ARTICLE

Optimized green extraction method for the recovery of phenolics from *bignay [Antidesma bunius* (L.) Spreng*]* pomace

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ABSTRACT

The main objective of this research was to optimize a green extraction technique for the recovery of phenolic compounds from freeze-dried *bignay* [*Antidesma bunius* (L.) Spreng.] pomace. Plackett-Burman design (PBD) was used to screen significant factors affecting the recovery of phenolic compounds from *bignay* pomace and Response Surface Methodology (RSM), specifically Box-Behnken design (BBD), was used to determine the optimum levels of the identified significant factors. The influence of solvent concentration (water/ethanol 0–100% [v/v]), sample concentration (1–15 % [w/v]), solvent pH (pH 2–7), incubation time (13–180 min), incubation temperature (30–70 °C), sonication time (0–90 min), and agitation (0–300 rpm) on the total phenolic compounds of the extract were investigated. The total monomeric anthocyanins, flavonoids, catechin contents, and antioxidant properties of the optimized extract were also determined. Results of PBD (adjusted R^2= 0.9820) and BBD (adjusted R^2= 0.8838) experiments were validated and the optimum conditions for phenolic compound extraction from *bignay* pomace were achieved with 10.41% sample in distilled water incubated at 30 °C for 13 min. Higher recovery of phenolics was attained when water was used as an extraction solvent under optimal extraction conditions. The *bignay* pomace extract features significant amounts of phenolic compounds and exhibits high antioxidant activities relative to other fruit peels reported in literature. The resulting extract is free of organic solvent residues and features significant polyphenolic compounds with high antioxidant activities, indicating its potential as a functional food ingredient. This optimized green extraction method offers an economical and sustainable alternative for recovering phenolic compounds from *bignay* pomace.

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INTRODUCTION

The bioactive compounds, functional food applications, and health benefits of indigenous berries have been well explored, as evidenced by the studies conducted by Butkhup and Samappito

KEYWORDS

bignay pomace; response surface methodology; green extraction

(2008), Barcelo et al. (2016), Recuenco et al. (2020), and Tengco et al. (2022). One of the indigenous berries that has been gaining attention in the Philippines is *Antidesma bunius* (L.) Spreng, locally known as *bignay* or *bugnay*. *Bignay* berries are high in bioactive compounds such as anthocyanin, flavonoids, phenolic acids, and antioxidants (Butkhup and Samappito 2008). The fruit is a cluster of 30–40 small berries that change from red to black when ripe (Belina-Aldemita et al. 2013). It is usually used to manufacture fruit juices, sugar preserves, and wine. During *bignay* wine processing, the puree is obtained with the use of a pulper machine. In this process, 70% of the raw materials are recovered as puree while the remaining 30% is discarded as a by-product known as the pomace (Seedarak and Lokaewmanee 2019). *Bignay* pomace, usually composed of fruit skin and seeds, can also be a source of bioactive compounds. *Bignay* seeds contain high amounts of plant secondary metabolites, such as condensed tannins (Gunun et al. 2014). While the *bignay* fruit itself is already used in a variety of agricultural products (Butkhup and Samappito 2008; Barcelo et al. 2016; Seedarak and Lokaewmanee 2019), the valorization of *bignay* pomace is not yet well studied.

The study of quantifying the phenolic metabolites from agricultural crops, like *bignay*, has recently gained considerable interest because of their health-promoting properties (Barcelo et al. 2016; Tengco et al. 2022). Concomitantly, the utilization of processing by-products from agri-food industries has also been a highlight of research and development (Dey et al. 2021), as addressed in the 12th United Nations Sustainable Development Goals, specifically the Responsible Consumption and Production (United Nations, 2015). The valorization of agricultural wastes as food ingredients offers an advantage because they are an economical source of phenolics, dietary fiber, and minerals. Several researches have been made to recover bioactive compounds from agricultural wastes, such as chestnut shells (Pinto et al. 2021), hazelnut shells (Stevigny et al. 2007), craft brewer's spent grain (Andres et al. 2020), and spent coffee grounds (Solomakou et al. 2022). The bioactive compounds present in these by-products can be used as functional food ingredients that confer desirable health benefits such as oxidative stress prevention associated with aging and some chronic diseases, cardiovascular disease, and cancer (Stevigny et al. 2007; Pinto et al. 2021).

In obtaining phenolic compounds for isolation and identification, the first and most critical step is the selection of the appropriate extraction technique to attain substantial yields (Alara et al. 2021a). The conventional phenolic compounds extraction from agricultural food by-products is conducted using organic solvents, like methanol and acetone, and usually involves manual labor-intensive procedures (Alara et al. 2021a; Solomakou et al. 2022). These methods are usually accompanied by agitation, sonication, centrifugation, and filtration. The disadvantages of using conventional methods include lengthy extraction times, excessive energy expenditure, the utilization of copious volumes of organic solvents, and thermal deterioration of some compounds. To address this, research that explores economical and "greener" techniques for extracting polyphenols from plants and their by-products has gained popularity for the past years (Panja 2017; Plaza et al. 2017; Cvjetko Bubalo et al. 2018). The application of "greener" extraction methods holds an advantage in terms of practicality, sustainability, and environmental impacts.

