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Optimization of Ultrasound-Assisted Extraction (UAE) for Simultaneous Determination of Individual Phenolic Compounds in 15 Dried Edible Flowers

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Abstract: Nowadays, dried edible flowers have become one of the eating habits of a healthy lifestyle. The most common way to consume dried flowers is via infused water (tisane). A number of studies on dried edible flowers have reported antioxidant activities mainly due to their phenolic compounds. This work has developed a new extraction method using ultrasound technology to determine phenolic compounds in 15 widely consumed edible flowers. Several extraction factors including pulse duty cycle (0.2, 0.6, 1.0 s^{-1}), temperature (10, 40, 70 °C), solvent-to-sample ratio (10:1, 20:1, 30:1 mL of solvent g^{-1} of sample), and solvent composition (0, 25, 50% methanol in water) have been optimized based on a Box–Behnken design coupled with response surface methodology. UPLC-PDA has been employed to quantify 12 major phenolic compounds (2,4,6-trihydroxy benzoic acid, protocatechuic acid, protocatechuic aldehyde, *p*-hydroxybenzoic acid, caffeic acid, vanillic acid, epicatechin, *p*-coumaric acid, ferulic acid, quercetin-3-rutinose, iso-ferulic acid, and quercetin-3-glucoside) in the extracts. The optimum extraction conditions for a 1 g sample were 30 mL of solvent (28% methanol in water) at 42 °C with 1.0 s^{-1} of pulse duty cycle. Based on the kinetic study, the optimal extraction time was 10 min. The method was validated with high precision (CVs of repeatability and intermediate precision were lower than 7%) and high accuracy (recovery higher than 90%). Additionally, the proposed ultrasound-assisted extraction was successfully applied in the determination of phenolic compounds in 15 dried edible flowers.

Keywords: Box–Behnken design; method development; UPLC; tisane; validation

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1. Introduction

Nowadays, edible flowers have become one of the choices in society's eating habits. The growth of functional food based on edible flowers and scientific interest in these commodities tend to increase the consumption of edible flowers [1]. The most popular way to prepare edible flowers is via infusions or the decoction of dry flowers in water, technically called a tisane. This approach has also been used in traditional medicine for years [2], considering the flowers' unique aroma and health benefits [3].

Edible flowers are potential sources for pharmaceutical substances [4] that positively contribute to human health as antioxidants with antiproliferative, antibacterial, anti-inflammatory, anti-cancer, anti-obesity, and neuroprotective effects [5,6]. These benefits are mainly a result of the phenolic compounds contained in the flowers [6]. Hence, it is essential to develop future studies using reliable methods for their analysis.

There are several challenges in developing an analytical extraction method for multiple analytes in a complex matrix due to the possibility of a rapid interaction between the analyte and the matrix and the time-consuming extraction procedure. One emerging technology

that can solve these issues is ultrasound-assisted extraction (UAE), which can speed up mass transfer and thereby improve the kinetics of the extraction.

In addition, former studies suggested that UAE requires lower solvent consumption while increasing the yield of phenolic compounds recovered from orange peel [7] and anthocyanin in *Hibiscus sabdariffa* [8] compared to heat-assisted extraction. This achievement can be explained due to the fact that UAE works at moderate temperatures, thus avoiding damage to the thermolabile phenolic compounds [6,9].

The applicability of UAE to recover bioactive compounds from flowers was reported for phenolic compounds in *Clitoria ternatea* [10], flavonoids in *Osmanthus fragrans* [11], and total phenolic compounds in *Santolina chamaecyparissus* [12]. However, several factors may influence the efficiency of UAE, such as the pulse duty cycle, solvent composition, temperature, and solvent-to-solid ratio [13–15]. Therefore, optimizing the extraction conditions which lead to the best responses from different matrix types in terms of phenolic compounds is necessary. The use of experimental design allows scientists to efficiently assess the effect of multiple factors on measures of response, which results in better resource management and thus lower experimental cost [16].

In this study, a Box–Behnken design was used to simultaneously evaluate the operating factors in the extraction efficiency of UAE. Subsequently, a response surface methodology followed with multi-response optimization and desirability functions were employed to define the optimum UAE condition. Finally, the optimized UAE was validated and applied to extract phenolic compounds from a number of dried edible flowers.

