Development of an aqueous ultrasound-assisted extraction process of bioactive compounds from beet leaves: A proposal for reducing losses and increasing biomass utilisation

Short title: Ultrasound-assisted extraction: A proposal for increasing biomass utilisation.

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ABSTRACT

BACKGROUND: Red beet plants are cultivated worldwide for the consumption of their roots, generating large amounts of unexploited by-products. In particular, beet leaves (BL) represent about 50% of the whole plant and are usually discarded as waste. This constitutes not only an economic issue, since multiple resources invested in the production will be wasted, but also an environmental problem because of the pollution associated with their disposal. However, BL comprise an important source of functional compounds (polyphenols and betalains) that could be recovered from the raw material, representing a sustainable solution for the underutilisation of this by-product. This study proposes the recovery of polyphenols and betalains using an aqueous ultrasound-assisted extraction (UAE) process at different powers (35, 50, and 100 W) that was characterised and optimised.

RESULTS: UAE significantly enhanced the recovery of bioactive compounds and shortened the time required for extraction in comparison to traditional macerations (35<50<100 W). During UAE the temperature of the systems increased as a function of the power applied, favouring the recovery of these phytochemicals. Additionally, Box-Behnken design and Response Surface Methodology were employed to optimise UAE conditions (90 W ultrasound power, 1:20 solid-liquid ratio, 16 min extraction time), under which the yields were 14.9 mg/g (polyphenols), 949.1 µg/g (betaxanthins), and 562.2 µg/g (betacyanins), consistent with the values predicted by the models.

CONCLUSION: This study enabled the development of a green-solvent UAE process that constitutes an effective post-harvest by-products strategy to minimize losses and increase biomass utilisation through the recovery of bioactive compounds from BL, promoting sustainability in the agri-food chain.

Keywords: Ultrasound-Assisted Extraction; Beet Leaves; Bioactive compounds; Biomass utilisation; Box-Behnken Design; Response Surface Methodology.

1. INTRODUCTION

The processing of fruits and vegetables generates significant amounts of by-products that constitute not only an environmental[1,2] but also an economic problem, since the efficiency of the production process is reduced.[3] Utilisation of these by-products comprises a sustainable strategy to minimize losses between production and consumption stages, enabling a comprehensive valorisation of the resources produced in the field. In addition, these agri-food by-products have gained considerable attention during the last decades because they represent an important and inexpensive source of valuable bioactive compounds,[1,4] known for their antioxidant, antiradical, and antibacterial activities.[2,5]

Extraction with organic solvents, such as hexane and methanol, is the most widespread methodology to recover bioactive compounds from different biological substrates.[6] Although organic solvents are especially effective in solubilizing phenolic compounds,[7] they are being deeply questioned due to their environmental impact as well as safety aspects associated with their handling.[8,9] In this context, the use of water as solvent comprises a satisfactory solution to the problem of solvent toxicity, in addition to the fact that it is inexpensive, highly available, and environmentally friendly.[10] The extraction capacity of water can be notoriously enhanced with the assistance of cleaner and safer green technologies,[11] such as ultrasound-assisted extraction (UAE), which allow effective and efficient extraction of bioactive compounds, while minimizing organic solvent use, extraction times, and energy consumption.[6] UAE has been successfully used as a postharvest by-products innovative technology for the recovery of several bioactive compounds like carotenoids,[12] betalains,[11] and polyphenols from different biological matrices[4,13,14], contributing to the reduction of biomass losses.

Red beet plants are cultivated worldwide for the consumption of their roots, generating large amounts of unexploited by-products. In particular, beet leaves (BL) represent about 50% of the whole plant and are usually discarded as waste, meaning that not only half of the biomass will be lost after harvest, but also multiple resources invested in the production will be wasted before reaching to consumers.[3] By-product discarding also constitutes an environmental problem because of the pollution induced by their disposal.[1,2] However, BL constitute an important source of functional compounds, *i.e.* polyphenols and betalains,[3] that could be recovered in order to improve the efficiency of plant utilisation and increasing sustainability within the production systems and agri-food chain. In fact, the extraction of phytochemicals from this vegetal matrix was previously studied by optimising temperature, ratio, and ethanol concentration during macerations.[15] In particular, the enrichment of a fruit and vegetable beverage with desolvated BL extract increased the shelf-life and antioxidant capacity of this product, demonstrating the potential of BL to develop high value-added foods.[16] However, to completely avoid the use of organic solvents (ethanol in this case) aqueous UAE of bioactive compounds from BL remains unexplored. In this regard, the present study aimed to characterise the aqueous UAE process of bioactive compounds from BL and to optimise this process in order to maximize the extraction yield.

2. MATERIALS AND METHODS

2.1. Plant material

Beet plants (*Beta vulgaris* L. var. conditiva), harvested at commercial maturity (achieved at around 80 days after planting), were acquired from a local market in Buenos

Aires, Argentina. They were transported to the laboratory under refrigerated conditions and were immediately processed. Leaves were cut into small pieces of around 3 mm^2 and were kept in a closed container, in the dark, and at 4 °C until all treatments were performed (to prevent sample dehydration and oxidative and enzymatic reactions). The moisture content of the samples was determined and results were expressed as dry weight (DW) basis.

