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Optimization of ultrasound-assisted extraction of antioxidant compounds from *Mucuna pruriens* pods using response surface methodology: a waste-to-value approach

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Mucuna pruriens, a leguminous plant known for the rich bioactive content of its seeds, also exhibits significant antioxidant potential in its pods, an underutilized agricultural by-product. This study aimed to optimize ultrasound-assisted extraction (UAE) conditions to maximize the recovery of phenolic compounds with antioxidant capacity (AOC) from *M. pruriens* pods. Optimization was conducted using response surface methodology (RSM), evaluating the effects of extraction time (10–20 min), ethanol concentration (0–100%), and ultrasound amplitude (0–80%). Ethanol concentration was identified as the most influential variable affecting both total phenolic content (TPC) and AOC. Optimal UAE conditions (10 min, 30% ethanol, 80% amplitude) yielded significantly higher TPC (274.21 ± 1.43 mg GAE per g) and AOC (DPPH: 2.41 ± 0.11, ABTS: 1.87 ± 0.09 and FRAP: 3.67 ± 0.08 mmol TEAC per g) compared to the traditional decoction method. HPLC-MS-based metabolite profiling tentatively identified 22 bioactive compounds in the pod and seed extracts. Furthermore, a notable L-Dopa content (5.8%) was quantified in the optimized pod extract, highlighting its potential as a valuable bioresource. These findings demonstrate the efficiency and sustainability of UAE in valorizing *M. pruriens* pods and support their potential application in food, nutraceutical, and pharmaceutical formulations.

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Sustainability spotlight

The processing of *Mucuna pruriens*, a legume valued for the nutritional and therapeutic properties of its seeds, generates large volumes of pod residue whose accumulation may lead to environmental impact while wasting their potential as source of valuable bioactives. This study promotes the valorization of this agro-industrial by-product by maximizing the antioxidant compound recovery through optimized ultrasound-assisted extraction (UAE), a green and efficient technology. The identification of L-Dopa and other health-promoting compounds reinforces the potential value of these residues for innovative applications in food, nutraceutical and pharmaceutical sectors. By reducing waste, enhancing resource efficiency, and supporting the principles of the circular economy, this work provides a more sustainable and responsible use of plant biomass in food and health-related industries.

1 Introduction

The generation of large volumes of agricultural waste is an unavoidable consequence of food production and processing. While most of this material is typically discarded in landfills, it also represents a valuable yet underutilized source of bioactive compounds with potential applications in different industries.[1] A wide variety of bioactive molecules, including phenolic acids, flavonoids, anthocyanins, and vitamins, has been successfully extracted from these residues.[2,3] Most of these compounds exhibit significant health-promoting properties, particularly due to their ability to scavenge free radicals, which help reduce oxidative stress, a key factor in the onset of chronic diseases.[4] As a result, these bioactive compounds have gained global recognition and are extensively incorporated as nutraceuticals, cosmeceuticals, and functional food for developing dietary supplements, energy beverages, and other health-enhancing products.

Legumes, members of the Fabaceae family, are widely cultivated for their nutritional value and therapeutic potential. *Mucuna pruriens* (Mp), also known as velvet bean, is a leguminous species found in tropical and subtropical regions, including northeastern Argentina.[5] Mp is a source of proteins, carbohydrates, minerals, fiber, and essential amino acids.[6] Moreover, it contains bioactive compounds that have demonstrated antioxidant, anti-inflammatory, hypocholesterolemic,

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and hypoglycemic properties in both *in vitro* and *in vivo* studies.[7–10] One of the most notable constituents of Mp seeds is L-Dopa, a non-protein phenolic amino acid, a dopamine precursor, and a key neurotransmitter in central nervous system regulation. Consequently, Mp has been widely used in Ayurvedic medicine for the treatment of Parkinson's disease.[11] Moreover, several studies confirmed its neuroprotective effects, suggesting its potential as a therapeutic agent not only for Parkinson's disease but also for other neurodegenerative disorders, including Alzheimer's disease, amyotrophic lateral sclerosis, and stroke.[12–14] However, seed extracts lacking detectable L-Dopa have also shown beneficial effects on erectile dysfunction, suggesting that other phenolic compounds (PCs) may contribute to its bioactivity.[15]

Although most research has focused on Mp seeds and their derivatives (flours and extracts), other plant parts also contain bioactive compounds with potential medicinal applications. The pods (Mp-p), which account for approximately 40% of the fruit, are typically discarded during seed processing, representing both a missed valorization opportunity and an environmental challenge. Their accumulation contributes to waste management issues and additional disposal costs, while practices such as open burning release harmful emissions that undermine sustainability goals. In this context, converting Mp-p into high-value products aligns with circular economy principles, as emphasized by the United Nations Agenda 2030 and the European Circular Economy Action Plan, which promote waste prevention, recycling, and reuse.[16]

Morphologically, these pods are firm and sigmoid in shape, characterized by longitudinal ridges and a surface covered by easily detachable reddish-orange hairs. As a non-starch lignocellulosic biomass, they comprise polymerized sugars (cellulose and hemicellulose), which can be hydrolyzed and fermented for bioethanol production.[17] Additionally, previous studies suggest that the pods of *Mucuna flagellipes* have potential applications in wastewater treatment, particularly for the removal of paint contaminants.[18] Beyond these uses, Mp-p are a valuable source of PCs, with concentrations increasing as the plant matures, while tannin levels decline.[17]

Recent research has explored the extraction of PCs from Mp-p using maceration with various solvents and the decoction method.[19] The extracts exhibited high phenolic content and substantial antioxidant activity (AOC), suggesting their potential as bioactive sources. However, both phenolic composition and AOC varied depending on the extraction method and solvent used, illustrating that extraction conditions influence their recovery.

Research on the extraction of bioactive compounds from natural sources has gained increasing attention due to the growing demand for natural antioxidants and their expanding applications in the food and cosmetic industries.[20,21] Ultrasound-assisted extraction (UAE) is an emerging technique in both applied research and industry. This method relies on acoustic cavitation, which disrupts cell walls and enhances mass transfer by facilitating solvent penetration into plant tissues, thereby improving the release of target compounds.[22] UAE offers several advantages over conventional methods, including cost-effectiveness, simplicity, enhanced extraction efficiency, and reduced processing time. Importantly, to our knowledge, no previous studies have reported the optimisation of UAE conditions for Mp-p, representing a novel approach compared with earlier work based solely on decoction or maceration.

To achieve chemically diverse extracts with potent biological activity, an optimized extraction approach is required. Response surface methodology (RSM) is a statistical tool enabling the optimization of extraction conditions by simultaneously evaluating the influence of multiple independent variables.[23] Studies on UAE for extracting bioactive and antioxidant compounds from plant by-products have shown that process parameters are commonly optimized using RSM.[24,25]

This study aimed to optimize high-intensity UAE conditions to obtain Mp-p extracts with enhanced phenolic content and AOC using RSM. In addition, bioactive compounds were tentatively identified in both the UAE-optimized and decoction extracts, and their profiles were compared with those of Mp seed extracts. Given the limited information available on L-Dopa content in Mp-p, its presence and concentration were also assessed and compared to that in the seed extracts. The findings of this study are expected to support the valorization of Mp-p, an underutilized agricultural by-product, thereby promoting more sustainable and comprehensive use of this crop.