The establishment of ideal conditions for phenolics extraction can be determined with the one-factor-at-a-time approach used in the studies of Mokrani and Madani (2016) and Benchikh and Louailèche (2014). This design is effective in establishing the effect of each factor, but can be costly, laborious, and time intensive. On the other hand, another statistical approach that can be conducted to establish ideal extraction conditions would be employing the Plackett-Burman design (PBD) followed by Response Surface Methodology (RSM). PBD identifies the factors with the greatest effect on the response variable. These factors will then be used in RSM for the sequential optimization of the extraction method for the specific commodity. Compared to employing the one-factor-at-a-time approach, the use of PBD and RSM economizes the number of runs while simultaneously taking account of variable interactions. Using empirical models on the obtained data and the experimental design, the impact of several factors and their interactions are assessed (Arruda et al. 2016). Studies conducted by Anastácio and Carvalho (2013), Borges et al. (2016), and Li et al. (2016) utilized PBD to screen for the factors affecting phenolic extraction. Conversely, Andres et al. (2020), Alara et al. (2021b), and Arruda et al. (2016) utilized RSM for the optimization of phenolic extraction from various commodities.

Thus far, there is insufficient data on optimization studies on the green extraction methods of phenolic compounds from *bignay* pomace. Additionally, this study can serve as one of the baselines that can be used to further the cause of processing by-product utilization in the Philippines. Therefore, this research aimed to identify the optimum green extraction parameters for maximum recovery of phenolic compounds from *bignay* pomace using RSM. Harnessing the capacity of *bignay* pomace as a cheap and safe source of antioxidants and phenolic compounds could be further proof that functional food ingredients can be extracted from agricultural and industrial by-products.

MATERIALS AND METHODS

Chemicals and reagents

All assays were conducted using analytical grade chemicals and reagents. Gallic acid, Trolox, and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Singapore).

Raw material

Ripe *bignay* berries, indicated by their black color, were harvested from the *bignay* trees in the vicinity of the Institute of Food Science and Technology (IFST) and the Philippine Rice Research Institute (PhilRice) at the University of the Philippines Los Baños. A pulper machine was used to separate the pomace from the puree. The pomace was frozen and subjected to freeze-drying. After which, the freeze-dried pomace was vacuum-packed and kept in the freezer (-20 °C) pending further analyses. The proximate composition of freeze-dried *bignay* pomace was as follows: moisture content - 6.09 %, ash - 6.23 %, crude protein - 7.88 %, crude fat - 4.36 %, and crude fiber - 43.17 %.

Extraction of phenolics from *bignay* pomace

The freeze-dried *bignay* pomace was ground and sieved (20-mesh) to obtain homogeneous samples. The samples were weighed into 125 mL Erlenmeyer flasks and the corresponding solvents were added. Samples were then incubated (Biobase Meihua Trading Co., Ltd., China), sonicated (Cole-Palmer, U.S.A.), and agitated (Benchmark Scientific, Inc., U.S.A.) according to the levels of factors indicated in the generated PBD and BBD (Tables 1 and 2). The extracts were filtered using a Miracloth (EMD Millipore Corp., USA) and Whatman® No. 1 filter paper (Sigma-Aldrich, U.S.A.) and stored in the freezer (-20 °C) pending analyses.

Optimization of phenolic compounds extraction

The optimum green conditions to maximize the phenolic compounds extraction from freeze-dried *bignay* pomace were determined using Response Surface Methodology (RSM). The Plackett-Burman design (PBD) of experiment was used to

determine the significant factors affecting the yield of phenolic extracts and the Box-Behnken design (BBD) was employed to establish the optimal levels/values of the significant factors identified. The experimental designs were generated using Design-Expert version 10 (Stat-Ease 2016).

Screening of significant factors using Plackett-Burman design (PBD)

Seven factors were considered in the optimization of phenolic extraction from *bignay* pomace. These were (A) solvent concentrations (water/ethanol 0–100 %, by volume), (B) sample concentration (1–11 %, weight by volume), (C) pH of the solvent (pH 2–7), (D) incubation time (13–120 min), (E) incubation temperature (30–70 °C), (F) sonication time (0–90 min), and (G) agitation (0–300 rpm). The pH of the solvent was adjusted accordingly using various concentrations of NaOH (0.2, 1, and 4N) and HCl (0.5 and 3N) solutions. The high and low values for the extraction parameters were selected based on previously published data on the phenolic extraction from fruits and processing by-products (Hammed et al. 2013; Tao et al. 2014; Arruda et al. 2016; Das and Eun 2018; He et al. 2018; Rocha et al. 2018; Vázquez-Espinosa et al. 2019; Aliaño-González et al. 2020; Kumar et al. 2021; Zubia et al. 2023). The samples were extracted using 125 mL Erlenmeyer flasks as containers and kept covered with parafilm and aluminum foil to prevent solvent evaporation especially when incubated at higher temperatures. The generated PBD resulted in a total of 15 runs with 3 center points. The total phenolic content (TPC), expressed as milligrams gallic acid equivalents per liter of extract (mg GAE/ L), was the response variable used.