2.2. Reagents

Folin-Ciocalteu reagent was acquired from Merk (Germany), gallic acid and sodium carbonate (Na_2CO_3) were purchased from Biopack (Argentina). For buffer McIlvaine preparation, disodium phosphate (Na_2HPO_4) and citric acid were provided by Anedra (Argentina).

2.3. Ultrasound-assisted extraction

Bioactive compounds from BL were extracted using an ultrasonic probe (Vibra-Cell VCX 750, Sonics & Materials Inc., USA) equipped with a flat tip of 13 mm diameter. The frequency of the ultrasound processor is 20 kHz and the highest power is 750 W. Amplitude was controlled to maintain fixed powers.

2.4. Characterisation of UAE process

In the first stage of this work the effect of different ultrasound powers (UP) on the extraction yield of bioactive compounds was studied. For this purpose, the evolution of polyphenol and betalain concentrations as well as the temperature profile developed during ultrasound treatments were evaluated.

2.4.1. Ultrasound and conventional extraction conditions

The sonication probe was immersed in a baker containing 20 g of the sample and 200 mL of water as extracting solvent. UAE was conducted during 30 min at three fixed UP, 35, 50, and 100 W. Samples were withdrawn at 0, 1, 2, 4, 6, 8, 10, 12, 14, 20, and 30 min after sonication started. Samples were centrifuged at 11200 *g* for 10 min at 5 °C (Eppendorf Centrifuge 5804R, Germany). Supernatants were collected and kept at 4 °C to subsequently determine bioactive compounds' concentration. Two conventional extractions (maceration) without ultrasonic irradiation were performed. One of the extractions was carried out at a constant temperature of 20 °C (coded as 0W*). The second extraction (coded as 0W) was performed using a thermostatic bath (Dubnoff, Vicking, Germany) to develop an extraction under a thermal profile similar to that registered during UAE at 35 W (20-65 °C). Samples were processed as mentioned for UAE.

2.4.2. Thermal profile modelling

In order to characterise the thermal profiles developed during UAE, temperature was registered using a thermometric probe (Part No. 830-00060, Sonics & Materials Inc., USA), while thermal profiles developed during macerations were registered using a mercury thermometer. An empirical equation (Eq. 1), with the Weibull form,[17] was proposed to model temperature changes during ultrasound processes. This model considers two terms given by a constant that indicates the initial temperature of the system, and a second term that reflects the exponential increases in this variable.

$$T = T_i + T_R\left(1 - e^{-k_R \cdot t^n}\right) \quad (1)$$

where T is the temperature at t time, T_i is the initial temperature, T_R is the change in temperature when the time approaches infinite, k_R represents the kinetic coefficient of temperature increase and n represents the shape factor that could be associated with the order of changes registered in temperature.[17]

2.5. Optimisation of UAE

In order to maximize polyphenols and betalains extraction from BL, simultaneous optimisation of UAE process was performed through Response Surface Methodology (RSM).

Numerous process variables are known to influence the extraction rate of bioactive compounds from food matrices, including extraction time, temperature, pH, solid-liquid ratio, solvent composition, matrix particle size, UP, among others.[15,18-20] In the present study, in which a green-solvent UAE process is under development (as conventionally used organic solvents were replaced by water) the process variables were UP ($X1$), solid-liquid ratio ($X2$), and extraction time ($X3$). Based on the results obtained in **3.2.** and to further explore the effect of ultrasound on this particular plant matrix, UP was studied in a wide range (10-90 W). Solid-liquid ratio values (1:20-3:20) were selected considering optimal ratios reported in the literature for UAE applied to other food matrices.[18,20] Finally, the extraction times evaluated (4-16 min) comprised the time interval at which the highest extraction yields of total polyphenols (TPC) and betaxanthins (BX) were observed (**3.2.**).

For the optimisation of UAE process, a three-level-three-factor Box-Behnken (BB) design was implemented with 15 experimental runs and the centre point made in

triplicate[21] (**Table 1**). TPC, BX, and betacyanins (BC) were determined for each condition.

2.5.1. Modelling and optimisation

The least-squares regression method was used to adjust data to second-order polynomial model as follows:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{2} \sum_{j=2,j>1}^{3} \beta_{ij} X_i X_j + \sum_{i=1}^{3} \beta_{ii} X_i^2 \quad (2)$$

where Y is the response variable (TPC, BX, or BC), β_0 is the model intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the coefficient for interaction terms, and X is a dimensionless coded value of the independent variable, x.

TPC, BX, and BC were determined for each essay and the second-order polynomial model (Eq. 2) was fitted to each response variable. To achieve the optimal conditions that maximize TPC, BX, and BC yield, simultaneous optimisation was carried out using the Desirability function with the Larger-the Best criteria, as described in Bengardino[15].