2 Materials and methods

2.1. Samples

Pods and seeds of *Mucuna pruriens* var.*utilis* were provided by the "El Sombrero-Corrientes" Experimental Station (National Institute of Agricultural Technology – INTA), Argentina. The samples were ground using an electric mill (KSM2 Braun, Naucalpan de Juárez, México) and then sieved to obtain particles ranging between 18 ASMT (1000 μm) and 100 ASMT (140 μm). The pod flours were stored at 10 °C until use.

2.2. Experimental design

To determine the optimal conditions for the UAE of PC and AOC, response surface methodology (RSM) was applied using Design Expert® software (Design Expert, Stat-Ease Inc., Minneapolis, MN). A Box–Behnken Design (BBD) with three independent variables (A = extraction time, B = ultrasound amplitude, and C = ethanol concentration (EC)), was employed. The effects of the independent variables were investigated within the following ranges: time (10–20 min), US amplitude (0–80%) and EC (0–100%), which were selected from the literature.[25] The response variables were total phenolic content (TPC) and AOC, assessed using the DPPH assay (AOCDPPH), (see Section 2.6.1). The experimental conditions for the 16 runs are outlined in Table 1. All experiments, including four central points, were conducted in a randomized order. The proposed quadratic model for each response variable (Y_i) is as follows:

$$Y_i = a + bA + cB + dC + eA^2 + fB^2 + gC^2 + hAB + iAC + jBC + \xi \quad (1)$$

Table 1 Box–Behnken experimental design of extraction parameters for the optimization of TPC and DPPH scavenging activities of Mp-p extracts obtained by UAE

	Independent variables[a]			Response variables[b,c]	
Run no.	*A* (min)	*B* (%)	*C* (%)	TPC (mg GAE per g)	DPPH (TEAC per g)
1	20	80	50	240.55 ± 7.08	1.58 ± 0.35
2	15	80	100	87.80 ± 2.77	0.57 ± 0.95
3	15	80	0	305.47 ± 3.37	1.58 ± 0.32
4	10	0	50	265.66 ± 5.54	1.63 ± 0.45
5	15	0	100	56.22 ± 1.40	0.43 ± 0.26
6	15	0	0	177.89 ± 6.29	1.48 ± 1.20
7	15	40	50	229.72 ± 6.14	1.88 ± 0.49
8	15	40	50	236.91 ± 2.34	1.55 ± 1.22
9	10	80	50	360.86 ± 6.42	2.50 ± 0.72
10	10	40	100	35.47 ± 1.00	0.26 ± 0.53
11	20	40	0	275.00 ± 0.86	0.74 ± 0.51
12	15	40	50	341.92 ± 0.73	1.35 ± 0.65
13	20	0	50	253.35 ± 2.53	1.83 ± 1.02
14	10	40	0	258.91 ± 4.87	1.40 ± 0.98
15	15	40	50	201.39 ± 6.36	1.61 ± 1.11
16	20	40	100	70.19 ± 1.41	0.25 ± 0.32

[a] *A*: extraction time, *B*: ultrasound amplitude and *C*: ethanol concentration. [b] TPC: total phenolic content and AOC_{DPPH}: antioxidant capacity, assessed using the DPPH assay. [c] Standard deviation values are included.

where *A*, *B*, and *C* are the independent variables; *a* is the intercept; *b*, *c*, and *d* are the linear coefficients; *e*, *f*, and *g* are the quadratic coefficients; *h*, *i*, and *j* are the two-factor interaction coefficients; and ξ is the error. The significant terms in the model were determined through analysis of variance (ANOVA) for each response variable. The Design Expert software's graphical and numerical optimization techniques were employed to optimize the response based on the desirability criterion. Three runs of the optimal solution were performed and incorporated into the model for point prediction.

2.3. Ultrasound-assisted extraction (UAE)

Pod flours were placed in a beaker, and solvent was added at a 1 : 10 ratio (100 mL). The solvent used was an ethanol/water mixture, characterized by its EC. High-intensity ultrasound was applied using an ultrasound probe (220-B, CV334 model, Sonics, USA) with a 13 mm tip diameter, connected to a high-intensity ultrasonic processor with temperature control (VCX500, Sonics, USA). The probe was immersed in the solution at a frequency of 20 kHz. Treatments were performed according to the conditions obtained from the experimental design. To prevent overheating, the beaker containing the pod flour dispersion was placed in an ice bath. Then, the dispersions were centrifuged at 3500×*g* for 25 min at 25 °C and vacuum filtered (using Buchner and filter paper) and the supernatants were subjected to a distillation evaporation process using a rotary evaporator (RE100-Pro, DragonLab, Beijing, China) until complete removal of the organic solvent, confirmed by the absence of residual ethanol odor. Finally, they were freeze-dried (model Christ Alpha 1–4 LO, Martin Christ, Osterode am Harz, Germany). The dispersions prepared with 100% ethanol were evaporated to dryness using a rotary evaporator and then subjected to vacuum drying. All the obtained powders were stored at 4 °C until use.

2.4. Decoction extraction

Two grams of pod flour were added to 100 mL of distilled water and boiled for 15 min. The resulting dispersions were then centrifuged (Sorvall ST 8R, Thermo Scientific, Germany) at 3000 rpm for 25 min and then vacuum-filtered. The solvent was evaporated using a rotary evaporator (RE100-Pro, DragonLab, China) and subsequently freeze-dried (CHRIST Alpha 1–4 LO). The dried extracts were stored at 4 °C until use.

2.5. Analysis of phenolic compounds (PCs)

2.5.1 Total phenolic content (TPC). The TPC was determined in the extracts, according to the Folin–Ciocalteu method as previously described by Avalos *et al.*[19] The results were expressed as mg of gallic acid equivalents (GAE) per g extract.

2.5.2 Total *ortho*-diphenol content (TODC). TODC was determined as described by Granato *et al.*[26] Briefly, 50 μL of extract (1 mg mL^{-1}) was mixed with 200 μL of 5% sodium molybdate reagent in 50% ethanol. After incubation in the dark for 25 min, the absorbance was measured at 370 nm using a microplate reader (Multiskan GO, Thermo Scientific, Finland). A calibration curve was constructed using a solution of caffeic acid (0–140 mg L^{-1}). The results were expressed as mg of caffeic acid equivalents (CAAE) per g extract.

2.5.3 Total flavonoid content (TFC). TFC was determined following the method proposed by Granato *et al.*[26] In a 96-well microplate, 100 μL of distilled water, 10 μL of 5% (w/v) $NaNO_2$ solution, and 25 μL of extract (0.5–1 mg mL^{-1}) were added. After 5 min, 25 μL of 10% (w/v) $AlCl_3 \cdot {}_6H_2O$ solution was added and allowed to react for another 5 min. Then, 50 μL of 1 M NaOH was added to each well, and the mixture was incubated for 5 min under continuous agitation. The absorbance was measured at 510 nm using a microplate reader (Multiskan GO, Thermo Scientific, Finland). A calibration curve was constructed using (+)-catechin as the standard (0.75 mg mL^{-1}). TFC was expressed as mg of (+)-catechin equivalent (CAE) per g extract.