Optimization of identified significant factors using Box-Behnken design (BBD)

The BBD was generated based on the results of PBD. The BBD consisted of 15 runs including three center points. Each run was analyzed for TPC. The predicted model and its validity were assessed. The optimal parameters for the extraction of phenolic compounds were determined based on its sustainability and suitability to be upscaled without compromising the amount of phenolics extracted from the pomace.

Model validation

Using the generated optimum conditions for extraction, the model was validated four times, wherein TPC of *bignay* pomace extracts was quantified. The experimental values and the predicted values were compared to verify the validity of the model.

Polyphenolic compounds analyses and antioxidant activities of *bignay* pomace extract

The quantitative determination of the polyphenolic compounds and antioxidant activities of *bignay* pomace extracts was conducted using a UV-Vis spectrophotometer (Shimadzu Corp., Japan).

Total phenolics content (TPC)

TPC was conducted according to Zubia et al. (2023). The standard used to plot the calibration curve (linearity range = 0–150 mg/ L, $R^2 > 0.9971$) was gallic acid. The TPC of the samples was conveyed as milligrams gallic acid equivalents per liter of extract (mg GAE/ L) during optimization and per gram dry weight (mg GAE/ g DW) during characterization.

Total Monomeric Anthocyanin content (TMAC)

TMAC was quantified using the pH differential method (AOAC, 2005). The total monomeric anthocyanin content (mg cyanidin-3-glucoside equivalents/ L) was computed based on the following equation below:

Total pigment (mg cyanidin-glucose equivalents/ L) =

$$(A \times MW \times DF \times 10^3) \,/\, (\varepsilon \times l)$$ /1/

Where A is $(A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$, MW is the molecular weight of cyanidin-3- glucoside (449.2 g/mol), DF is the dilution factor, ε is the molar extinction coefficient of cyanidin 3-glucoside (26,900 L x mol^{-1}x cm^{-1}), l is the cell path length (1 cm), and 10^3 is the factor conversion from g to mg.

The TMAC value was then converted to mg cyanidin-3-glucoside equivalents/ g dry weight (mg C3GE/ g DW).

Total flavonoids content (TFC)

TFC was determined as per Fattahi et al. (2014). The standard curve (linearity range = 0–500 µg/ mL, $R^2 > 0.9995$) was plotted using catechin. The values were conveyed as mg catechin equivalents per gram DW (mg CE/ g DW).

Condensed tannin content (CTC)

CTC assay was conducted as per Medini et al. (2014). The standard curve (linearity range = 0–400 µg/ mL, $R^2 > 0.9972$) was plotted using catechin. The values were conveyed as mg catechin equivalents per gram DW (mg CE/ g DW).

Determination of antioxidant properties

The DPPH• scavenging activity assay was conducted according to Marinova and Batchvarov (2011) with some modifications. The $ABTS^{\bullet+}$ and FRAP assay was based on Castillo-Israel et al. (2020) with modifications. A calibration curve was obtained using Trolox as the standard for all antioxidant assays (DPPH: linearity range: 0–7.2 µg/ mL, $R^2 > 0.9963$; ABTS: linearity range: 0–40 µg/ mL, $R^2 > 0.9972$; FRAP: linearity range: 0–40 µg/ mL, $R^2 > 0.9950$). The results were conveyed as mg Trolox equivalents per gram dry weight (mg TE/ g DW).

Statistical analysis

The regression equations, analyses of the response surface and contour plots, and statistical analysis of the experimental design were conducted using Stat-Ease® Design-Expert version 10 (Ease 2016). The results were expressed as mean ± standard deviation for the screening and optimization studies (n=4), polyphenolic compounds analysis (n=3), and antioxidant assays (n=5).

RESULTS AND DISCUSSIONS

Phenolic compounds are ubiquitous in various fruits and vegetables and are captivating the interest of researchers because of their antioxidant capacities and other potential health benefits. These compounds are commonly extracted using copious amounts of organic solvents, long incubation times, and immense dependency on the operator (Plaza et al. 2017). These conventional extraction methods were efficient enough, but the resulting extracts are unsafe for human consumption since the solvents can be dangerous upon ingestion (Che Sulaiman et al. 2017). Green extraction methods, utilizing the least amount of organic solvents possible, are desirable to minimize the environmental and health effects as well as maximize the yield of polyphenols (Panja 2017).

Screening of factors affecting the extraction of phenolics from *bignay* pomace

Plackett-Burman design (PBD) of experiment was employed to identify significant factors affecting the phenolic extraction from *bignay* pomace. The impact of solvent concentration (A), sample concentration (B), pH of the solvent (C), incubation time (D), incubation temperature (E), sonication time (F), and agitation (G) were evaluated.

Table 1 shows the PBD generated for each run and its corresponding experimental TPC values. The TPC values obtained ranged from 25.84 to 903.48 mg GAE/ L. The lowest value was observed on the run with the following parameters: (A) 100 % ethanol, (B) 1 g sample, (C) pH 7, (D) 120 min incubation, (E) 30 °C, (F) 90 min sonication, and (G) 300 rpm agitation. In contrast, the highest value was given by the treatment with (A) 0 % ethanol, (B) 11 g sample, (C) pH 2, (D) 120 min incubation, (E) 70 °C, (F) no sonication, and (G) 300 rpm agitation. The huge variation in the TPC values observed is an indication of the need for the determination of significant factors that directly contribute to the response variable.

