that reported increased TPC with rising temperature and extended extraction time (Jurinjak Tušek et al., 2016). According to these findings, a higher amount of Y_2 (mg/g dw) can be extracted by employing high temperatures, moderate ethanol concentrations, and extended extraction durations.

Similar to the PCA results in HAE, solvent concentration plays a significant role in the extraction of phenolic compounds. The most effective results were achieved when using ethanol concentrations ranging from 50% to 80%. Conversely, the least favorable results were obtained when employing either 0% or 100% ethanol, as indicated in Table 3A and Fig. 2A. These findings can be explained by the inverse relationship between ethanol concentration and the solvent's dielectric constant. As ethanol concentration increases, the dielectric constant of the solvent decreases, thereby facilitating better solvent diffusion within the matrix (Pimentel-Moral et al., 2020). This points out that most of the phenolic compounds present in AM flowers had an intermediate polarity and their extraction is favored when using mixtures of water/ethanol. This quadratic effect of the solvent has also been observed in previous studies involving the extraction of phenolic compounds from various sources, such as wild thyme (Jovanović et al., 2017), pumpkin peels (Leichtweis et al., 2023) or elderberries (Domínguez et al., 2020).

In addition to the findings from the PCA analysis, the RSM approach revealed that temperature was another crucial factor influencing the extraction of phenolic compounds. The effect of this parameter can be attributed to its ability to rupture cell membranes, enhance solvent diffusion, and disrupt interactions between phenolics and other matrix compounds. As a result, this leads to an increased release of cellular content into the solvent.

On the other hand, Fig. S2 displays the predicted values for each response variable as determined by the models (**Eq. (1)**). These values exhibited a high correlation with the experimental results, affirming the models' effectiveness in describing the extraction yield and TPC of AM samples. Moreover, it is worth noting that the residuals were evenly distributed around zero for all extraction variables.

3.3.3. Effect of UAE conditions on response variables

Fig. 3 displays three-dimensional surface plots resulting from the RSM analysis for AM samples subjected to UAE.

Like HAE results, the solvent concentration had the most significant influence on extraction yield. The linear effect had a strong negative effect, indicating that higher ethanol concentration led to lower yield (Table 3A and Fig. 3A). In addition, ultrasonic power positively affected extraction yield, regardless of the time and solvent used since its linear term was statistically significant (Fig. 3A). These results are consistent with previous studies conducted on *Taraxacum officinale* (Sun et al., 2014) and *Achillea kellalensis* (Yancheshmeh et al., 2022), other species belonging to the Asteraceae family, which reported an increase in extraction yield when intermediated ethanol concentrations and elevated ultrasonic power were employed.

Regarding Y_2 (mg TPC/g dw), the UAE model exhibited greater complexity compared to the HAE model. Ultrasonic power was the crucial parameter affecting Y_2 as its linear term was highly significant ($p < 0.001$), indicating that higher power levels led to increased TPC. These results agree with phenolics' recovery from *A. kellalensis* (Yancheshmeh et al., 2022). Solvent concentration, on the other hand, had a significant quadratic effect, demonstrating that intermediate ethanol concentrations were necessary to increase TPC (Table 3A). The influence of time remained less clear. While neither the linear nor the quadratic effects were statistically significant for Y_2, it is noteworthy that some interactive terms demonstrated statistical significance. For example, when examining the relationship between time and power, a curve with a minimum point becomes noticeable when the ultrasonic power is at its lowest level. Conversely, a curve with a peak is evident when the highest power settings are employed (Fig. 3A). These curves remain consistent within an ethanol concentration range of 0–80%, implying that higher TPC is achieved when higher power levels are applied with intermediate extraction time. However, when the ethanol concentration surpasses 80% and reaches 100%, the curvature associated with the highest power level transitions into a positive linear effect, signifying that increasing all three critical parameters increases TPC.

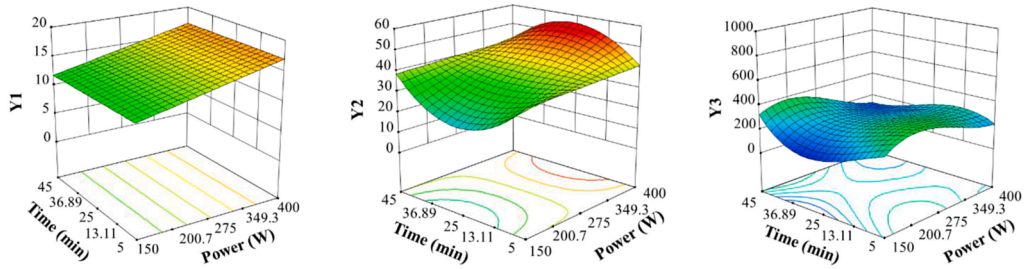
Differing from the results obtained through PCA in UAE, the use of the RSM approach reveals a substantial influence of ultrasonic power and a complex interaction between the extraction time and intensity of ultrasound on TPC. These factors dominated the extraction patterns of phenolic compounds in UAE. To obtain the highest phenolic content, it is necessary to use high levels of ultrasonic power, solvent concentration, and time durations within the 13 to 45-min range. Ultrasonic waves enhance mass transfer from the matrix to the solvent through cavitation, which results in the disruption of cell membranes (Gajic et al., 2019; Thilakarathna et al., 2023). When comparing TPC values (Y_2, mg/g dw) using both methods, UAE was less efficient, recovering approximately 26% fewer