2.5.2. Validation

The model was validated performing a set of three independent experiments using the extraction conditions obtained after simultaneous optimisation. The experimental values were then compared to those predicted by the fitted models.

2.6. Determination of total polyphenol content

TPC was determined according to Zhang[22] with some modifications. Briefly, 70 µL of each sample solution were placed on a 96-well microplate and 100 µL Folin-Ciocalteau regent (diluted 1:10 in distilled water) were added. After 5 min of incubation, 30 µL Na_2CO_3 7,5% (w/v) were added. The reaction mixture was homogenized and incubated in the dark at room temperature for 2 h. Absorbance was measured at 765 nm using a spectrophotometric microplate reader (Multiskan GO, Thermo Scientific, Finland). A calibration curve was made using gallic acid and results were expressed as mg of gallic acid equivalents (GAE) per g DW of BL (mg GAE/g DW).

2.6.1. Determination of betaxanthin and betacyanin content

The content of betalains was assessed spectrophotometrically following the method described by Fernandez[16]. The extracts were previously diluted in buffer McIlvaine and absorbance was measured at 476, 536 and 600 nm. BX and BC concentrations were expressed as µg vulgaxanthin-I and µg betanin per g DW (µg/g DW), respectively.

2.7. Statistical analysis

All analyses were carried out in triplicate and were expressed as mean ± standard deviation. Data from different experimental studies were analysed using STATISTICA 7.1 software (Statsoft Inc., 2005). Kinetic parameters were adjusted with non-linear least squares routines. The statistical analysis of extraction processes was performed using the analysis of variance (ANOVA) including the F-ratio, which established the model global significance and the determination coefficient R^2.

3. RESULTS AND DISCUSSION

3.1. Thermal profile modelling

The thermal profiles obtained during extraction treatments indicated that maceration carried out at 20 °C (0W*), as expected, showed a constant temperature during the entire process **(Fig. 1)**. Meanwhile, maceration performed using the thermal bath (0W) revealed a thermal profile similar to that obtained for UAE at 35 W, as it was designed for this purpose. Furthermore, during ultrasound treatments, the temperature of the systems increased as a function of the UP applied. It is well known that, when ultrasound is applied, electrical energy is used to generate oscillations of the piezoelectric crystal (the ultrasonic probe), which is transformed into mechanical energy and, then, into acoustic energy, in the form of ultrasonic waves that propagate through the liquid medium giving rise to the phenomenon of cavitation. This phenomenon is characterised by the generation, growth, and collapse of gas and vapour bubbles[23] within the liquid with the consequent release of thermal energy in the form of heat that is absorbed by the medium, increasing its temperature.[24] In particular, treatments performed at 35 and 50 W presented a similar trend, with temperature increases almost linear during the first 18 and 15 min, respectively (at mean rates of 1.8 and 2.2 °C/min), with a slow attenuation thereafter. On the other hand, at the highest power applied (100 W) a rapid increase in temperature was detected during the first 8 min (at a mean rate of 5.2 °C/min), reaching values near 70 °C. The attenuation of increments was more evident under this condition. This behaviour was modelled with a Weibull form-model (Eq. 1) showing a good degree of fitting (**Fig. 1**)**,** where lines represent models obtained for each condition and dots represent experimental data.

Model parameters together with R^2 are presented in **Table 2**. T_R values for 35 and 50 W treatments were similar, while T_R value was significantly higher for the 100 W treatment. This parameter gives an idea of the temperature stabilization value ($T_i + T_R$) if time elapses indefinitely. These values are associated with the thermal equilibrium that would be achieved between the generation of heat in the liquid medium due to ultrasound and the loss of heat to the environment through the walls of the container. Thus, for samples treated at 35 and 50 W, this value would be about 83 °C, meanwhile for the sample treated at 100 W, this value would reach about 94 °C. On the other hand, *k* value also showed a relationship with the UP. This parameter, which represents the speed of temperature, presented a linear relationship with UP, increasing around 4.10^{-3} every 10 W. Finally, *n* parameter, which is related to the shape of the curve, indicated approximate first-order kinetics for the temperature increases when the lowest UP (35 and 50 W) were applied; meanwhile, for the treatment at 100 W the value of *n* was 20% higher, indicating a higher order in temperature changes.

Numerous studies have addressed the extraction of bioactive compounds with ultrasound assistance, but thermal profiles associated with high-intensity ultrasound treatments have not always been reported. In fact, some authors claim to have a precise control of the system temperature carrying out isothermal treatments using jacketed glasses,[13,19,25,26] ice, water, or ethanol baths.[12,25] Others, reported that, despite working with similar systems, increases in temperature were indeed registered.[27,28] To the best of our knowledge, only Parniakov[14], studying the UAE of polyphenols and chlorophylls from the microalgae *Nannochloropsis* spp., presented a thermal profile associated to ultrasound treatments performed under non-isothermal conditions, and these profiles follow the same pattern as the one presented in this study.