Table 1: Plackett-Burman experimental design for variable screening and the corresponding experimental TPC values.

Run	A	B	C	D	E	F	G	H
1	100	1	7	120	30	90	300	25.84 ± 1.13
2	100	11	2	13	30	90	0	503.19 ± 12.11
3	100	11	2	120	70	90	0	900.42 ± 11.60
4	0	1	2	13	30	0	0	82.48 ± 1.23
5	0	11	7	13	70	90	300	691.22 ± 38.54
6	50	6	4.5	66.5	50	45	150	646.22 ± 9.87
7	50	6	4.5	66.5	50	45	150	643.17 ± 13.61
8	100	11	7	13	30	0	300	371.74 ± 5.81
9	0	1	2	120	30	90	300	103.04 ± 6.22
10	50	6	4.5	66.5	50	45	150	615.72 ± 14.53
11	0	11	2	120	70	0	300	903.48 ± 9.80
12	0	11	7	120	30	0	0	247.31 ± 2.46
13	100	1	7	120	70	0	0	69.44 ± 3.51
14	100	1	2	13	70	0	300	100.21 ± 3.30
15	0	1	7	13	70	90	0	102.86 ± 0.23

Solvent concentrations in percent ethanol (A), sample concentration in percent (B), pH of the solvent (C), incubation time in min (D), incubation temperature in °C (E), sonication time in min (F), and agitation in rpm (G). Total phenolic content (TPC) in mg GAE/L extract (H) presented as mean ± standard deviation (n=4)

To determine which of the seven factors evaluated had a significant effect on the response variable, PBD was performed following the standard procedure using the Design-Expert software. The values with the highest contribution were selected. This could be evaluated using a half-normal plot or the contribution table. The values under the ANOVA table were also evaluated, which included the *p*-value and the model's lack of fit. Initial assessment showed that the lack of fit was significant, which is not desirable since the model must fit. Thus, the necessary data transformations suggested by the software were performed and the graphs on the diagnostic tab were evaluated. The graphs on the diagnostic tab, i.e., normal probability plot, residuals vs. predicted plot, and residuals vs. run plot, were evaluated to assess whether the assumptions of ANOVA were met. After which, the lack of fit was deemed not significant, and thus, among the seven factors evaluated, three extraction parameters were found to significantly impact the amount of phenolics recovered. The factors that had significant effects (*p*-value<0.05) on the TPC were sample concentration, incubation temperature, and incubation time. From these three significant factors identified, sample concentration had the most significant effect on the response, with 65.38% contribution. Meanwhile, incubation temperature accounts for 11.97% contribution, followed by incubation time (8.62%), and other remaining variables (14.03%). The Pareto chart (Fig. 1) corroborated the results wherein the *t*-value of the significant factors exceeded the Bonferroni limit. The values above the threshold of the Bonferroni limit are considered to evoke significant effects while those below the t-limit are insignificant (Hu et al. 2016). Values that exceeded the *t*-limit but not the Bonferroni limit may or may not be significant and their inclusion in the optimization experiment may be based on the discretion of the researcher. Sample concentration, incubation temperature, and incubation time elicited a significantly positive effect on the extraction of phenolics from *bignay* pomace as graphically presented in Fig. 2. Similar trends were observed in studies conducted by Anastácio and Carvalho (2013) and Han et al. (2011) wherein they reported that solvent-to-solid ratio positively affected the phenolic compounds extraction from sweet potato and betel nut seed, respectively. This can be attributed to the principles of mass transfer indicating that the main driving force of the reaction is brought about by the gradient observed between the sample and the solvent (Belwal et al. 2016).

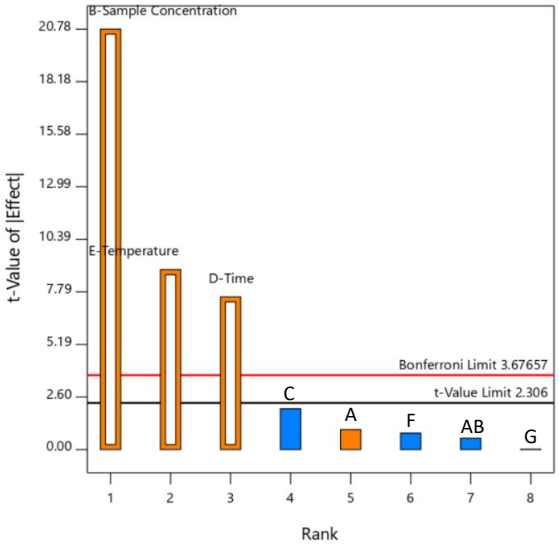

Figure 1: Pareto chart representing the effect of each variable on the total phenolic content (TPC) of freeze-dried *bignay* pomace extracts in descending order from greatest to lowest contribution. A: solvent concentration, B: sample concentration, C: pH, D: time, E: temperature, F: sonication time, G: agitation, and AB: 2-factor interaction AB

Meanwhile, Santos and Meireles (2011) reported that incubation temperature positively affected the amount of phenolics extracted from Brazilian jabuticaba skins. Comparable results were also reported in studies that extracted phenolics from the leaves of Himalayan Quercus species (Pandey et al. 2021) and carob pulp (Benchikh and Louailèche 2014) showing a positive linear relationship between phenolic content and incubation temperature. While polyphenol recovery can be improved by increasing the temperature, it should not exceed the temperature (>100 °C) at which the compounds degrade and become ineffective (Belwal et al. 2016; Solomakou et al. 2022).