ULTRASOUND-ASSISTED EXTRACTION

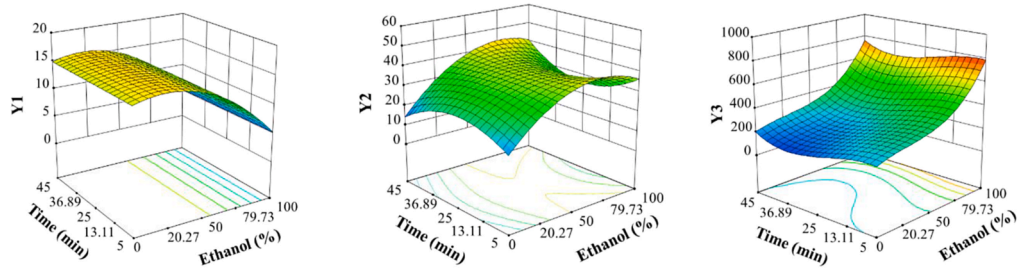
Y_1 (mg extract/ 100 g dw)	Y_2 (mg / g dw)*	Y_3 (mg / g extract)**

A) Response surface plots

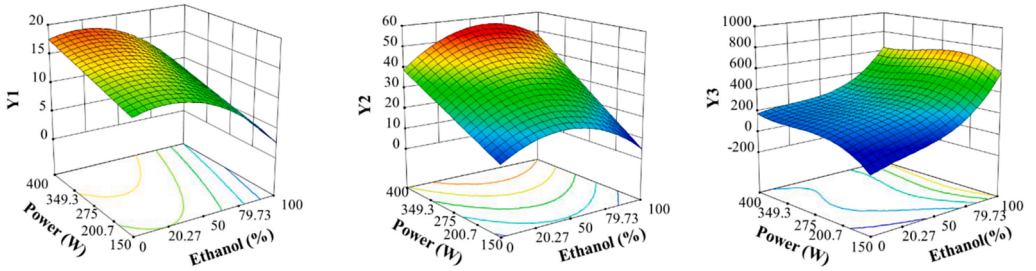
x_3= 50% ethanol



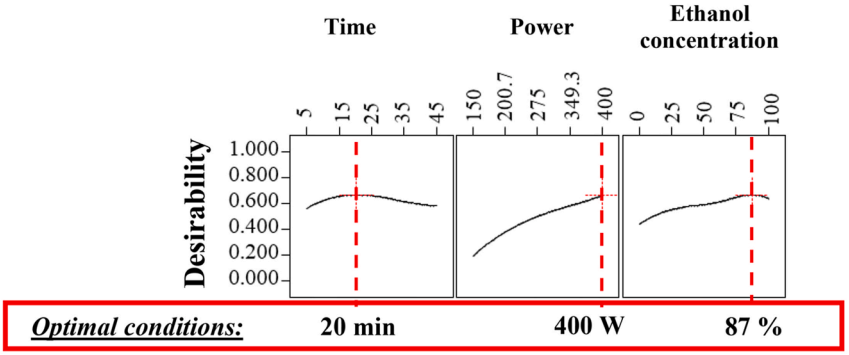
x_2= 275 W



x_1= 25 min



B) Simultaneous optimization



* TPCwas calculated by quantifying all phenolic compounds in *A. montana* extracts using LC-MS.
** TPC given in milligrams of all phenolic compounds per gram of dry extract.

Fig. 3. Response surface methodology analysis in AM extracts using UAE. A) Response surface plots depicting the combined influence of two independent variables, with the third variable held constant at its midpoint. B) Predicted values of response variables under optimal conditions and Desirability function.

phenolic compounds than those observed for HAE (Table 2). As previously mentioned, this disparity may be attributed to the fact that employing ultrasonic waves at room temperature is less effective than using elevated temperatures for extracting the phenolic compounds found in AM.

In line with the preceding subsection, Fig. S2 shows the predicted values for each response variable as determined by the models (**Eq. (1)**). Similar to HAE results, these values exhibit a high degree of correlation with the experimental data, and for every extraction variable, the residuals are evenly distributed around zero.