2.6. Antioxidant capacity (AOC) assays

2.6.1 DPPH radical scavenging. The DPPH free radical scavenging assay was conducted according to Brand-Williams *et al.*[27] A stock solution of DPPH was prepared by dissolving 20 mg of DPPH in 100 mL of 80% (v/v) methanol. A working solution was obtained by diluting the stock solution (1 : 5) with 80% (v/v) methanol. In a 96-well microplate, 20 μL of the extract (5 mg mL^{-1}) was mixed with 280 μL of the DPPH working solution and incubated in the dark. After 60 min, the absorbance at 517 nm was recorded using a microplate reader (Multiskan GO, Thermo Scientific, Finland). A calibration curve was constructed using a standard solution of Trolox (2 mg dissolved in 10 mL of 80% methanol). The antioxidant activity was

expressed as mmol of Trolox equivalents antioxidant capacity (mmol TEAC) per g extract.

2.6.2 ABTS radical cation scavenging activity. The activity of the antioxidants was determined by detecting their ability to scavenge the free radical of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) using the method described by Re *et al.*[28] The $ABTS^{\bullet+}$ radical was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in the dark at room temperature (25 °C) for 16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm. Ten μL of the sample (0.5 mg mL^{-1}) and 1 mL of $ABTS^{\bullet+}$ solution were mixed in an Eppendorf tube. After incubation for 45 min, 300 μL of the mixture was transferred into a 96-well microplate. The absorbance of initial and endpoints was measured at 734 nm in a microplate reader (Multiskan GO, Thermo Scientific, Finland). A calibration curve was constructed using a standard solution of Trolox (2 mg dissolved in 10 mL of 80% methanol). The antioxidant activity was expressed as mmol of Trolox equivalents antioxidant capacity (mmol TEAC per g extract).

2.6.3 Ferric reducing antioxidant power (FRAP) assay. FRAP was estimated according to the method described by Pérez-Burillo *et al.*[29] The FRAP reagent was prepared by mixing 2.50 mL of 10 mM 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) solution in 40 mM HCl, 2.50 mL of 20 mM $FeCl_3 \cdot 6H_2O$, and 25 mL of 0.3 M acetate buffer (pH 3.6). In a 96-well microplate, 280 μL of the FRAP reagent was mixed with 20 μL of the extract. After incubation in the dark for 30 min, the absorbance was measured at 593 nm using a microplate reader (Multiskan GO, Thermo Scientific, Finland). A calibration curve was constructed using Trolox solutions (0.01–0.40 mg mL^{-1}). The results were expressed as Trolox equivalents antioxidant capacity (mmol TEAC per g extract).

2.7. Analysis of individual phenolic compounds by LC-MS

Identification of PCs was performed using a Shimadzu LCMS-8040 Triple Quadrupole Mass Spectrometer (Shimadzu Corporation, Japan) using electrospray ionization, equipped with a quaternary pump (LC-20AD), degassing device, autosampler (SIL-20A), PDA detector (SPD-M20A), and LC and LC/MS system software. The dry extracts were reconstituted in the corresponding extraction solvent and filtered through a 0.45 μm nylon membrane. The injection volume was 10 μL. The separation of the compounds was performed on a Kinetex C18-EVO (C18 column, 5 μm particle size, 150 × 4.6 mm i.d., Phenomenex, CA, USA) thermostated at 35 °C. The chromatographic conditions of the method used were a mobile phase consisting of water/trifluoroacetic acid (0.1%) as solvent A and acetonitrile as solvent B. The gradient program at a flow rate of 1.3 mL min^{-1} was as follows: 0–100% A (0.1% trifluoroacetic acid) for 3 min, 4–30% B (acetonitrile) for 50 min, 30–98% B for 5 min, and isocratic 98% B for 2 min. The compounds were detected by absorbance scanning from 280 to 600 nm. HPLC-mass spectrometry (HPLC-MS) conditions were sheath gas flow, 15 mL min^{-1}; capillary voltage, 4500 V; and capillary temperature, 250 °C. Spectra were obtained in positive ion mode between *m*/*z* 100 and 2000 amu and in full scan mode from 1.5 to 60 min. The phenolic compounds were characterized by using their M+ and fragmentation patterns.

2.8. Identification and quantification of L-Dopa

The standards and samples were dissolved in UPLC-MS-grade ultrapure water acidified with formic acid. They were then filtered and diluted in an 80 : 20 water/acetonitrile mixture, achieving a final sample concentration of 100 ppm. A total of 5 μL was injected into a Shimadzu UPLC coupled to a Sciex Q-TRAP 4500 mass spectrometer with an ESI ionization source. Standard and sample analyses were performed on an UPLC system consisting of a LC-40 D solvent delivery system, a DGU-403 degasser, a CTO-40 S column oven, a SIL-40 C autosampler and a CBM-20A controller (Shimadzu). UPLC conditions were optimized using a Shim-pack GIST C18 column (Shimadzu 2.1 × 100 mm, 2 μm). Gradient elution was performed with mobile phases A (water with 0.1% formic acid) and B (acetonitrile). The following gradient elution was used at a flow rate of 0.4 mL min^{-1}: where B was maintained at 10% from 0 to 5 min, 26% from 5 to 7 min, 40% from 7 to 9 min, 65% from 9 to 12 min, 95% from 12–14 min and 10% from 14 to 16 min. The column temperature was maintained at 40 °C and the autosampler temperature was held at 4 °C. Spectral data acquisition and analysis were conducted using the software MultiQuant. L-Dopa was identified by monitoring precursor-product ionic transitions at *m*/*z* 198.0 → 152.3 (primary transition), 198.0 → 181.0 (secondary transition), and 198.0 → 138.0 (tertiary transition). Quantification was based on the primary transition (198.0 → 152.3) and achieved by comparison to a calibration curve generated using a standard compound. Turboionspray source was operated in positive ionization mode. Mass spectrometric analysis was conducted in multiple reaction monitoring (MRM) mode and the optimized mass spectrometric parameters were as follows: an ion spray voltage of 5500 V; turboionspray temperature of 300 °C; curtain gas of 25 psi; nebulizing gas of 50 psi; declustering potential of 40 V; entrance potential of 10 V; collision cell exit potential of 12 V; collision energy of 17 eV for 198.0 → 152.3, 15 eV for 198.0 → 181.0 and 23 eV for 198.0 → 138.0. The dwell time was 20 ms. The acquisition time of the mass spectrometer was the same as the chromatographic run time.