Likewise, in previous studies conducted by Che Sulaiman et al. (2017) and Muzolf-Panek and Stuper-Szablewska (2021), incubation time was also found to have a positive linear relationship with the extraction of polyphenols. The extended interaction between the pomace and the solvent may have allowed ample time for the polyphenolic compounds to leach out to the solvent (Che Sulaiman et al. 2017). However, Benchikh and Louailèche (2014) pointed out that an excessive incubation time would not be useful since, at a certain point, the final equilibrium between solute and solvent would be reached. This phenomenon is called the Fick's second law of diffusion (Mokrani and Madani 2016).

The *F*-test is used to compare the source mean square to the residual mean square. In the study, the *F*-test elicited that the regression model displayed a high *F*-value (F = 183.10) and a low *p*-value (p<0.0001), stipulating that the model is highly significant. The lack of fit (the variation due to model inadequacy) *F*-value of the model is not significant (*F*-value = 0.1117), indicating that there is no evidence that the model does not adequately explain the variation in the responses.

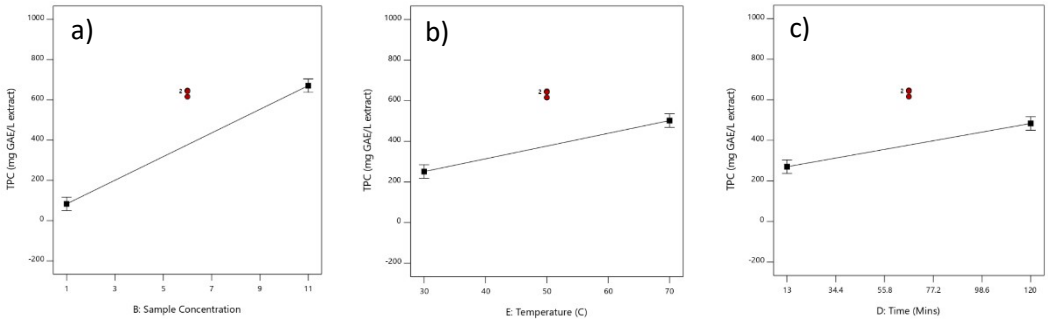

Figure 2: Single factor effects plot for (a) sample concentration, (b) incubation temperature, and (c) incubation time on the total phenolic content (TPC) of freeze-dried *bignay* pomace extracts using Plackett-Burman design.

The model recorded a coefficient of determination (R^2) of 0.9874, adjusted R^2 of 0.9820, and predicted R^2 of 0.9578. The R^2 illustrates the quality of model fit. R^2 is the ratio of the explained variation to the total variation (Arruda et al. 2016) wherein the experimental data is more acceptable as it approaches 1. The predicted R^2 value stipulates the ability of the model to predict the response values whilst the adjusted R^2 shows the descriptive power of the regression model since it only considers the significant model terms (Che Sulaiman et al. 2017). In this research, the high value of the adjusted and predicted R^2 indicates that the experimental and the theoretical values are in reasonable agreement since the difference between them is less than 0.2. A model is considered as effective when the difference between the predicted and adjusted R^2 is less than 0.2 (Che Sulaiman et al. 2017). The model equation developed through PBD showing the dependence of TPC on sample concentration, time, and temperature is as follows:

TPC= -422.34088 + 58.72161*Sample Concentration + 1.99251*Time + 6.28208*Temperature /2/

Optimization of significant factors identified

Based on the results obtained after screening various extraction factors, the Box-Behnken design (BBD) of experiment was performed to optimize the levels of significant parameters identified on the extraction of phenolics from *bignay* pomace. The factors considered for optimization were sample concentration, incubation temperature, and incubation time. On the other hand, the factors that were determined to be insignificant were kept constant. The solvent concentration used was 0 % ethanol (distilled water). Solvent pH was kept constant and was recorded at 6.67. Sonication time and agitation were no longer applied as they were not significant based on PBD results. The utilization of water as an extracting solvent is highly favorable. Water is considered a "green solvent", readily available, cheap, easily recycled, non-flammable, and non-toxic, making it both economically and environmentally advantageous (Cvjetko Bubalo et al. 2018; Andres et al. 2020). Furthermore, there have been previous studies that utilized water as the solvent for phenolic extractions, such as in chestnut shells (subcritical water)(Pinto et al. 2021), craft brewer's spent grains (Andres et al. 2020), sweet potato peels (Anastácio and Carvalho 2013), and Brazilian jaboticaba skins (acidified, high pressure CO_2 assisted) (Santos and Meireles 2011).