2. Materials and Methods

2.1. Chemicals and Reagents

Analytical grade standard compounds (2,4,6-trihydroxy benzoic acid, protocatechuic acid, protocatechuic aldehyde, *p*-hydroxybenzoic acid, caffeic acid, vanillic acid, epicatechin, *p*-coumaric, ferulic acid, quercetin-3-rutinose, iso-ferulic acid, quercetin-3-glucoside), ethyl acetate, and ethanol were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO, USA). The ultrapure water for the experiments was obtained from a Milli-Q water purification system by EMD Millipore Corporation (Bedford, MA, USA). The methanol (Fisher Scientific, Loughborough, UK), acetonitrile (Fisher Scientific, Loughborough, UK), and acetic acid (Scharlab, S.L., Sentmenat, Barcelona, Spain) were HPLC grade.

2.2. Plant Material

The dried flowers were obtained from an artisan floral tea producer (Elif Tea and Tisane, Cirebon, Indonesia). Fifteen flowers were used, such as *Calendula officinalis, Dianthus caryophyllus, Lilium bulbiferum, Chrysanthemum morifolium, Osmanthus fragrans, Prunus persica, Jasminum sambac, Clitoria ternatea, Rosa gallica* (bud), *Rose mengyin* (bud), *Malva sylvestris, Hibiscus sabdariffa, Chrysanthemum morifolium* (bud), *Malus* sp., and *Paeonia suffruticosa*. The size of tisane flowers was reduced using an ML130 grinder (Jata, Bilbao, Spain) with 30 s on and 30 s off and repeated for 5 times. Subsequently, the ground flower was passed through a 1 mm screen mesh using a vibratory sieve shaker (AS 200, Retsch GmbH, Germany). A homogenous composite sample consisting of the same portion of each flower powder was prepared by tumbling the mixture for method development experiments. Meanwhile, the remaining flower powders were stored individually for real sample application in an airtight container at 4 °C.

2.3. Ultrasound-Assisted Extraction (UAE)

Ultrasonic system Sonopuls HD 4200 (20 Hz, 200 W, BANDELIN electronic GmbH & Co KG, Heinrichstrabe, Berlin, Germany) with TS 104 probe, diameter 4.5 mm, was used for assisting the extraction. The sample was weighed (0.5 g) and placed in 50 mL centrifuge tubes. Based on the experimental design, a varied solvent composition (0, 25, 50% methanol in water) was added to reach the defined solvent-to-sample ratios (10:1, 20:1, 30:1 mL of solvent g^{-1} of sample). The extraction was performed at a range of pulse duty

cycles (0.2, 0.6, 1.0 s^{-1}) and temperatures (10, 40, 70 °C) controlled by Frigiterm system (J.P. Selecta, Barcelona, Spain). After the extraction, the extracts were centrifuged (Centrofriger, J.P. Selecta) at 4000 rpm. Then, the necessary amount of methanol-water was added up to a 25 mL final volume. The extracts were kept in closed vials wrapped with aluminum foil and stored at 4 °C until analysis.

2.4. Analysis of the Phenolic Compounds by UPLC-PDA

The extracts were analyzed by ultra-high performance liquid chromatography coupled with a photodiode array detector (UPLC-PDA) (Acquity UPLC Waters Corporation, Milford, MA, USA). The chromatographic separation was carried out in a C18 solid-core based reverse-phase column (1.7 µm, 2.1 × 100 mm, CORTECS UPLC, Waters Corporation, Ireland). The column temperature was set at 47 °C. The mobile phase consisted of phase A (2% acetic acid in ultrapure water) and phase B (2% acetic acid in acetonitrile). The gradient of elution (time, % solvent B) was 0 min, 0%; 1 min, 0%; 3 min, 5%; 4 min, 10%; 4.5 min, 10%; 5 min, 20%; 7 min, 20%; 8 min, 30%; 9 min, 100%; 12 min, 100%; 13 min, 0%. The flow rate was 0.55 mL min^{-1}. The extracts were filtered through a 0.22 µm nylon syringe filter (Filter-Lab, Barcelona, Spain) before the injection into the chromatographic system. The resulting chromatogram was processed utilizing Empower 3 software (Waters). For identifying the compounds, a full scanning for the spectra (200–400 nm) was performed. While for the quantification, a specific wavelength was chosen at the maximum absorbance for the corresponding compound: 260 nm for 2,4,6-trihydroxy benzoic acid, protocatechuic acid, protocatechuic aldehyde, quercetin-3-rutinose, quercetin-3-glucoside, *p*-hydroxybenzoic acid, and vanillic acid; 280 nm for epicatechin; 310 nm for *p*-coumaric acid, and 320 nm for caffeic acid, ferulic acid, and iso-ferulic acid.