2.9. Statistical analysis

Assays described in sections 2.5 and 2.6 were performed in triplicate. For each parameter evaluated, differences between extracts obtained from pods and seeds were analyzed by analysis of variance (ANOVA) followed by the least significant difference test LSD Fisher ($\alpha = 0.05$). All statistical analyses were conducted using Infostat software.[30]

3 Results and discussion

3.1. Fitting the models

The optimal extraction conditions depend on the specific plant matrix under investigation. In this study, RSM was utilized to

Table 2 ANOVA for response surface models: the estimated regression model of the relationship between response variables (TCP and AOC_{DPPH}) and independent variables[a]

TPC	Sum of squares	Degree of freedom	Mean square	*F*-Value	*p*-Value	
Model	120 200.00	2	60 120.80	24.21	<0.0001	Significant
C	73 649.30	1	73 649.30	29.66	0.0001	
C^2	46 592.30	1	46 592.30	18.76	0.0008	
Residual	32 282.19	13	2483.25			
Lack of fit	20 912.04	10	2091.20	0.55	0.7915	Not significant
Pure error	11 370.14	3	3790.05			
Cor total	152 500.00	15				
R^2	0.7883					
Adjusted R^2	0.7558					
Predicted R^2	0.6915					
Adeq precision	9.4483					

AOC_{DPPH}	Sum of squares	df[b]	Mean square	*F*-Value	*p*-Value	
Model	6.0400	6	1.0100	30.58	<0.0001	Significant
A	0.2415	1	0.2415	7.33	0.0241	
B	0.0940	1	0.0940	2.85	0.1254	
C	1.7300	1	1.7300	52.50	<0.0001	
AB	0.3158	1	0.3158	9.59	0.0128	
B^2	0.4064	1	0.4064	12.34	0.0066	
C^2	3.2600	1	3.2600	98.88	<0.0001	
Residual	0.2964	9	0.0329			
Lack of fit	0.1538	6	0.0256	0.54	0.7617	Not significant
Pure error	0.1425	3	0.0475			
Cor total	6.3400	15				
R^2	0.9532					
Adjusted R^2	0.9221					
Predicted R^2	0.8780					
Adeq precision	20.1847					

[a] *A*: extraction time, *B*: ultrasound amplitude and *C*: ethanol concentration. [b] TPC: total phenolic content and AOC_{DPPH}: antioxidant capacity assessed using the DPPH assay.

optimize key extraction parameters. The selection of solvents was influenced by the promising results observed from macerating Mp-p with ethanol–water mixtures, in addition to their sustainability and compatibility for formulating food ingredients.[19] A solid-to-solvent ratio of 1 : 10 was maintained across all experiments, in line with the established reference.

The values obtained for TPC and AOC_{DPPH}, evaluated across the 16 runs suggested by the experimental design, are shown in Table 1, while in Table 2 the significant coefficients and equivalent *p*-values are presented, all below 0.05, demonstrating the significant effect of these coefficients on relevant response variables. Model validity was further confirmed through lack-of-fit tests, which showed being not significant ($p > 0.05$) for either response variable, indicating that the model was suitable. For TPC and AOC_{DPPH}, the coefficients of determination (R^2) were 0.7883 and 0.9532, respectively, indicating that the regression models effectively explained their variability. The lower R^2 for TPC may be due to matrix effects from other pod constituents, which can interfere with phenolic quantification, and the contribution of non-phenolic antioxidants such as alkaloids, saponins, flavonoids, coumarins, and alkylamines.[6] Despite this, the R^2 value remains acceptable for model adequacy ($R^2 > 0.75$)[31] and reflects system complexity.[32]

The close similarity between R^2 and adjusted R^2 values in both models suggested a strong correlation between observed and predicted values, confirming the model robustness. The models' adequacy was further validated by the signal-to-noise ratio, measured as the "adeq. precision". A ratio greater than 4 is desirable, indicating sufficient signal strength relative to the noise. The ratios of 9.4483 for TPC and 20.1847 for AOC_{DPPH} confirmed that the models provide an adequate signal to effectively navigate the design space, ensuring reliable predictions and optimization within the experimental framework.

3.2. Analysis of TPC

By applying multiple regression analysis on the experimental data, the response TPC, independent variables, and the test variables were related by a second-order polynomial equation incorporating the significant terms, which is shown in eqn (2) (for coded factors):

$$Y_{TPC} = +266.30 - 95.95C - 107.93C^2 \quad (2)$$

Statistical analysis revealed that TPC was significantly influenced by EC, both as a linear term (*C*) and a quadratic term (C^2) ($p < 0.05$), whereas extraction time and ultrasound

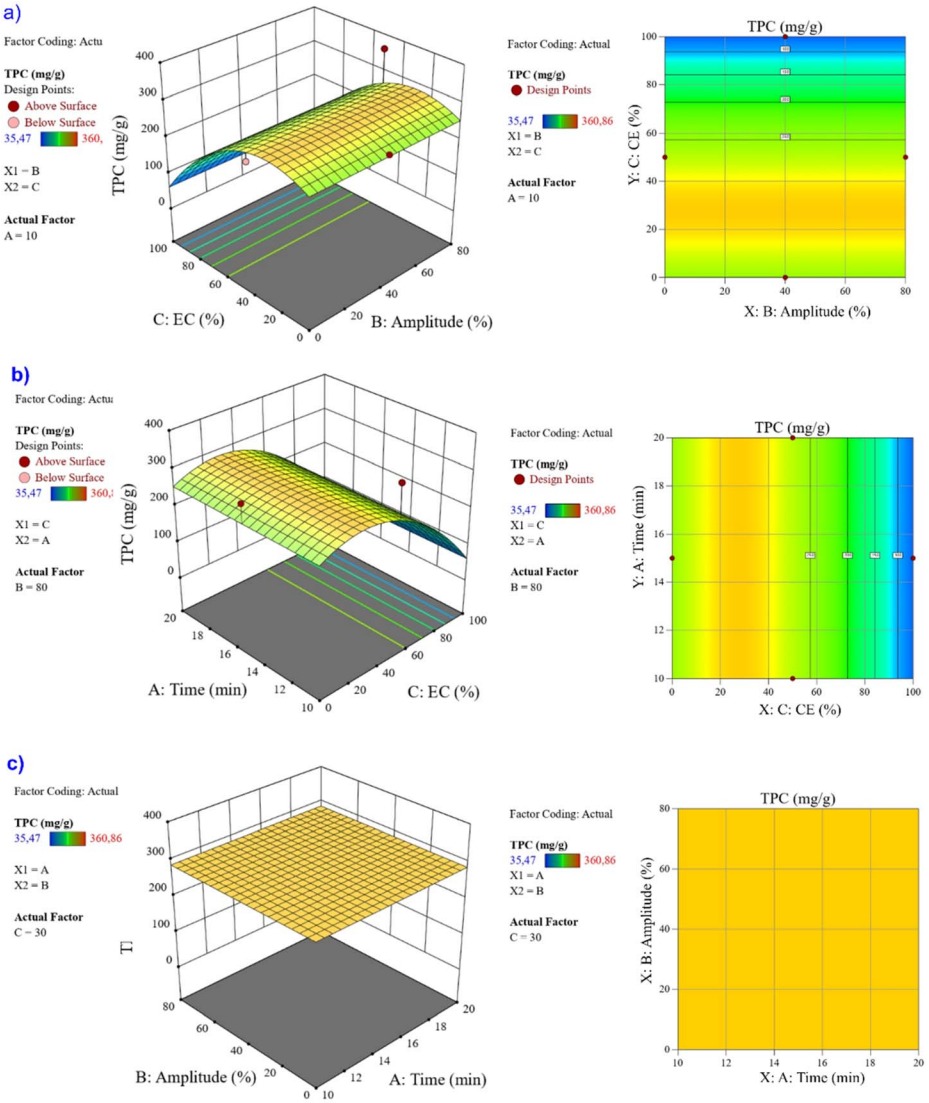

Fig. 1 Response surface plots for the effect of (a) amplitude and ethanol concentration, (b) ethanol concentration and time, and (c) time and amplitude, on the total phenolic content.

amplitude did not have a statistically significant effect ($p > 0.05$). As shown in eqn (2), EC negatively affected the TCP recovery (−95.95), with an even stronger inhibitory effect in its quadratic form (−107.93).