Table 2 shows the Box-Behnken experimental conditions with the corresponding TPC values. The range of factors used in the optimization was adjusted because variable screening showed a positive effect on the response. The sample concentration used was adjusted to 1–15% w/v and incubation time was 13–180 minutes. Incubation temperature was retained, as phenolic compounds are sensitive to thermal degradation, and exposure to temperatures higher than 70°C should be avoided.

The TPC ranged from 86.37 to 890.90 mg GAE/ L extract. The highest TPC value was observed in run 14 (Table 2) with the highest sample concentration (15% w/v) and incubated at 50 °C for 13 min. On the other hand, the lowest value was seen in run 1 where the extraction was conducted at 30 °C for 96.5 min, using the lowest sample concentration (1% w/v). The experimental data on the TPC resulted in a wide range of values. This stipulates that extraction optimization is vital to attain high yields in the most economical way possible.

Table 2: Box-Behnken experimental design with three independent variables and the corresponding experimental TPC values.

Run	A	B	C	D
1	1	30	96.5	86.37 ± 2.69
2	8	50	96.5	432.16 ± 12.60
3	8	70	180	673.95 ± 4.73
4	8	70	13	504.83 ± 15.04
5	8	50	96.5	498.77 ± 53.88
6	15	70	96.5	670.76 ± 17.44
7	8	30	13	531.13 ± 19.55
8	15	50	180	842.31 ± 50.80
9	8	30	180	522.99 ± 15.04
10	8	50	96.5	599.27 ± 10.70
11	1	50	13	93.36 ± 3.94
12	1	50	180	93.55 ± 4.26
13	15	30	96.5	753.13 ± 26.69
14	15	50	13	890.90 ± 12.27
15	1	70	96.5	101.69 ± 3.99

Sample concentration in percent (A), incubation temperature in °C (B), and incubation time in min (C). Total phenolic content (TPC) in mg GAE/L extract (D) presented as mean ± standard deviation (n=4)

BBD was conducted following standard procedure using the Design-Expert software. No data transformations were conducted prior to data analysis. ANOVA revealed that only sample concentration (p-value<0.0001) had a significant positive effect on TPC during optimization. Incubation temperature (p-value = 0.8326) and incubation time (p-value = 0.6805) were found to be insignificant. The results are graphically presented in Fig. 3. Similar observations were described by Anastácio and Carvalho (2013), Han et al. (2011) and Belwal et al. (2016) on the effect of sample concentration. These studies observed that TPC of extracts and sample-to-solvent ratio have a positive linear relationship. In contrast, Andres et al. (2020) detailed that using a higher solid-to-liquid ratio resulted in lower TPC values. As for the incubation temperature and times, these are inconsequential during optimization. The behavior of incubation temperature contradicts the findings of Hou et al. (2016) on phenolic and antioxidant compounds extraction from *Melaleuca bracteata* leaves. Their study indicated that TPC continued to rise with increasing incubation temperature for the test range, 30–70 °C. On the other hand, the effect of incubation time was in agreement with Andres et al. (2020) and Belwal et al. (2016), where extraction time had no effect on phenolic compounds extraction from craft brewer's spent grain and *Berberis asiatica* fruits, respectively.

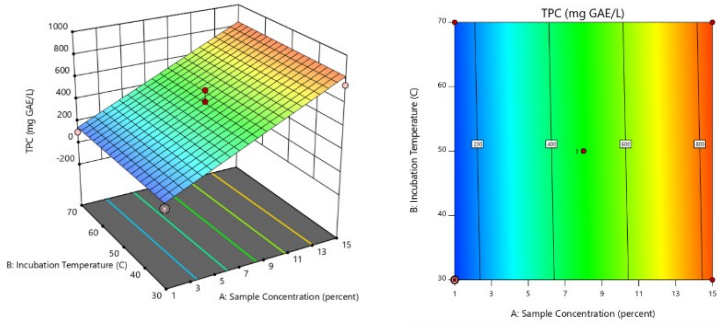

Figure 3: Response surface plot (a) and contour plot (b) showing the interactive effect of sample concentration, incubation temperature, and incubation time on the total phenolic compounds of freeze-dried *bignay* pomace extracts

The coefficient estimate value for sample concentration (A) was 49.68095, incubation temperature (B) was 0.360007, and incubation time (C) was 0.168525 while the intercept was 54.63238. The regression equation as expressed as the actual values/equation is as follows:

TPC= 54.63238 + 49.68095*Sample Concentration + 0.360007 *Incubation Temperature + 0.168525*Incubation time /3/

The actual equation depicts the dependence of the response variable on the factors used.

The model generated was found to be adequate for accurately predicting the results of the response parameter through the evaluation of these statistical measures. For the model to be a good fit, the R^2 should be at least 0.8000. In this study, the R^2 is 0.9087. Furthermore, the predicted (R^2 = 0.8192) and adjusted R^2 (R^2 = 0.8838) are in reasonable agreement. It can be classified as strong since the adjusted R^2 was relatively close to 1 and the difference between the values is less than 0.2. According to Dahmoune et al. (2015), the adjusted R^2 value should be near the R^2 to have a statistically good model. Model adequacy can also be evaluated based on *F*- and *p*-values. The model has an *F*-value of 36.49 and a *p*-value of <0.0001, which elicits that the model is significant. Dahmoune et al. (2015) reported that there is only a 0.01% chance that the large *F*-value of the model is due to noise. Conversely, the lack of fit *F*- and *p*-values (*F*-value = 1.31; *p*-value = 0.5066) of the model is not significant relative to pure error, hence verifying the soundness of the model. The inconsequential value of the lack of fit is desirable since the model must fit. Furthermore, the adequate precision value obtained, which is the measure of signal to noise, was 14.89. A value of more than 4 is considered desirable since this indicates an appropriate signal-to-noise ratio and an adequate model.