The temperature increase cannot be neglected or unknown, especially when ultrasound is applied as a non-thermal technology. However, when used as extracting technology, the heat generated in the medium may be beneficial for improving extraction yields since high temperatures have been proven to accelerate and maximize polyphenol extraction from BL.[15]

3.2. UAE profile of bioactive compounds from beet leaves

In order to explore the effect of ultrasound assistance on the extraction yield of bioactive compounds throughout time, the evolution of TPC, BX, and BC concentrations was evaluated under different UP.

3.2.1. Total polyphenol content

The TPC extracted from BL using maceration and ultrasound as extracting methodologies are presented in **Figure 2a.**

As expected, extraction yields were considerably low in conventional extractions (0W* and 0W). Macerations performed at 20 °C (0W*) yielded only 2.5 mg GAE/g DW after 30 min. Meanwhile, a progressive increase in temperature from 20 to 65 °C (0W) (**Fig. 1**) favoured the extraction of these compounds, doubling the yield ($p<0.05$). It is well known that increasing temperature promotes the recovery of polyphenols from vegetal sources,[15] mainly by altering the membrane structure of plant cells, facilitating the diffusivity of the extracted molecules into the solvent, and increasing polyphenol solubility.[13]

In regard to UAE treatments, it is noteworthy that a significant ($p<0.05$) improvement in polyphenol extraction yield was achieved even when the lowest UP was

applied. In fact, TPC of extracts obtained after 35 W ultrasound treatment doubled those obtained at 0 W, despite presenting similar thermal profiles. This performance improvement is, therefore, exclusively attributed to ultrasound treatment. Cavitation effect produced by ultrasound leads to swelling and hydration of vegetal matrices, producing micro-jets and micro-bubbles that disrupt the matrix and favour the entry of the solvent into plant cells. This event facilitates the release of bioactive compounds, increasing the extraction yield.[29,30]

Furthermore, increases in UP had a positive effect on the extraction of polyphenols (35<50<100 W) and considerably shortened extraction times; only 8, 6, and 2 min of ultrasound treatment at 35, 50, and 100 W, respectively, were required to reach a comparable yield to that obtained after 30 min of maceration (0W) (5.5 mg GAE/g DW). In fact, reducing UAE duration is highly recommended since it contributes to minimizing energy consumption. Maximum extraction yields (10.1 and 11.8 mg GAE/g DW) were achieved at 12 and 8 min at 50 and 100 W, respectively, and were 1.7 and 2.0-fold higher than those obtained after 30 min of maceration (0W). These improvements are attributed to both the cavitation phenomenon and the thermal effect associated with energy propagation produced during ultrasonic treatments. Nonetheless, prolonged exposition times under these UP had a negative impact on TPC. These results were in accordance to Carrera[31], who described a decrease in anthocyanin extraction yield from grapes when ultrasound extraction times increased from 6 to 15 min. Long sonication periods may lead to the generation of free radicals from water, which promote the degradation of polyphenols by triggering radical chain reactions.[14,31]

Finally, although the yield of these compounds relies mainly on their concentrations in the raw material, our results were comparable to those reported for

other vegetable by-products recovered using aqueous UAE processes,[19,25] confirming the potential of this resource to be revalued.

3.2.2. Betaxanthin and betacyanin content

The BX and BC contents obtained from BL using maceration and ultrasound as extracting methodologies are presented in **Figures 2b and 2c**.Differently from TPC, the profiles of BX yield obtained from conventional extractions (0W* and 0W) were similar, despite having different thermal profiles. Both treatments exhibited an average concentration of 187.0 µg/g DW after 30 min of maceration. These results are in accordance with those described by Bengardino[15], who reported that temperature was not a determinant factor for BX when selecting the most significant variables for extraction optimisation of bioactive compounds from BL, employing maceration with organic solvents.

When applying the lowest UP (35 W), the treatment improved the extraction yield of BX respect to 0 W, despite having the same thermal profile. As for TPC, this result is attributed to the cavitation phenomenon. Furthermore, increasing UP resulted in increasing yields as well as significant reductions in extraction times. In fact, the BX yield obtained at 30 min of maceration was achieved at only 3, 2, and 1 min of ultrasound exposure at 35, 50, and 100 W, respectively. The recovery of this pigment from BL reached maximum values after 14, 10, and 8 min of extraction at UP of 35, 50, and 100 W, respectively, in which the amount of BX was 2.7, 6.0, and 8.5-fold higher than those extracted during maceration. For all UP applied, longer extraction times led to a decrease in BX concentration. Our results agree with Maran[29], who observed a decrease in the extraction yield of betalains from *Bougainvillea glabra* flowers after 25 min of ultrasound

treatment. BX susceptibility has been reported by numerous authors,[7,15] being this behaviour attributed to different degradation and transformation mechanisms like hydrolysis, decarboxylation, isomerization, and dehydrogenation.

As reported for BX, the extraction profiles of BC obtained during conventional extractions (0W* and 0W) were similar, with an average BC concentration of 135.5 µg/g DW after 30 min of maceration.