3.3.4. Simultaneous optimization and validation of optimal HAE and UAE conditions

While TPC (Y_2) exhibited a comparable pattern to extraction yield (Y_1) in both HAE and UAE techniques, the optimal range of extraction conditions varied slightly between these two responses, primarily because not all dry extract corresponds to phenolic compounds. Additionally, TPC (Y_3) displayed distinct optimal values compared to the other variables, justifying the application of simultaneous optimization. In this regard, findings from the Desirability function pointed out that for HAE, the optimal conditions to maximize both yield and TPC were $t = 60$ min, $T = 40$ °C and $S = 77\%$ ethanol, whereas for UAE, the optimal conditions were $t = 20$ min, $P = 400$ W and $S = 87\%$ ethanol (Table 3B). Under these optimal conditions, the predicted values for all the responses were calculated using **Eq. (1)**, considering the significant coefficients (Table 3A). The results are outlined in part B of Table 3. When comparing the predicted values for the three response variables in both techniques, HAE resulted in a higher extraction yield and enabled the extraction of 26% more phenolic compounds compared to UAE. The values of Y_3 were slightly higher when using the UAE method, as expected, given that Y_3 is a combination of both Y_2 and yield (Table 3B). To our knowledge, no previous studies have focused on the optimization of AM flowers using either HAE or UAE. However, it is worth noting that other research has indicated that HAE has shown greater efficacy in recovering phenolic compounds from other matrices when compared to UAE (Albuquerque et al., 2017; Leichtweis et al., 2023; López et al., 2018).

The results from the new set of AM extracts, experimentally prepared under optimal HAE and UAE conditions determined through simultaneous optimization, are presented in Table 3B. The statistical analysis indicated that, for HAE, experimental values for Y_1 and Y_3 closely matched their predicted values, while Y_2 exceeded the expected values. In the case of UAE, all response variables obtained experimentally aligned well with their predicted values, affirming the reliability of the models.

3.4. Evaluation of multiple extraction cycles and comparison with Soxhlet extraction

In another study, we investigated how the number of extraction cycles affected the recovery of phenolic compounds. Examining the potential benefits of using extraction cycles with fresh solvent is relevant here because the sample's characteristics might impede solvent diffusion and cause some bioactive compounds to be retained (Álvarez-Casas et al., 2014). The results of the sequential extraction under the optimized conditions for both extraction methods are depicted in Table 3C.

Both extraction techniques demonstrated a significant improvement ($p < 0.05$) in extraction yield and TPC with the application of successive extraction cycles. Specifically, when focusing on TPC (Y_2) in HAE, the content increased as extraction cycles were applied, showing an increase of 65% and 24%. However, no significant differences were observed ($p > 0.05$) between the second and third cycles. On the other hand, in the case of UAE, the second and third cycles increased the content by 54% and 28%, respectively. Significant differences ($p < 0.05$) between all the cycles were reported. Consequently, these findings indicate that employing two extraction cycles is the most efficient strategy for enhancing the recovery of phenolic compounds with HAE, while three cycles are preferred for UAE.

Following the selection of the optimal extraction cycles, a new statistical analysis was conducted to compare the results of both techniques with the Soxhlet method, established as the reference extraction technique. Regarding the most critical response, TPC (Y_2), both techniques significantly improved the content of phenolic compounds recovered compared to the Soxhlet method, which allowed to obtain 84.42 mg/g dw. For HAE, the TPC increased a 29%, while it augmented a 38% for UAE (Table 3C). It is noteworthy that both techniques offer advantages over Soxhlet extraction. Firstly, Soxhlet extraction required 3.5h, in contrast to the 2-h duration for two cycles of HAE and the 1-h duration for three cycles of UAE. Secondly, these extractions are conducted at lower temperatures, resulting in reduced energy consumption. Furthermore, it is worth noting that two cycles of HAE used a smaller amount of solvent compared to both the Soxhlet method and three cycles of UAE. Given these benefits, both techniques could be considered for further studies aimed at scaling up the extraction process.

4. Conclusion

In this work, AM flowers were demonstrated to be a valuable source of phenolic compounds primarily comprising flavonols and hydroxycinnamic acids. The major compound identified within them was dicaffeoylquinic acid, which, together with other phenolic compounds, has demonstrated various bioactive properties, especially antioxidants.

When unsupervised multivariate analysis was applied to the LC-MS/MS data derived from AM samples treated with HAE and UAE, it became evident that the ethanol concentration had a significant impact on the extraction of specific phenolic compounds. These compounds tended to cluster together based on the solvent concentration. The RSM approach reinforced these findings, highlighting that temperature and ultrasonic power also played critical roles in influencing the total phenolic content in HAE and UAE, respectively. The models created for all response variables using RSM offered valuable insights into the complex interactions among extrac-

tion conditions, in particular, in the UAE models. On the other hand, optimal HAE (60 min, 40 °C, 77% ethanol) and UAE (20 min, 400 W, 87% ethanol) conditions that maximized all response variables were successfully found by applying simultaneous optimization with the Desirability function. The validation experiments confirmed that the values obtained experimentally under optimal conditions closely matched those statistically predicted, affirming the robustness of the models. These results further validate that RSM is a dependable method for optimizing extraction parameters and improving efficiency, thereby contributing to a deeper comprehension of the process.