Fig. 1 displays the 3D response surface plots for TPC. As shown in Fig. 1a and b, a clear dependence on EC is observed, with a significant increase in TPC values ($p < 0.05$) up to approximately 50% ethanol, followed by a substantial decline at higher ethanol levels. Notably, increasing the EC up to 100% resulted in a marked decrease in the TPC value. Furthermore, Fig. 1c illustrates that when EC is maintained at 30%, varying ultrasound amplitude and extraction time does not affect TPC, as high values are consistently observed across all data points.

This behaviour can be attributed to changes in the solubility and diffusivity of bioactive compounds, which are strongly influenced by EC. While moderate EC improves the extraction of certain PCs, excessive ethanol levels may reduce the recovery of a broader range of PCs, as previously reported.[33,34] These findings align with earlier studies, in which maceration of Mp-p using either pure ethanol or water yielded relatively low TPC values, underscoring the importance of optimizing solvent composition to enhance extraction efficiency.[19].

3.3. Analysis of DPPH radical scavenging activity

By applying multiple regression analysis on the experimental data, the response AOC_{DPPH} and the independent variables, the test variables were related by a second-order polynomial equation incorporating the significant terms, which is shown in eqn (3) (for coded factors):

$$Y_{AOC} = +1.58 - 0.17A + 0.11B - 0.46C - 0.28AB + 0.32B^2 - 0.90C^2 \quad (3)$$

Statistical analysis revealed that the linear and quadratic terms of EC (C and C^2, respectively), the linear term of extraction time (A), and the quadratic term of ultrasound amplitude

(B^2) were statistically significant ($p < 0.05$). Although the linear term of ultrasound amplitude (B) was not significant ($p > 0.05$), it was retained in the model due to the significance of its quadratic term and its interaction with extraction time (AB).

Fig. 2 displays the 3D response surface plots illustrating the significant interaction effects on the AOCDPPH variable. Unlike the TPC variable, which was primarily influenced by ethanol concentration, AOC_{DPPH} showed a more complex dependence on the process variables. As shown in the plots, the lowest AOC_{DPPH} values were observed at the extremes of ethanol concentration, *i.e.*, pure water and 100% ethanol, with a peak achieved at around 50% ethanol.

Fig. 2a and c demonstrate that an increase in ultrasound amplitude enhances the extraction of compounds with radical scavenging activity, where the highest AOC_{DPPH} values were achieved at an 80% amplitude. This result confirmed the positive effect of this technology on the recovery of bioactive antioxidants.

An interesting finding was that the highest AOC_{DPPH} values were achieved with shorter extraction times, while prolonged extraction times led to a significant decrease in AOC_{DPPH} (Fig. 2b and c). In previous studies, conventional extraction of Mp-p with 50% ethanol required 24 h of maceration at room temperature to yield extracts with significant AOC.[19] Typically, extraction methods using water and organic solvent mixtures require maceration times of at least one hour or more.[35] Therefore, the significant reduction in extraction time achieved in this study highlights the efficiency of ultrasound as a green technology for bioactive compound recovery.

The range extraction time selected in this study was based on previous research.[25] However, for more precise optimization of the process, upcoming studies could evaluate even shorter extraction times, such as 5 min, to assess their effects on extraction efficiency and the stability of bioactive compounds in the final extract.

3.4. Optimization and model validation

To identify the extraction conditions that simultaneously maximize the TPC and AOC, a multi-response optimization was

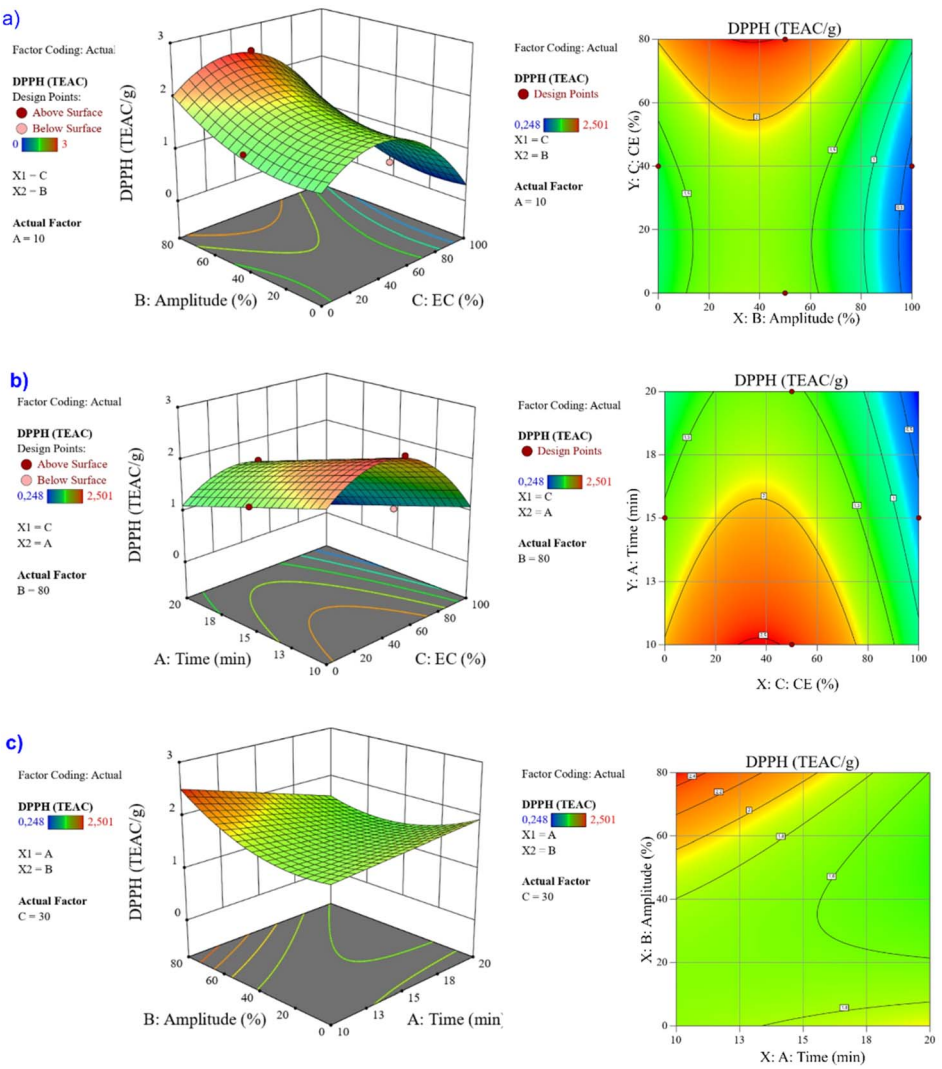

Fig. 2 Response surface plots for the effect of (a) amplitude and ethanol concentration, (b) ethanol concentration and time, and (c) time and amplitude, on the antioxidant activity evaluated by DPPH assay.