In line with TPC and BX results, 35W-ultrasound treatments yielded higher concentrations of BC than 0W-maceration extractions, despite having similar thermal profiles. This effect is, again, attributed to the cavitation phenomenon. In addition, increases in UP had a positive effect on extraction yield, which incremented progressively during the 30 min-treatment, reaching concentrations between 1.7 and 2.7-fold higher than those obtained in conventional extractions. Different from BX, BC was not affected during the ultrasonic treatment, which is consistent with findings reported in the literature.[5,7] This stability is mostly attributed to BC glycosylation, which confers higher redox potential and, thus, lower reactivity with molecular oxygen.[5,32]

3.3. UAE optimisation of bioactive compounds from beet leaves

After exploring the kinetics of aqueous UAE of bioactive compounds, RSM was used to study the effect of UP, solid-liquid ratio, and extraction time on TPC, BX, and BC yields. **Table 1** shows the content of TPC, BX, and BC obtained in the 15 experimental runs of the BB design. It can be observed from the table that the three response variables exhibited maximum yield at the same experimental conditions, which corresponded to run 8, *i.e.*, 90 W UP (X_1), 2:20 solid-liquid ratio (X_2), and 16 min extraction time (X_3). The results obtained for each response variable in the experimental runs were then fitted to

the second-order polynomial models, and the regression coefficients obtained are presented in **Table 3**.

The yields of TPC and BX were significantly influenced by the three independent variables, as the linear regression coefficients were significant ($p<0.05$) for these models. Ultrasound power (X_1) and extraction time (X_3) exerted a positive effect on these response variables, meaning that higher yields are reached at the highest UP and extraction time under evaluation. Similar results were reported by other authors optimising polyphenol[33] and betalain extractions[34] from different vegetal by-products. In contrast, solid-liquid ratio had a negative impact on TPC and BX yields, since the linear (X_2, negative) regression coefficient was significant for this variable, which indicates higher yields at lower solid-liquid ratios. Working in the optimisation of bioactive compounds extraction from BL by maceration, Bengardino[15] also obtained negative coefficients for the linear term of solid-liquid ratio in the polynomial model of TPC. At low solid-liquid ratios, a higher volume of the solvent is available to penetrate the plant matrix allowing bioactive compounds to dissolve and, in turn, increasing the recovery yield.[15] Furthermore, the interaction term between UP and extraction time (X_1X_3) significantly influenced ($p<0.05$) the yields of TPC and BX, meaning that at different UP the evolution of these compounds along the extraction process is different, as it was observed for the extraction kinetics profile of TPC and BX (**Fig. 2**).

On the other hand, like TPC and BX, BC were significantly affected ($p<0.001$) by the linear coefficient of UP (X_1), which exerted a positive effect on BC extraction rate. Additionally, the solid-liquid ratio influenced the yield of BC, as linear (X_2, negative) and quadratic (X_2^2) coefficients were significant ($p<0.01$) in the polynomial equations. As for TPC and BX, lower solid-liquid ratios enhanced the extraction yield. Furthermore, the

extraction time had an impact on BC, since both the linear (X_3, positive) and quadratic (X_3^2) terms were significant ($p<0.001$). Additionally, BC were significantly affected ($p<0.01$) by the interactions between UP and extraction time (X_1X_3) and by solid-liquid ratio and extraction time (X_2X_3). This indicates that BC varies along the extraction process in a different way depending on the power or the ratio.

Once the quadratic models were obtained, simultaneous optimisation was conducted to predict the experimental extraction conditions that maximize TPC, BX, and BC yields. The optimal conditions for aqueous UAE of bioactive compounds from BL were 90 W UP (X_1= 1), 1:20 solid-liquid ratio (X_2= -1), and 16 min extraction time (X_3= 1), with a Desirability value of 0.9574 (**Fig. 3**). The Desirability surfaces obtained as a function of each independent variable are presented in **Fig. 4**. Under optimal conditions the predicted yields for the response variable were 12.3 mg GAE/g DW (TPC), 966.7 μg/g DW (BX), and 453.9 μg/g DW (BC). The reliability of the models was then evaluated performing three independent experiments under the optimal conditions, at which the experimental values obtained for TPC, BX, and BC were 14.9 ± 0.6 mg GAE/g DW, 949.1 ± 28.4, and 562.2 ± 41.3 μg/g DW, respectively. These values were in accordance with the predicted concentrations of bioactive compounds found by the models, confirming the versatility of RSM and Desirability function to find the optimal conditions that maximize the recovery of bioactive compounds. Differences between experimental and predicted values were attributed to natural variations in the concentration of bioactive compounds in the raw material coming from different batches. In beet plants, these variations are influenced by numerous factors such as cultivar, harvesting term, and crop conditions (mainly high temperatures).[35]