Upon comparing the predicted values for the three response variables in both methods, it became evident that HAE not only yielded higher extraction efficiency but also facilitated the extraction of approximately 26% more phenolic compounds compared to UAE.

This study also highlights the efficacy of employing multiple extraction cycles to enhance the extraction efficiency of phenolic compounds. Regarding HAE, conducting two extraction cycles under optimal conditions leads to a significant improvement in TPC, while in the case of UAE, three cycles are necessary to achieve the same result. In both instances, TPC exceeds what can be obtained through Soxhlet extraction, thereby enhancing overall performance and reducing the time, energy, and solvent usage.

Overall, these optimized methods are suitable for industrial scaling and can be applied to obtain phenolic-rich extracts from an underutilized plant for the development of bio-based applications.

CRediT authorship contribution statement

Paula Garcia-Oliveira: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Franklin Chamorro:** Validation, Software, Methodology, Formal analysis. **Jesus Simal-Gandara:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Miguel A. Prieto:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Lucía Cassani:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgment

L. Cassani (ED481D-2024-002) acknowledges Consellería de Educación, Ciencia, Universidades e Formación Profesional (Xunta de Galicia) for the financial support. The authors thank the EU-FORA Fellowship Program (EUBA-EFSA-2022-ENREL-02) that supports the work of F. Chamorro. Financial support for this research was also provided by the Agency for the Scientific and Technological Promotion (ANPCyT, Argentina) through the project Scientific and Technological Research Projects (PICT) (2020)/1602 and the Ibero-American Program of Science and Technology for Development (CYTED-IBEROCIRCULAR, 323RT0142). L. Cassani acknowledges Prof. Dr. Héctor Goicochea of Laboratorio de Desarrollo Analítico y Quimiometría (LADAQ, CONICET) at the Universidad Nacional del Litoral (Santa Fe, Argentina) for his kindly support in developing the experimental design in Design Expert software.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2024.101722.

References

Albuquerque, B.R., Heleno, S.A.8., Oliveira, M.B.P.P., Barros, L., Ferreira, I.C.F.R., 2021. Phenolic compounds: current industrial applications, limitations and future challenges. Food Funct. 12 (1), 14–29. https://doi.org/10.1039/d0fo02324h.

Albuquerque, B.R., Prieto, M.A., Barreiro, M.F., Rodrigues, A.E., Curran, T.P., Barros, L., Ferreira, I.C.F.R., 2017. Catechin-based extract optimization obtained from *Arbutus unedo* L. fruits using maceration/microwave/ultrasound extraction techniques. Ind. Crop. Prod. 95, 404–415. https://doi.org/10.1016/j.indcrop.2016.10.050.

Alcázar Magaña, A., Kamimura, N., Soumyanath, A., Stevens, J.F., Maier, C.S., 2021. Caffeoylquinic acids: chemistry, biosynthesis, occurrence, analytical challenges, and bioactivity. Plant J. 107 (5), 1299–1319. https://doi.org/10.1111/tpj.15390.

Álvarez-Casas, M., García-Jares, C., Llompart, M., Lores, M., 2014. Effect of experimental parameters in the pressurized solvent extraction of polyphenolic compounds from white grape marc. Food Chem. 157, 524–532. https://doi.org/10.1016/j.foodchem.2014.02.078.

Backes, E., Pereira, C., Barros, L., Prieto, M.A., Genena, A.K., Barreiro, M.F., Ferreira, I.C.F.R., 2018. Recovery of bioactive anthocyanin pigments from *Ficus carica* L. peel by heat, microwave, and ultrasound based extraction techniques. Food Res. Int. 113, 197–209. https://doi.org/10.1016/j.foodres.2018.07.016.

Barral-Martinez, M., Garcia-Oliveira, P., Nuñez-Estevez, B., Silva, A., Finimundy, T.C., Calhelha, R., Nenadic, M., Sokovic, M., Barroso, F., Simal-Gandara, J., Ferreira, I.C.F.R., Barros, L., Prieto, M.A., 2021. Plants of the family Asteraceae: evaluation of biological properties and identification of phenolic compounds. Chemistry Proceedings 51. https://doi.org/10.3390/csac2021-10486.

Benali, T., Bakrim, S., Ghchime, R., Benkhaira, N., El Omari, N., Balahbib, A., Taha, D., Zengin, G., Hasan, M.M., Bibi, S., Bouyahya, A., 2022. Pharmacological insights into the multifaceted biological properties of quinic acid. Biotechnol. Genet. Eng. Rev. 1–30. https://doi.org/10.1080/02648725.2022.2122303.