Table 3 Optimum UAE conditions for Mp-p as predicted by the statistical model and those obtained experimentally[a,b]

	A (min)	*C* (%)	*B* (%)	TPC (mg GAE per g)	AOC_{DPPH} (TEAC per g)
Predicted	10	30	80	287.54	2.50
Experimental	10	30	80	274.21 ± 1.43	2.41 ± 0.11
%RSD				3.97	4.40

[a] *A*: extraction time, *B*: ultrasound amplitude and *C*: ethanol concentration. [b] TPC: total phenolic content and AOC_{DPPH}: antioxidant capacity, assessed using the DPPH assay. The results are expressed as mean ± sd of three independent experiments.

Table 4 Extraction yield, total phenolic content (TPC), *ortho*-diphenolic content (TODC), total flavonoid content (TFC), and antioxidant capacity of p-US, p-DE, and s-US extracts[a]

Extract	Yield (%)	TPC (mg GAE per g)	TODC (mg CAAE per g)	TFC (mg CAE per g)
p-US	13.80 ± 0.12b	274.21 ± 1.43a	59.30 ± 1.29a	69.78 ± 0.90b
p-De	12.51 ± 0.22c	162.25 ± 5.50c	53.32 ± 3.85b	57.02 ± 0.92c
s-US	24.50 ± 0.67a	271.65 ± 0.03b	31.46 ± 0.45c	130.58 ± 10.3a

[a] Different letters (a, b, and c) show values that are statistically significantly different within a column at the 95% confidence level ($p < 0.05$), expressed in terms of mean ± SD.

performed, assigning equal statistical weight to both response variables.

Statistical optimization determined the optimal conditions to be 10 min extraction time, 80% amplitude and 30% ethanol concentration. Based on these conditions, the proposed mathematical model predicted the values for the response variables, which are presented in Table 3. To validate the model, three independent experiments were conducted under optimized conditions, measuring both TPC and AOC_{DPPH}.

The results confirmed the validity of the model, as evidenced by the relatively low residual deviation (%RSD), which indicates a high degree of agreement between the experimental and predicted values. Moreover, the experimental model identified multiple variable combinations with statistically acceptable desirability for maximising response variables. The validated optimal conditions exhibited a high desirability score (0.880) (see Fig. S1 in the SI).

3.5. Extract characterization

The analyses were conducted on the extract obtained under optimized UAE conditions (p-US). Additionally, the pod extract obtained *via* the decoction method (p-De) and the seed extract of Mp obtained under optimized UAE conditions (s-US) were evaluated for comparative assessment. The selection of the decoction method was based on its previously reported effectiveness in enhancing AOC and its widespread use in the preparation of herbal infusions and tinctures.[19,36] The seed extract was obtained under optimized UAE conditions to facilitate a direct comparison between matrices and leverage the more extensive data available for seed-derived compounds.

3.5.1 Phenolic compound content. Table 4 shows the TPC, TODC, and TFC values, determined for the three samples under study. The highest extraction yield was observed in s-US (24.50%), followed by p-US (13.80%) and p-De (12.51%). The greater yield in s-US may be attributed to the presence of additional components such as proteins and soluble carbohydrates, which are abundant in this matrix.[6] The yields of the pod extracts obtained by both methods were similar, although higher than those obtained by maceration.[19]

p-US exhibited significantly higher TPC, TODC, and TFC values than p-De, indicating that UAE enhanced PC recovery. TODC, which contributes to radical stability *via* intramolecular hydrogen bonding,[37] represented a significant proportion of the TPC (56% and 52% in p-US and p-De, respectively). Interestingly, the s-US extract exhibited lower TPC and TODC values than p-US, yet a significantly higher TFC content, nearly twice that of p-US. This suggests that Mp seeds are richer in flavonoids; however, the influence of UAE on matrix composition cannot be ruled out.

Extracts obtained with 95% ethanol using Soxhlet extraction for 4 h from *M. pruriens* var. *utilis* and var. *pruriens* seeds exhibited TPC values of 252.96 ± 3.27 mg GAE per g and 223.54 ± 9.33 mg GAE per g, respectively, comparable to those obtained in the present study.[38] In contrast, leaf extracts of *M. pruriens* obtained by Soxhlet extraction with ethanol and methanol over 36 h yielded lower TPC values (155.89 and 72.11 mg GAE per g, respectively) compared to both pod and seed extracts.[10] These findings demonstrate the influence of the plant part and extraction method on the recovery of phenolic compounds.

Comparatively, our findings are consistent with previous reports demonstrating the potential of UAE as a green extraction technique. Although pressurized liquid extraction (PLE) has achieved higher yields in cowpea pod[39] and range waste,[40] the TPC obtained by UAE in our study was superior, indicating its effectiveness for extracting specific bioactives from *M. pruriens* pods. Compared with microwave-assisted extraction, UAE operates under milder thermal conditions, which reduces degradation of thermolabile compounds while maintaining

Table 5 Antioxidant capacity of p-US, p-DE, and s-US extracts evaluated by DPPH, FRAP, and ABTS assays[a]

Extract	DPPH (mmol TEAC per g)	FRAP (mmol TEAC per g)	ABTS (mmol TEAC per g)
p-US	2.41 ± 0.11b	3.67 ± 0.08b	1.87 ± 0.09b
p-DE	0.88 ± 0.04c	1.26 ± 0.07c	0.46 ± 0.02c
s-US	3.83 ± 0.09a	3.96 ± 0.05a	3.17 ± 0.11a

[a] Different letters (a, b, and c) show values that are statistically significantly different within a column at the 95% confidence level ($p < 0.05$), expressed in terms of mean ± SD.

competitive efficiency.[41] These observations emphasise that extraction efficiency and phenolic recovery are strongly influenced by the interaction between the extraction technique and the matrix composition.

From a practical standpoint, UAE is promising for scale-up due to its short processing times, low operational costs, and use of food-grade solvents. While industrial implementation faces challenges, particularly regarding equipment capacity and energy requirements for high-intensity ultrasound, UAE remains a sustainable and feasible strategy for the valorisation of agro-industrial by-products relative to other emerging extraction methods.

3.5.2 Antioxidant capacity (AOC). The AOC of plant extracts is influenced by multiple mechanisms, which can be evaluated using different analytical methods.[26] The DPPH, ABTS and FRAP assays are widely employed to evaluate AOC. The DPPH assay assesses a compound's ability to neutralize free radicals through hydrogen donation, while the ABTS assay quantifies the reduction of the $ABTS^{\bullet+}$ radical cation based on decolourisation. Additionally, the FRAP assay assesses the extract's capacity to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. The AOCs of the extracts under study, as determined by these assays, are presented in Table 5.