4. CONCLUSIONS

This is, to our knowledge, the first scientific report evaluating the efficiency of aqueous UAE to recover bioactive compounds from underutilised BL. UAE technology enhanced the extraction yield of polyphenols and betalains (35<50<100 W) avoiding the use of unsafe organic solvents and shortening extraction times in comparison to traditional macerations. Nonetheless, ultrasound exposition times should be carefully optimised in order to avoid the degradation of such compounds. During ultrasound treatment the temperature of the systems increased as a function of the power applied, contributing to the recovery of these phytochemicals. Simultaneous optimisation of three selected variables (UP, solid-liquid ratio, and extraction time) was successfully performed through RSM to obtain the optimal conditions that maximize polyphenol and betalain yields. This study enabled the development of a green-solvent UAE process that constitutes an effective post-harvest by-products strategy to minimize losses and increase biomass utilisation through the recovery of bioactive compounds from BL, promoting sustainability in the agri-food chain. Further studies will be oriented to the direct use of the organic solvent-free BL extracts to enrich and add value to natural foods.

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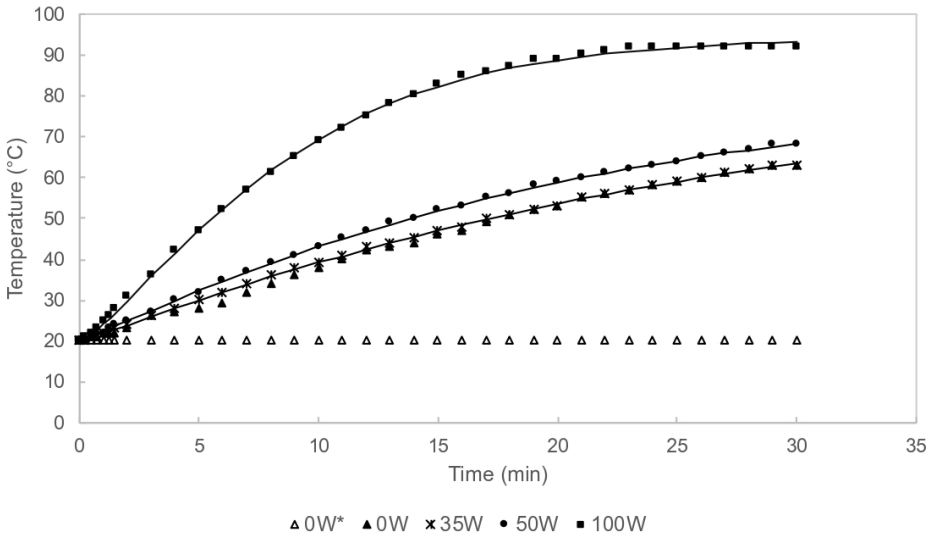
7. FIGURE CAPTIONS

Fig. 1. Thermal profiles during extraction processes. Symbols represent experimental data and lines represent the fitted models. 0W* extraction without ultrasound at 20°C; 0W extraction without ultrasound with a temperature increase from 20 to 65 °C; 35W, 50W, and 100W extractions assisted by ultrasound at powers of 35, 50, and 100 W.

Fig. 2. Effect of ultrasound power on the extraction yield of TPC (a), BX (b), and BC (c) from beet leaves. 0W* extraction without ultrasound at 20°C; 0W extraction without ultrasound with a temperature increase from 20 to 65°C; 35W, 50W, and 100 W extraction assisted by ultrasound at powers of 35, 50, and 100 W.

Fig. 3. Profiles for predicted values of TPC, BX, BC, and Desirability as function of ultrasound power (X_1), solid-liquid ratio (X_2), and extraction time (X_3).

Fig. 4. Desirability surfaces as function of ultrasound power (X_1), solid-liquid ratio (X_2), and extraction time (X_3).



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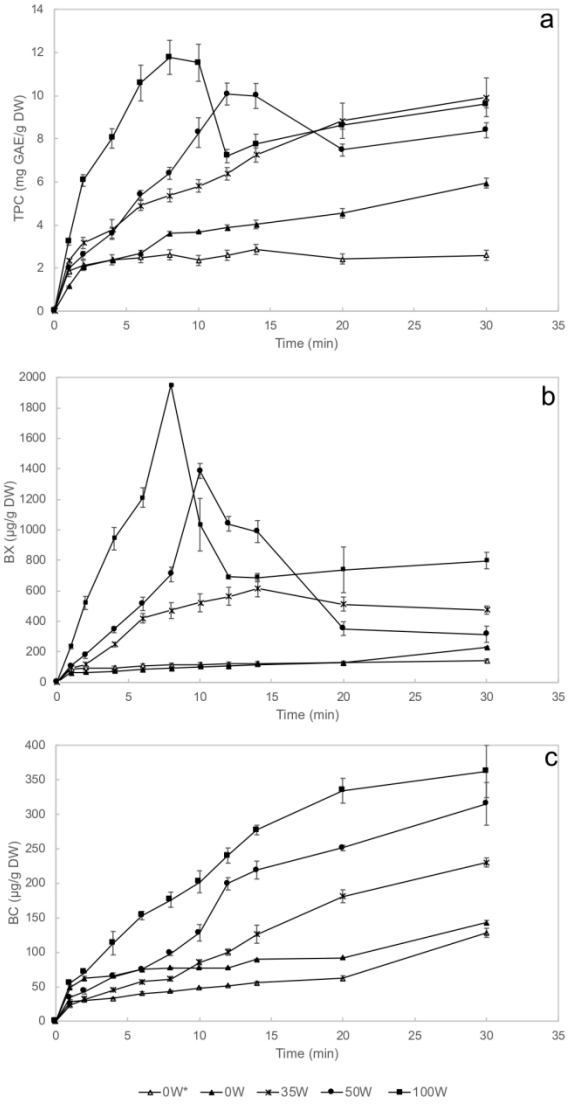
200x150mm (150 x 150 DPI)