Benarfa, A., Gourine, N., Hachani, S., Harrat, M., Yousfi, M., 2020. Optimization of ultrasound-assisted extraction of antioxidative phenolic compounds from *Deverra scoparia* Coss. & Durieu (flowers) using response surface methodology. J. Food Process. Preserv. 44 (7), 1–10. https://doi.org/10.1111/jfpp.14514.

Cassani, L., Lourenço-Lopes, C., Barral-Martinez, M., Chamorro, F., Garcia-Perez, P., Simal-Gandara, J., Prieto, M.A., 2022. Thermochemical characterization of eight seaweed species and evaluation of their potential use as an alternative for biofuel production and source of bioactive compounds. Int. J. Mol. Sci. 23 (4), 2355. https://doi.org/10.3390/IJMS23042355/S1.

Cassani, L., Tomadoni, B., Moreira, M.R., Agüero, M.V., 2018. Improving quality parameters of functional strawberry juices: optimization of prebiotic fiber enrichment and geraniol treatment. Food Bioprocess Technol. 11 (11), 2110–2124. https://doi.org/10.1007/s11947-018-2170-x.

Chen, Y.L., Hwang, T.L., Yu, H.P., Fang, J.Y., Chong, K.Y., Chang, Y.W., Chen, C.Y., Yang, H.W., Chang, W.Y., Hsieh, P.W., 2016. *Ilex kaushue* and its bioactive component 3,5-dicaffeoylquinic acid protected mice from lipopolysaccharide-induced acute lung injury. Sci. Rep. 6 (1), 1–12. https://doi.org/10.1038/srep34243.

Clauser, M., Aiello, N., Scartezzini, F., Innocenti, G., Dall'Acqua, S., 2014. Differences in the chemical composition of *Arnica montana* flowers from wild populations of north Italy. Nat. Prod. Commun. 9 (1), 3–6. https://doi.org/10.1177/1934578x1400900102.

Clifford, M.N., Johnston, K.L., Knight, S., Kuhnert, N., 2003. Hierarchical scheme for LC-MSn identification of chlorogenic acids. J. Agric. Food Chem. 51 (10), 2900–2911. https://doi.org/10.1021/jf026187q.

Clifford, M.N., Knight, S., Kuhnert, N., 2005. Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSn. J. Agric. Food Chem. 53 (10), 3821–3832. https://doi.org/10.1021/jf050046h.

de Athayde, A.E., de Araujo, C.E.S., Sandjo, L.P., Biavatti, M.W., 2021. Metabolomic analysis among ten traditional "Arnica" (Asteraceae) from Brazil. J. Ethnopharmacol. 265, 113149. https://doi.org/10.1016/J.JEP.2020.113149.

Deng, J., Qi, X.L., Guan, Z.Z., Yan, X.M., Huang, Y., Wang, Y.L., 2013. Pretreatment of SH-SY5Y cells with dicaffeoylquinic acids attenuates the reduced expression of nicotinic receptors, elevated level of oxidative stress and enhanced apoptosis caused by β-amyloid peptide. J. Pharm. Pharmacol. 65 (12), 1736–1744. https://doi.org/10.1111/jphp.12096.

Domínguez, R., Zhang, L., Rocchetti, G., Lucini, L., Pateiro, M., Munekata, P.E.S., Lorenzo, J.M., 2020. Elderberry (*Sambucus nigra* L.) as potential source of antioxidants . Characterization , optimization of extraction parameters and bioactive properties. Food Chem. 330, 127266. https://doi.org/10.1016/j.foodchem.2020.127266.

Enomoto, H., 2020. Mass spectrometry imaging of flavonols and ellagic acid glycosides in ripe strawberry fruit. Molecules 25 (20), 4600. https://doi.org/10.3390/MOLECULES25204600.

Flórez-Fernández, N., Ferreira-Anta, T., Torres, M.D., Domínguez, H., 2021. Valorization of *Arnica montana* wastes after extraction of the ethanol tincture: application in polymer-based matrices. Polymers 13 (18). https://doi.org/10.3390/polym13183121.

Gai, Q.Y., Jiao, J., Wang, X., Fu, Y.J., Lu, Y., Liu, J., Wang, Z.Y., Xu, X.J., 2021. Simultaneous quantification of eleven bioactive phenolic compounds in pigeon pea natural resources and in vitro cultures by ultra-high performance liquid chromatography coupled with triple quadrupole mass spectrometry (UPLC-QqQ-MS/MS). Food Chem. 335, 127602. https://doi.org/10.1016/j.foodchem.2020.127602.

Gajic, I.S., Savic, I., Boskov, I., Žeraji, S., 2019. Optimization of ultrasound-assisted extraction of phenolic compounds from black locust (*Robiniae pseudoacaciae*) flowers and comparison with conventional methods. Antioxidants 8 (248), 1–14.