In the pod extracts, all three assays used to evaluate AOC revealed a marked difference between the UAE and decoction methods. The p-US extract exhibited significantly higher AOC, with values of 2.41 ± 0.11 mmol TEAC per g in the DPPH assay, 3.67 ± 0.08 mmol TEAC per g in the FRAP assay, and 1.87 ± 0.09 mmol TEAC per g in the ABTS assay. In contrast, the p-De extract showed markedly lower values, at 0.88 ± 0.04 mmol TEAC per g, 1.26 ± 0.07 mmol TEAC per g, and 0.46 ± 0.02 mmol TEAC per g, respectively. These results are consistent with the higher phenolic content measured in p-US compared to p-De, supporting the notion that UAE enhances the recovery of compounds with antioxidant potential. However, it is also important to consider that the high temperatures required during decoction may lead to the degradation of PC or a reduction in their AOC.[42]

The s-US extract exhibited higher AOC than the pod extracts across all three assays. Although this matrix showed lower TPC and TODC values than p-US, the increased AOC may be attributed to its higher TFC content. In this regard, previous studies

Table 6 Tentatively identified compounds in pod extracts (p-US and p-DE) and seed extracts (s-US) by HPLC-DAD-MS[a]

		RT (min)			
Compound	Formula	p-US	p-DE	s-US	$[M + H]^+$
Ferulic acid	$C_{10}H_{10}O_4$	49.934	49.885	49.911	195
Ursolic acid	$C_{30}H_{48}O_3$	10.083	n.d.	n.d.	457
Catechin	$C_{15}H_{14}O_6$	15.488	15.56	n.d.	291
L-Dopa	$C_9H_{11}NO_4$	45.672	45.664	45.648	197
Quercetin	$C_{15}H_{10}O_7$	53.101	52.742	50.773	303
Gallocatechin	$C_{15}H_{14}O_7$	55.306	55.302	55.303	307
Chlorogenic acid	$C_{16}H_{18}O_9$	53.343	54.141	52.74	355
Gallocatechin gallate	$C_{22}H_{18}O_{11}$	54.144	54.277	55.303	459
Stigmasterol	$C_{29}H_{48}O$	55.529	n.d.	45.648	413
Daucosterol	$C^{35}H_{60}O_6$	54.144	54.141	54.14	577
Glutathione	$C_{10}H_{17}N_3O_6S$	55.529	55.302	55.303	308
6-Methoxyharman	$C_{13}H_{16}N_2O$	57.373	n.d.	n.d.	213
Stizolamine	$C_7H_{11}N_5O_2$	n.d.	13.234	n.d.	198
Daidzein	$C_{15}H_{10}O_4$	57.056	56.421	55.963	255
Asperglaucide	$C_{27}H_{28}N_2O_4$	n.d.	24.848	n.d.	445
3-Hydroxy-9-methoxypterocarpane	$C_{16}H_{14}O_5$	n.d.	n.d.	40.768	271
Genistein	$C_{15}H_{10}O_5$	n.d.	n.d.	18.036	271
Prunetin (7-*O*-Methyl-genistein)	$C_{16}H_{12}O_5$	38.014	n.d.	n.d.	285
Procyanidin dimer	$C_{30}H_{26}O_{12}$	52.627	52.742	52.74	579
Serotonin	$C_{10}H_{12}N_2O$	57.373	57.624	n.d.	177
Kaempferol	$C_{15}H_{10}O_6$	50.795	50.779	50.773	287
β-Sitosterol	$C_{29}H_{50}O$	53.343	52.742	52.74	415

[a] n.d., not detected; p-US, pod extracts obtained by ultrasound, p-DE, pod extracts obtained by decoction; s-US, seed extracts obtained by ultrasound.

have shown that flavonoid-rich extracts from *M. pruriens* seeds exhibit enhanced radical-scavenging activity.[10] Furthermore, other bioactive compounds, such as amino acids and alkaloid, have also been shown to contribute to antioxidant mechanisms in *Mucuna* spp. seeds.[43] These findings suggest that s-US extracts may contain a wider range of antioxidant compounds. Nevertheless, the considerable AOC exhibited by p-US remains highly promising, particularly given that these pod materials are regarded as agricultural waste.

3.6. LC-MS analysis

Qualitative analysis using HPLC-MS/MS was conducted to tentatively identify bioactive compounds in Mp-p extracts (p-US and p-De) by comparing the results with literature data. Similarly, the bioactive compounds present in the seed extract (s-US) were analysed to provide a comprehensive comparison.

Table 6 displays the 22 compounds tentatively identified in the analyzed samples. The identified metabolites include phenolic acids, flavonoids, alkaloids, sterols, and other bioactive compounds, many of which have been previously reported in *Mucuna* species seeds and other plant parts from various geographical regions using different extraction methods.[44] However, the presence of these compounds in pod-derived extracts remains less explored. L-Dopa, the primary bioactive compound of *M. pruriens*, was detected across all extracts, highlighting its consistent presence and pharmacological relevance, particularly for the treatment of neurodegenerative disorders.[11]

Among the phenolic acids, ferulic acid and chlorogenic acid were present in all extracts, reinforcing their potential antioxidant contributions. However, gallic acid and caffeic acid, which have been previously reported in *M. pruriens* pods,[19] were not identified in any of the samples. This absence may suggest lower concentrations of these compounds in the analyzed extracts or possible variations in plant chemotypes.

Serotonin was exclusively detected in the pod-derived extracts (p-US and p-De), supporting previous findings that serotonin accumulation is specific to *Mucuna pruriens* pods.[45]

Additionally, glutathione, a well-known antioxidant tripeptide, was detected across all samples, reinforcing the antioxidant profile of *M. pruriens* pods.[44]

Flavonoids such as quercetin, kaempferol, gallocatechin, and its gallate derivatives were consistently detected in all extracts while catechin was found exclusively in the pod extracts (p-US and p-De), indicating a chemical difference between the pod and seed extracts. Some of these flavonoids, such as epigallocatechin gallate, kaempferol, and catechin have demonstrated have both antioxidant and neuroprotective effects, including the inhibition of α-synuclein aggregation, which is a key factor in neurodegenerative diseases.[11]

Additionally, the antioxidant and oestrogenic activities of ethanolic extracts of *Mucuna sempervirens* were attributed to the presence of flavonoids, including kaempferol, quercetin, and their derivatives.[46]

Isoflavones, including daidzein, genistein, and prunetin, exhibited differential distribution across extracts. Daidzein was detected in all samples,[47] while prunetin was found exclusively in p-US, and genistein was only detected in s-US. Similarly, medicarpin (3-hydroxy-9-methoxypterocarpane), an isoflavonoid derivative with α-glucosidase inhibitory activity, was detected solely in s-US.[48]

Procyanidin dimers, known for their strong radical-scavenging ability and potent antioxidant properties against lipid peroxidation, were detected in all extracts.[49]

Ursolic acid, a triterpenoid with neuroprotective effects comparable to L-Dopa,[50] and 6-methoxyharman, an alkaloid previously identified in *M. pruriens* leaves,[44] were exclusively detected in the p-US extract.