Optimal conditions were determined by minimizing the incubation time and temperature, keeping the sample concentration within the experimental range, and maximizing TPC. The factors were then assigned various priority levels. Stat-Ease® Design-Expert version 10 showed a list of numerical values that would fit the desired criteria.

The incubation time (*F*-value: 0.1789; *p*-value: 0.6805) and temperature (*F*-value: 0.0468; *p*-value: 0.8326) were statistically not significant in the model; thus, these factors were set to a minimum to economize the extraction process. Faster incubation time would mean faster extract production and less energy consumption. As for the incubation temperature, it is known to influence compound solubility, diffusion coefficient, and solvent viscosity, and even weaken the phenol linkages with proteins and polysaccharides (Andres et al. 2020). However, phenolic compounds are also denatured at certain temperatures. Furthermore, the study conducted by Andres et al. (2020), where they also used water as the extracting solvent for brewer's spent grain, showed that at lower temperatures, TPC extraction was higher. Thus, in this study, the temperature was set to a minimum. The sample concentration was set to be within the experimental range despite having a positive effect on the TPC. To economize the extraction process, the amount of sample used must be enough to have a substantial yield without using excessive raw materials. An optimized sample-to-solvent ratio is vital to identify a balance in extraction costs and solvent waste and to prevent saturation effects (Pandey et al. 2021). Lastly, the TPC was set to a maximum since higher amounts of phenolic compounds being extracted are desirable to maximize raw material utilization and process efficiency.

Validation of the optimized extraction protocol for phenolics extraction

The optimum extraction conditions were identified based on the response surface plots (Fig. 3). With a desirability index of 0.91, the optimal phenolic compound extraction method was determined to be at a sample concentration of 10.41 %, incubated at 30 °C for 13 min. The optimal sample concentration and incubation temperature obtained were in accordance with the optimal conditions described by Andres et al. (2020) from the optimization of phenolic and antioxidant extraction conditions in craft brewer's spent grains. The only difference was the incubation time wherein they utilizedutilized 121.9 min. This can be attributed to the difference in the plant tissue matrix of the raw materials.

To validate the adequacy of the model, four independent experiments using the optimum conditions were conducted. The predicted TPC mean for the optimized method was 584.31 mg GAE/ L extract. The model suggested that at 95 % confidence, the data mean should fall between the predicted interval of 426.37 to 742.24 mg GAE/ L extract. The validated experimental results showed a mean value (572.65 mg GAE/ L) within the prediction interval with a relative error of 2 %. This proves that the model generated is valid and adequate. Thus, RSM was able to effectively predict the TPC extracted from *bignay* pomace.

Polyphenolic compounds characterization using optimum conditions

The optimized extraction conditions resulted in high levels of phenolic compounds. Table 3 shows the polyphenolic compounds characterization of freeze-dried *bignay* pomace obtained using the optimized extraction conditions. The TPC of the extract was found to be 7.37 $\pm$ 0.05 mg GAE/ g DW which is higher compared to the values obtained by (Andres et al. 2020) on craft brewer's spent grains and Muzolf-Panek and Stuper-Szablewska (2021) on herbs and spices. The observed TPC in this study is higher than the phenolic content of banana, dates, sweet cherry, sour cherry, and white grape, but lower than red onion scales, purple sunflower hull, and buckwheat hulls (Shi et al. 2005). Namiesnik et al. (2013) also reported that the polyphenol content of gooseberry water extract was 5.37 $\pm$ 0.6

mg GAE/ g DW, indicating a higher recovery of phenolics in *bignay* pomace under optimal extraction conditions.

Table 3: Polyphenolic compounds characterization of freeze-dried *bignay* pomace obtained using the optimized extraction conditions.

Analysis	Values
Polyphenolic compounds analysis	
TPC, mg GAE/ g DW[a]	7.37 ± 0.05
TMAC, mg C3GE/ g DW[a]	2.35 ± 0.16
TFC, mg CE/ g DW[a]	2.88 ± 0.17
CTC, mg CE/ g DW[a]	10.61 ± 0.22
Antioxidant activity	
ABTS, mg TE/ g DW[b]	18.93 ± 0.17
FRAP, mg TE/ g DW[b]	15.46 ± 1.15
DPPH, mg TE/ g DW[b]	2.68 ± 0.10

GAE=gallic acid equivalents; C3GE=cyanidin-3-glucoside equivalents; CE=catechin equivalent; TE=Trolox equivalent; DW=dry weight
[a]Values are expressed as average ± standard deviation (n=3)
[b]Values are expressed as average ± standard deviation (n=5)