Ganzera, M., Egger, C., Zidorn, C., Stuppner, H., 2008. Quantitative analysis of flavonoids and phenolic acids in *Arnica montana* L. by micellar electrokinetic capillary chromatography. Anal. Chim. Acta 614 (2), 196–200. https://doi.org/10.1016/j.aca.2008.03.023.

Garcia-Oliveira, P., Barral, M., Carpena, M., Gullón, P., Fraga-Corral, M., Otero, P., Prieto, M.A., Simal-Gandara, J., 2021. Traditional plants from Asteraceae family as potential candidates for fuctional food industry. Food Funct. 12, 2850–2873. https://doi.org/10.1039/d0fo03433a.

Gaspar, A., Craciunescu, O., Trif, M., Moisei, M., Moldovan, L., 2014. Antioxidant and anti-inflammatory properties of active compounds from *Arnica montana* L. Romanian Biotechnological Letters 19 (3), 9353–9365.

Gawlik-Dziki, U., Świeca, M., Sugier, D., Cichocka, J., 2011. Comparison of in vitro lipoxygenase, xanthine oxidase inhibitory and antioxidant activity of *Arnica montana* and tinctures. Acta Scientiarum Polonorum, Hortorum Cultus 10 (3), 15–27.

Gil-Martín, E., Forbes-Hernández, T., Romero, A., Cianciosi, D., Giampieri, F., Battino, M., 2022. Influence of the extraction method on the recovery of bioactive phenolic compounds from food industry by-products. Food Chem. 378. https://doi.org/10.1016/j.foodchem.2021.131918.

Gong, G., Guan, Y.Y., Zhang, Z.L., Rahman, K., Wang, S.J., Zhou, S., Luan, X., Zhang, H., 2020. Isorhamnetin: a review of pharmacological effects. Biomed. Pharmacother. 128, 110301. https://doi.org/10.1016/j.biopha.2020.110301.

Granato, D., Santos, J.S., Escher, G.B., Ferreira, B.L., Maggio, R.M., 2018. Use of principal component analysis (PCA) and hierarchical cluster analysis (HCA) for multivariate association between bioactive compounds and functional properties in foods: a critical perspective. Trends Food Sci. Technol. 72, 83–90. https://doi.org/10.1016/j.tifs.2017.12.006.

Gulsunoglu, Z., Purves, R., Karbancioglu-Guler, F., Kilic-Akyilmaz, M., 2020. Enhancement of phenolic antioxidants in industrial apple waste by fermentation with *Aspergillus* spp. Biocatal. Agric. Biotechnol. 25, 101562. https://doi.org/10.1016/j.bcab.2020.101562.

Han, X., Wang, S., Yang, X.-X., Li, T.-J., Zhao, H.-J., Zhou, L.-P., Lin Zhao, |, Yong-Rui Bao, |, Xian-Sheng Meng, |, Bao, Y., Zhao, L., 2019. Analysis of plasma migration components in *Patrinia villosa* (Thunb.) Juss. effective parts by UPLC-Q-TOF-MS. Biomed. Chromatogr. 34, e4701. https://doi.org/10.1002/bmc.4701.

Jahromi, S., 2019. Extraction techniques of phenolic compounds from plants. In: Soto-Hernández, M., García-Mateos, R., Palma-Tenango, M. (Eds.), Plant Physiological Aspects of Phenolic Compounds. IntechOpen. https://doi.org/10.5772/intechopen.84705.

Jaiswal, R., Kuhnert, N., 2011. Identification and characterization of two new derivatives of chlorogenic acids in arnica (*Arnica montana* L.) flowers by high-performance liquid chromatography/tandem mass spectrometry. J. Agric. Food Chem. 59 (8), 4033–4039. https://doi.org/10.1021/jf103545k.

Jaramillo, S., Lopez, S., Varela, L.M., Rodriguez-Arcos, R., Jimenez, A., Abia, R., Guillen, R., Muriana, F.J.G., 2010. The flavonol isorhamnetin exhibits cytotoxic effects on human colon cancer cells. J. Agric. Food Chem. 58 (20), 10869–10875. https://doi.org/10.1021/jf102669p.

Jovanović, A.A., Đorđević, V.B., Zdunić, G.M., Pljevljakušić, D.S., Šavikin, K.P., Gođevac, D.M., Bugarski, B.M., 2017. Optimization of the extraction process of polyphenols from *Thymus serpyllum* L. herb using maceration, heat- and ultrasound-assisted techniques. Separation and Purification Technology 179, 369–380. https://doi.org/10.1016/j.seppur.2017.01.055.

Jurinjak Tušek, A., Benković, M., Belščak Cvitanović, A., Valinger, D., Jurina, T., Gajdoš Kljusurić, J., 2016. Kinetics and thermodynamics of the solid-liquid extraction process of total polyphenols, antioxidants and extraction yield from Asteraceae plants. Ind. Crop. Prod. 91, 205–214. https://doi.org/10.1016/J.INDCROP.2016.07.015.