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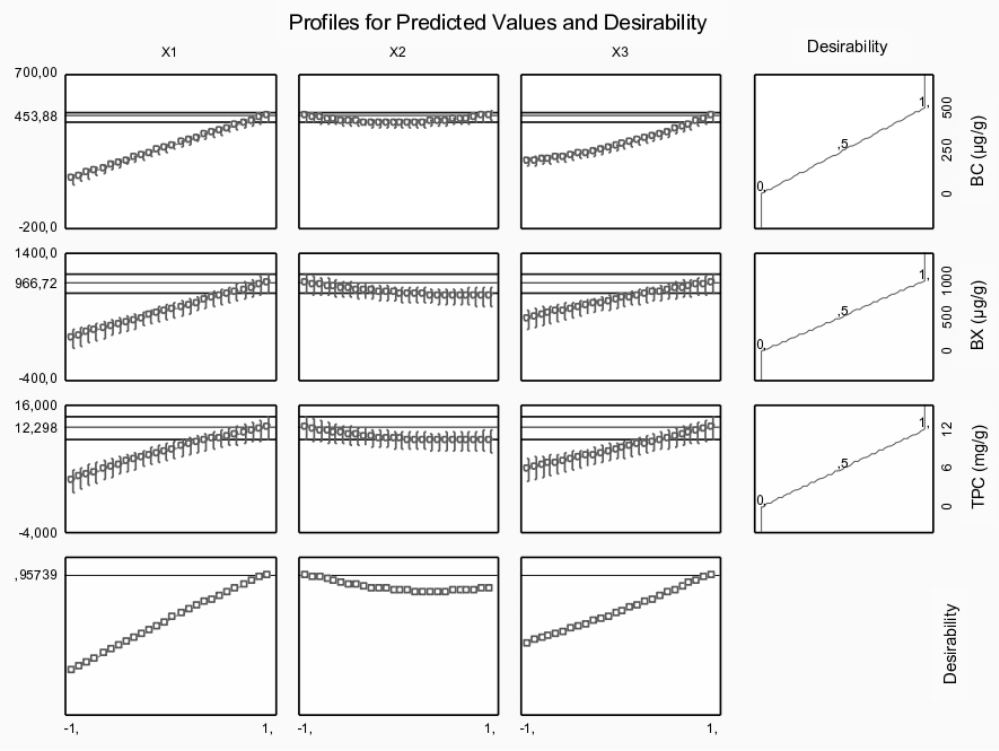

Fig. 3. Profiles for predicted values of TPC, BX, BC, and Desirability as function of ultrasound power (X1), solid-liquid ratio (X2), and extraction time (X3).

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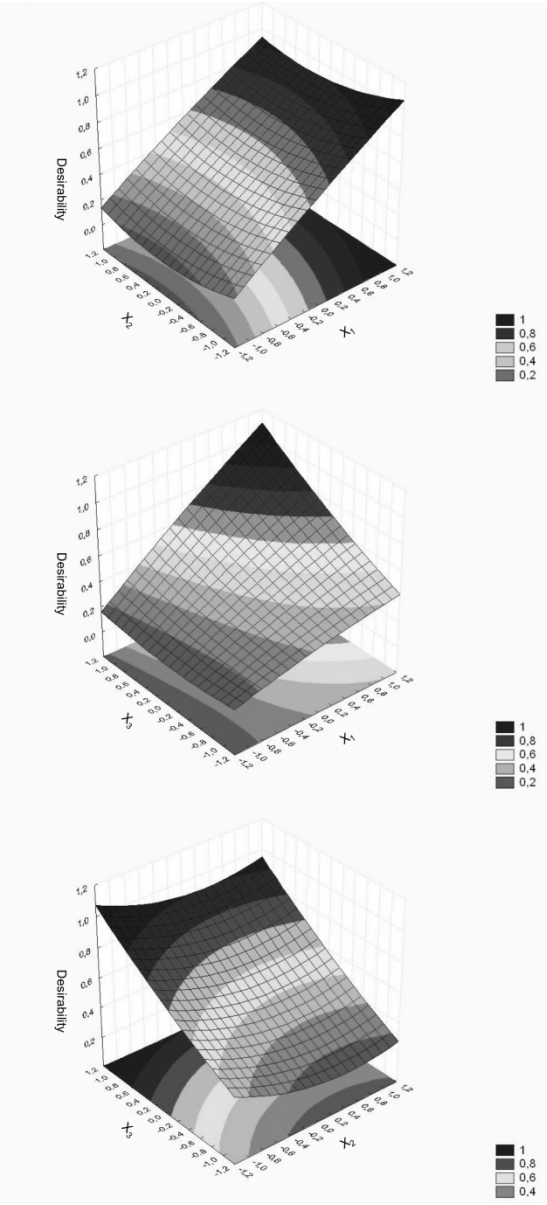