On the other hand, a higher value of TMAC was obtained in *bignay* pomace compared to Brazilian jaboticaba skins (Santos and Meireles 2011) extracted using acidified water under a high-pressure carbon dioxide assisted extraction and that of different varieties of strawberries (Zitouni et al. 2020) extracted using ethanol. On the contrary, gooseberry extracts (Namiesnik et al. 2013) showed a higher TMAC compared to *bignay* pomace. Meanwhile, the TFC value obtained is higher compared to garlic, nutmeg, and onion, but lower than cloves, oregano, thyme, and different varieties of strawberries (Namiesnik et al. 2013; Pandey et al. 2021). As for the CTC, the values obtained were higher compared to gooseberry (Namiesnik et al. 2013) and comparable with different strawberry varieties (Zitouni et al. 2020). However, the TMAC, TFC, and CTC were lower compared to Barcelo et al. (2016) and Recuenco et al. (2020) on fully ripe *bignay* berries.

The *bignay* pomace extract was also found to exhibit significant amounts of antioxidants based on ABTS, FRAP, and DPPH assays. Three types of assays were conducted since a single method would not accurately give a good measure of the antioxidant activity of a sample. The reason is that the mechanism of chemical reactions involved in each assay and their capacity to react with specific radical species differ. The antioxidant properties of *bignay* pomace were determined using three types of assays namely $DPPH^{\bullet}$ (2,2-Diphenyl-1-picrylhydrazyl) scavenging assay, $ABTS^{\bullet+}$ [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] scavenging activity and ferric-reducing antioxidant power (FRAP). According to Munteanu and Apetrei (2021), on the basis of chemical reactions involved, antioxidant activity assays can either be classified as hydrogen atom transfer (HAT) or single electron transfer (SET). Furthermore, Munteanu and Apetrei (2021) also differentiated DPPH, ABTS, and FRAP. DPPH assay is a common test applied to assess the antioxidant activity of plant extracts. It is based on the mechanism of electron donation from antioxidants to neutralize the DPPH radical. It is classified under the mixed mode tests wherein HAT and SET mechanisms can both be present at varied proportions, which is dependent on the reaction conditions (i.e. pH). DPPH is insoluble in water but soluble in different organic solvents. On the other hand, ABTS measures the capacity of antioxidants to neutralize 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). Its solubility in water and organic solvents makes it appropriate to be used to determine the antioxidant activity of lipophilic and hydrophilic compounds. It is classified under mixed mode methods. Lastly, FRAP is a SET-based method wherein the antioxidant capacity is measured under acidic conditions and is characterized by the reduction of (Fe^{3+})-ligand to the blue ferrous complex. It is recommended to use FRAP together with other assays to further distinguish the dominant antioxidant mechanism.

The obtained ABTS and FRAP values in the study were higher in comparison with different fruit peels such as apple, banana, avocado, dragon fruit, mango, pomegranate, and plum (Suleria et al. 2020). For the FRAP, the values were in range with the results of Andres et al. (2020) using water as an extraction solvent for craft brewer's spent grains. The DPPH values obtained in the study were higher compared to different fruit peels such as banana, dragon fruit, melon, nectarine, and plum while the values were lower compared to the peels of apples, avocado, grapefruit, and mango (Suleria et al. 2020). Furthermore, the results of all the assays conducted are lower compared to Zubia et al. (2023) wherein aqueous ethanol acidified with citric acid was utilized as the extraction solvent for *bignay* pomace.

CONCLUSIONS

In this study, RSM was used to identify the optimum green extraction parameters for maximum recovery of phenolic compounds from *bignay* pomace. The application of mathematical models was found to be effective in optimizing and predicting extraction conditions, which can be used as an alternative to conventional methods. The results of this study provided data that reinforced the idea of valorization of *bignay* pomace. The study also showed that polyphenolic and antioxidant compounds can be recovered from *bignay* pomace using water as a green extraction solvent with minimal incubation time and temperature. Thus, the optimized method shows promising economic and environmental advantages. It is also important to note that this extraction method is very easy to set up and implement on an industrial scale (compared to the use of organic solvents). Furthermore, this study could serve as one of the baselines that can be used to further the cause of processing by-product utilization in the Philippines.

The valorization of *bignay* pomace as a potential functional food ingredient would be profitable for manufacturers since it results in maximum raw material utilization. Moreover, exploring the possible application of by-products of the food and nutraceutical industry supports economic and environmental sustainability and decreases food waste. The determination of optimized extraction conditions is necessary to establish cost-effective and efficient recovery processes when applied at an industrial scale.

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CONFLICT OF INTEREST

The authors would like to disclose that there is no conflict of interest regarding this publication.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Gilda Melanie O. Babaran: Contributed to gathering, processing, and interpreting of data, performing assays, writing the original draft article, and critical revision. Sheba Mae M. Duque: Contributed to the experimental design of the work, experiment supervision, laboratory protocols, project leadership, acquisition of funds, resources, manuscript review and critical revision, and final approval of manuscript. Claire S. Zubia: Contributed to the conception or design or work, methodology, resources, manuscript review, and critical revision. Florencio C. Reginio Jr.: Contributed to the conception and design of work, resources, manuscript review, and critical revision.

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