Kimel, K., Godlewska, S., Krauze-Baranowska, M., PobŁocka-Olech, L., 2019. HPLC-DAD-ESI/MS analysis of Arnica TM constituents. Acta Poloniae Pharmaceutica - Drug Research 76 (6), 1015–1027. https://doi.org/10.32383/appdr/112187.

Leichtweis, M.G., Molina, A.K., Petropoulos, S.A., Carocho, M., Pires, T.C.S.P., Dias, M.I., Calhelha, R., Oliveira, M.B.P.P., Pereira, C., Barros, L., 2023. Valorization of pumpkin peel as a source of bioactive compounds: optimization of heat- and ultrasound-assisted extraction. Molecules 28 (7). https://doi.org/10.3390/molecules28073168.

Lesjak, M., Beara, I., Simin, N., Pintać, D., Majkić, T., Bekvalac, K., Orčić, D., Mimica-Dukić, N., 2018. Antioxidant and anti-inflammatory activities of quercetin and its derivatives. J. Funct.Foods 40, 68–75. https://doi.org/10.1016/j.jff.2017.10.047.

Li, Z.H., Guo, H., Xu, W. Bin, Ge, J., Li, X., Alimu, M., He, D.J., 2016. Rapid identification of flavonoid constituents directly from PTP1B inhibitive extract of raspberry (*Rubus idaeus* L.) leaves by HPLC-ESI-QTOF-MS-MS. Journal of Chromatographic Science 54 (5), 805–810. https://doi.org/10.1093/chromsci/bmw016.

Lin, L.Z., Harnly, J.M., 2008. Identification of hydroxycinnamoylquinic acids of arnica flowers and burdock roots using a standardized LC-DAD-ESI/MS profiling method. J. Agric. Food Chem. 56 (21), 10105–10114. https://doi.org/10.1021/jf802412m.

Lin, Y., Wu, B., Li, Z., Hong, T., Chen, M., Tan, Y., Jiang, J., Huang, C., 2012. Metabolite identification of myricetin in rats using HPLC coupled with ESI-MS. Chromatographia 75 (11–12), 655–660. https://doi.org/10.1007/s10337-012-2239-z.

Liu, C., Wahefu, A., Lu, X., Abdulla, R., Dou, J., Zhao, H., Aisa, H.A., Xin, X., Liu, Y., 2022. Chemical profiling of kaliziri injection and quantification of six caffeoyl quinic qcids in beagle plasma by LC-MS/MS. Pharmaceuticals 15 (6), 663. https://doi.org/10.3390/PH15060663.

Liu, W., Li, J., Zhang, X., Zu, Y., Yang, Y., Liu, W., Xu, Z., Gao, H., Sun, X., Jiang, X., Zhao, Q., 2020. Current advances in naturally occurring caffeoylquinic acids: structure, bioactivity, and synthesis. J. Agric. Food Chem. 68 (39), 10489–10516. https://doi.org/10.1021/acs.jafc.0c03804.

López, C.J., Caleja, C., Prieto, M.A., Barreiro, M.F., Barros, L., Ferreira, I.C.F.R., 2018. Optimization and comparison of heat and ultrasound assisted extraction techniques to obtain anthocyanin compounds from *Arbutus unedo* L. fruits. Food Chem. 264, 81–91. https://doi.org/10.1016/j.foodchem.2018.04.103.

Nishikawa, M., Kada, Y., Kimata, M., Sakaki, T., Ikushiro, S., 2022. Comparison of metabolism and biological properties among positional isomers of quercetin