Fig. 4. Desirability surfaces as function of ultrasound power (X1), solid-liquid ratio (X2), and extraction time (X3).

209x460mm (150 x 150 DPI)

Table 1. Box-Behnken design matrix for the optimization of UAE of TPC, BX, and BC from beet leaves.

	Coded variables			Decoded variables			Response variables		
Run	X_1	X_2	X_3	UP (watt)	S-L ratio	Time (min)	TPC (mg GAE/g DW)	BX (μg/g DW)	BC (μg/g DW)
1	0	-1	-1	50	1:20	4	4.9 ± 0.1	281.4 ± 37.5	164.6 ± 20.6
2	0	-1	1	50	1:20	16	9.3 ± 0.9	602.2 ± 28.6	273.5 ± 45.3
3	0	1	-1	50	3:20	4	3.2 ± 0.3	170.0 ± 6.8	89.5 ± 3.8
4	0	1	1	50	3:20	16	7.0 ± 0.5	448.8 ± 13.5	282.9 ± 15.7
5	-1	0	-1	10	2:20	4	1.7 ± 0.1	69.1 ± 1.6	33.5 ± 0.8
6	1	0	-1	90	2:20	4	4.4 ± 0.2	314.6 ± 15.8	98.1 ± 3.4
7	-1	0	1	10	2:20	16	2.6 ± 0.2	134.6 ± 15.8	59.6 ± 9.7
8	1	0	1	90	2:20	16	10.1 ± 0.9	780.0 ± 14.9	416.4 ± 23.8
9	-1	-1	0	10	1:20	10	2.6 ± 0.2	123.0 ± 6.3	54.2 ± 3.4
10	-1	1	0	10	3:20	10	1.7 ± 0.1	107.7 ± 6.6	50.6 ± 3.4
11	1	-1	0	90	1:20	10	9.0 ± 0.6	753.3 ± 60.2	274.0 ± 44.4
12	1	1	0	90	3:20	10	8.0 ± 0.4	623.2 ± 27.3	244.4 ± 18.8
13	0	0	0	50	2:20	10	5.3 ± 0.1	353.3 ± 18.3	117.0 ± 11.2
14	0	0	0	50	2:20	10	5.0 ± 0.3	336.0 ± 22.4	110.9 ± 8.6
15	0	0	0	50	2:20	10	5.0 ± 0.3	337.2 ± 35.1	115.8 ± 14.2

UP: ultrasound power, S-L: solid-liquid, TPC: polyphenols, BX: betaxanthins, BC: betacyanins.

Table 2. Model parameters of thermal profiles associated to each ultrasound treatment.

Model parameter	Ultrasound power		
	35 W	**50 W**	**100 W**
TR	63.06	61.13	74.13
k	0.03131	0.03926	0.05972
n	1.06434	1.08298	1.26435
R^2	0.99964	0.99976	0.99944

Table 3. Regression coefficients and the ANOVA for the second-order polynomial models for TPC, BX, and BC from beet leaves.

	TPC		BX		BC	
	Coefficient	**p-Value**	**Coefficient**	**p-Value**	**Coefficient**	**p-Value**
β_0	5.1*	0.000023	342.2*	0.000051	114.6*	0.000003
X_1	2.9*	0.000035	254.7*	0.000019	104.4*	0.000000
$X_1 \times X_1$	-0.6	0.111488	4.4	0.862442	-4.7	0.344640
X_2	-0.8*	0.015006	-51.3*	0.025469	-12.4*	0.010323
$X_2 \times X_2$	0.8	0.044129	55.3	0.069388	46.0*	0.000162
X_3	1.9*	0.000291	141.4*	0.000336	80.8*	0.000002
$X_3 \times X_3$	0.2	0.622568	-21.9	0.403940	42.1*	0.000248
$X_1 \times X_2$	0.0	0.966151	-28.7	0.268153	-6.5	0.197130
$X_1 \times X_3$	1.2*	0.010531	100.2*	0.007373	73.1*	0000014
$X_2 \times X_3$	-0.2	0.615733	-10.5	0.667591	21.1*	0.004743

X_1: ultrasound power, X_2: solid-liquid ratio, X_3: extraction time, TPC: polyphenols, BX: betaxanthins, BC: betacyanins. * Coefficients with p-Value<0.05 were considered significant and used in the second-order polynomial models.