Sterol analysis revealed the presence of stigmasterol, daucosterol, and β-sitosterol, compounds recognized for their pharmacological properties.[51] While daucosterol and β-sitosterol were present in all extracts, stigmasterol was found exclusively in the UAE-derived extracts (p-US and s-US).

The differential distribution of plant metabolites across tissues was reflected in our findings. Most compounds were identified in pod extracts, whereas medicarpin and genistein were exclusively detected in seed extracts (s-US), suggesting tissue-specific localization or concentrations in pods below the detection limit.

Regarding the extraction methodology, compounds such as ursolic acid, stigmasterol, 6-methoxyharman, and prunetin were detected in the p-US extract. This suggests that UAE facilitates their release, while these compounds may otherwise degrade or exhibit poor solubility under the high-temperature conditions used in decoction.[52,53] Conversely, asperglaucide (aurantiamide acetate), a dipeptide alkaloid with known anti-inflammatory, antibacterial, antioxidant, and anticancer activities, and stizolamine, a pyrazine derivative associated with pollinator signals, were exclusively detected in the p-De extract.[54]

Thus, the compounds detected in the p-US extract not only support its AOC but also suggest additional potential applications, highlighting the efficiency of UAE as a sustainable and effective extraction technique.

In multiple reaction monitoring (MRM) analysis, the use of standards is essential for accurate quantification, correction of matrix effects, and maintaining consistent ionization efficiency. In this study, however, the application of MRM was limited to L-Dopa, a major bioactive compound in *Mucuna pruriens*, due to the limited availability and high cost of reference standards.

3.7. Quantification of L-Dopa

Several plant species are recognised as natural sources of L-Dopa, with Mp seeds exhibiting the highest concentrations, typically ranging from 1.2–10%.[55] Reported values for other plant tissues include 0.16–0.36% in leaves, 0.20–0.30% in stems, and 0.13–0.17% in roots.[56] However, data on L-Dopa levels in Mp-p remain limited. A review reported that whole pods may contain up to 4% L-Dopa, with concentrations decreasing during maturation; nonetheless, that study did not specify the *Mucuna* species, extraction methods, or experimental conditions, making it difficult to validate or compare the findings.[44]

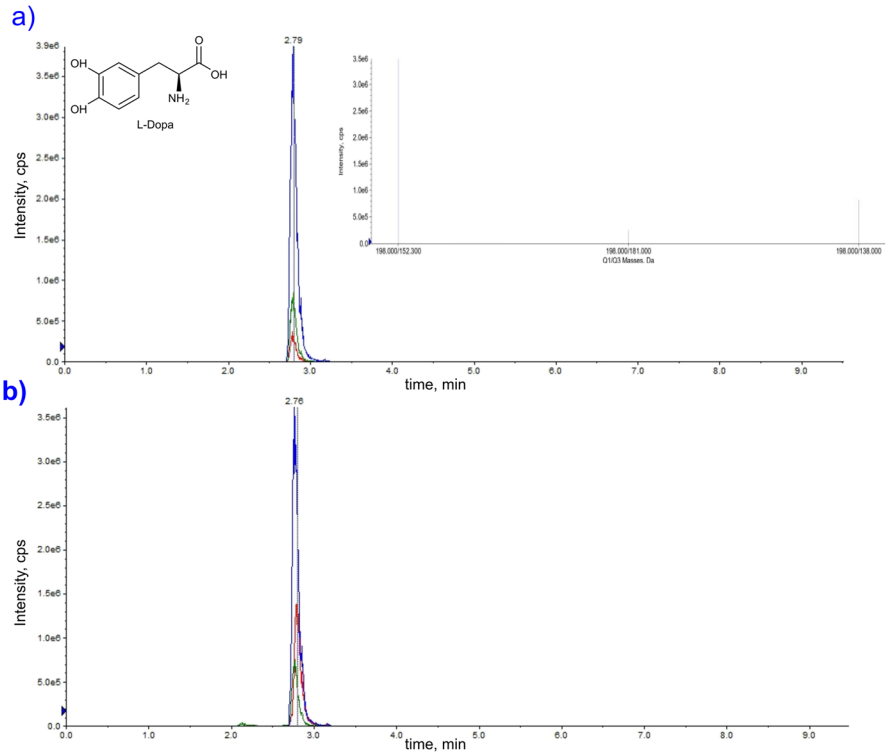

Fig. 3 (a) Extracted ion chromatogram (XIC) and multiple reaction monitoring (MRM) chromatogram of the L-DOPA standard (1 ppm); (b) XIC of L-DOPA detected in the p-US extract.

In this study, qualitative analysis using UPLC-MS/MS confirmed the presence of L-Dopa in both pod and seed extracts. Representative chromatograms are shown in Fig. 3, illustrating the detection of L-Dopa in the standard solution and in the p-US extract.

Quantification results revealed that the p-US extract contained a substantial amount of L-Dopa (5.80%), although this was lower than the concentration observed in the seed extract (9.50%). In contrast, L-Dopa was not quantifiable in the p-DE extract under the conditions used. These findings align with previous reports indicating that L-Dopa degrades significantly under heat treatments such as cooking and autoclaving.[57] The detection of L-Dopa in p-US extracts is particularly noteworthy, as it highlights the potential of this often-overlooked by-product as a valuable alternative source of this bioactive compound.

4 Conclusion

This study demonstrated that ultrasound-assisted extraction (UAE) is an efficient and environmentally sustainable method for recovering bioactive compounds from *Mucuna pruriens* pods, an underutilized agricultural by-product. By optimising UAE parameters (ethanol concentration, ultrasound amplitude, and extraction time), reductions in ethanol concentration and extraction time were achieved, leading to improved recovery of phenolic compounds and antioxidant activity in the optimized extracts compared to those obtained by traditional decoction methods.

Phytochemical analysis confirmed the presence of key bioactive compounds, including a significant concentration of L-Dopa (5.80%) in the optimised extracts, highlighting the potential value of *Mucuna pruriens* pods as a source of health-promoting ingredients.

Beyond improving extraction efficiency, this approach provides a sustainable strategy for agro-industrial waste valorization, with promising applications in the food, nutraceutical, and pharmaceutical industries.

Author contributions

Beatriz I. Avalos: data curation, investigation, methodology, writing – original draft. Belén A. Acevedo: conceptualization, formal analysis, funding acquisition, writing – review &editing. Juan P. Melana Colavita: investigation, methodology. Romina Curbelo: investigation, methodology. Eduardo Dellacassa: investigation, methodology, writing – review & editing. Margarita M. Vallejos: conceptualization, formal analysis, funding acquisition, writing – review & editing.

Conflicts of interest

The authors declare no competing interest.

Data availability

Data will be made available on request.

Supplementary information: contour plots of desirability. See DOI: https://doi.org/10.1039/d5fb00206k.

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