glucuronide in LPS- and RANKL-challenged RAW264.7 cells. Bioscience, Biotechnology and Biochemistry 86 (12), 1670–1679. https://doi.org/10.1093/bbb/zbac150.
Oliveira, R.N., Paranhos da Silva, C.M., Moreira, A.P.D., Mendonça, R.H., Thiré, R.M.S.M., McGuinness, G.B., 2017. Comparative analysis of PVA hydrogels incorporating two natural antimicrobials: *Punica granatum* and *Arnica montana* tinctures. J. Appl. Polym. Sci. 134 (41). https://doi.org/10.1002/app.45392.
Osorio-Tobón, J.F., 2020. Recent advances and comparisons of conventional and alternative extraction techniques of phenolic compounds. J. Food Sci. Technol. 57 (12), 4299–4315. https://doi.org/10.1007/s13197-020-04433-2.
Paşayeva, L., Eruygur, N., Ayaz, F., Tugay, O., Fatullayev, H., 2021. Assessment of the antioxidant and enzyme inhibition activities of *Cousinia iconica* with focus on phytochemical investigation by LC-MS/MS. Plant Biosyst. 155 (6), 1178–1188. https://doi.org/10.1080/11263504.2020.1829736.
Pimentel-Moral, S., Borrás-Linares, I., Lozano-Sánchez, J., Alañón, M.E., Arráez-Román, D., Segura-Carretero, A., 2020. Pressurized GRAS solvents for the green extraction of phenolic compounds from *Hibiscus sabdariffa* calyces. Food Res. Int. 137, 109466. https://doi.org/10.1016/j.foodres.2020.109466.
Pljevljakušić, D., Janković, T., Jelačić, S., Novaković, M., Menković, N., Beatović, D., Dajić-Stevanović, Z., 2014. Morphological and chemical characterization of *Arnica montana* L. under different cultivation models. Ind. Crop. Prod. 52, 233–244. https://doi.org/10.1016/j.indcrop.2013.10.035.
Rocchetti, G., Blasi, F., Montesano, D., Ghisoni, S., Marcotullio, M.C., Sabatini, S., Cossignani, L., Lucini, L., 2019. Impact of conventional/non-conventional extraction methods on the untargeted phenolic profile of *Moringa oleifera* leaves. Food Res. Int. 115, 319–327. https://doi.org/10.1016/j.foodres.2018.11.046.
Röhrl, J., Piqué-Borràs, M.R., Jaklin, M., Werner, M., Werz, O., Josef, H., Hölz, H., Ammendola, A., Künstle, G., 2023. Anti-inflammatory activities of *Arnica montana* planta tota versus flower extracts: analytical, in vitro and in vivo mouse paw oedema model studies. Plants 12 (6), 1348. https://doi.org/10.3390/PLANTS12061348/S1.
Sharma, S., Arif, M., Nirala, R.K., Gupta, R., Thakur, S.C., 2016. Cumulative therapeutic effects of phytochemicals in *Arnica montana* flower extract alleviated collagen-induced arthritis: inhibition of both pro-inflammatory mediators and oxidative stress. J. Sci. Food Agric. 96, 1500–1510. https://doi.org/10.1002/jsfa.7252.
Shehata, M.G., Abd El Aziz, N.M.A., Youssef, M.M., El-Sohaimy, S.A., 2021. Optimization conditions of ultrasound-assisted extraction of phenolic compounds from orange peels using response surface methodology. J. Food Process. Preserv. 45 (e15870), 1–10. https://doi.org/10.1111/jfpp.15870.
Suganthy, N., Devi, K.P., Nabavi, S.F., Braidy, N., Nabavi, S.M., 2016. Bioactive effects of quercetin in the central nervous system: focusing on the mechanisms of actions. Biomed. Pharmacother. 84, 892–908. https://doi.org/10.1016/j.biopha.2016.10.011.
Sun, Z., Su, R., Qiao, J., Zhao, Z., Wang, X., 2014. Flavonoids extraction from *Taraxacum officinale* (dandelion): optimisation using response surface methodology and antioxidant activity. J. Chem. 2014. https://doi.org/10.1155/2014/956278.
Thilakarathna, R.C.N., Siow, L.F., Tang, T.K., Chan, E.S., Lee, Y.Y., 2023. Physicochemical and antioxidative properties of ultrasound-assisted extraction of mahua (*Madhuca longifolia*) seed oil in comparison with conventional Soxhlet and mechanical extractions. Ultrason. Sonochem. 92, 106280. https://doi.org/10.1016/j.ultsonch.2022.106280.
Tsai, S.W., Lin, C.C., Lin, S.C., Wang, S.P., Yang, D.H., 2019. Isorhamnetin ameliorates inflammatory responses and articular cartilage damage in the rats of monosodium iodoacetate-induced osteoarthritis. Immunopharmacol. Immunotoxicol. 41 (4), 504–512. https://doi.org/10.1080/08923973.2019.1641723.
Yancheshmeh, B.S., Panahi, Y., Allahdad, Z., Abdolshahi, A., Zamani, Z., 2022. Optimization of ultrasound-assisted extraction of bioactive compounds from Achillea kellalensis using response surface methodology. Journal of Applied Research on Medicinal and Aromatic Plants 28, 100355. https://doi.org/10.1016/J.JARMAP.2021.100355.
Zhou, Y., Fu, X., Guan, Y., Gong, M., He, K., Huang, B., 2020. 1,3-Dicaffeoylquinic acid targeting 14-3-3 tau suppresses human breast cancer cell proliferation and metastasis through IL6/JAK2/PI3K pathway. Biochem. Pharmacol. 172, 113752. https://doi.org/10.1016/J.BCP.2019.113752.
Žitek, T., Postružnik, V., Knez, Ž., Golle, A., Dariš, B., Knez Marevci, M., 2022. *Arnica montana* L. supercritical extraction optimization for antibiotic and anticancer activity. Front. Bioeng. Biotechnol. 10, 1–9. https://doi.org/10.3389/fbioe.2022.897185.