2.5. Experimental Design

In this work, a Box–Behnken experimental design (BBD) was used to measure the effects of four independent factors, i.e., pulse duty cycle (X_1), temperature (X_2), solvent-to-solid ratio (X_3), and solvent composition (X_4) on the total of benzoic acid derivatives, cinnamic acid derivatives, and flavonoids. As the design included four factors with three levels −1 (low), 0 (medium), and 1 (high), thus, the experimental design consisted of 27 treatments with three repetitions at their center points (Table 1).

Table 1. Box–Behnken design with normalized measured responses * and the prediction errors.

Run	Factors				Responses (%)					
	X_1	X_2	X_3	X_4	Benzoic Acid Derivatives		Cinnamic Acid Derivatives		Flavonoids	
					Observed	Error	Observed	Error	Observed	Error
1	−1	−1	0	0	58.71	5.11	71.01	1.11	73.29	3.22
2	1	−1	0	0	71.45	1.83	77.91	0.42	89.37	2.68
3	−1	1	0	0	73.50	1.59	85.81	3.73	87.44	2.96
4	1	1	0	0	72.09	4.74	85.57	3.17	89.61	2.40
5	0	0	−1	−1	79.17	1.00	54.48	18.34	71.94	0.95
6	0	0	1	−1	100.00	4.94	69.98	23.90	89.43	7.19
7	0	0	−1	1	50.17	8.41	74.23	11.97	71.68	8.12
8	0	0	1	1	57.47	1.59	100.00	4.79	100.00	1.01
9	−1	0	0	−1	86.65	0.71	55.70	8.66	79.05	7.91
10	1	0	0	−1	84.32	2.68	35.68	23.71	73.75	7.88
11	−1	0	0	1	55.04	2.95	99.61	15.28	97.57	11.02
12	1	0	0	1	53.96	2.44	96.15	2.36	87.36	2.70
13	0	−1	−1	0	63.54	0.02	68.27	1.93	78.39	5.82
14	0	1	−1	0	65.84	3.53	79.56	1.12	82.24	2.92

Table 1. *Cont.*

Run	Factors				Responses (%)					
	X_1	X_2	X_3	X_4	Benzoic Acid Derivatives		Cinnamic Acid Derivatives		Flavonoids	
					Observed	Error	Observed	Error	Observed	Error
15	0	−1	1	0	72.73	2.34	86.56	1.45	91.10	1.15
16	0	1	1	0	77.11	0.94	92.30	0.90	98.16	0.95
17	−1	0	−1	0	77.05	6.46	61.37	11.86	71.67	5.49
18	1	0	−1	0	66.22	5.01	80.25	10.66	80.95	4.08
19	−1	0	1	0	67.89	3.57	71.05	15.72	84.80	6.82
20	1	0	1	0	78.12	4.90	91.72	3.07	98.90	1.16
21	0	−1	0	−1	78.19	1.64	36.10	15.66	65.55	6.00
22	0	1	0	−1	88.66	0.99	50.14	14.63	81.99	2.35
23	0	−1	0	1	53.37	2.95	96.31	3.29	87.65	1.19
24	0	1	0	1	55.23	3.66	96.32	1.24	90.48	1.29
25	0	0	0	0	73.45	0.74	67.82	12.08	94.24	2.19
26	0	0	0	0	74.11	0.15	88.21	14.37	93.40	1.28
27	0	0	0	0	74.44	0.59	86.90	12.67	89.03	3.46

* The relative value to maximum response (%) of the phenolic compounds in the samples.

Once the BBD was completed, Minitab software (Minitab Ltd., Brandon Curt, UK) was used for data analysis. The statistical significance of the studied factor and the evaluation of the fitting quality of the polynomial model were defined based on the analysis of variance (ANOVA). A second-order polynomial equation including all possible main, interaction, and quadratic effects was applied as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{44} {X_4}^2 \quad (1)$$

where Y was the dependent variable while X_1, X_2, X_3, and X_4 were the independent variables. β_0 corresponded to the ordinate, β_i represented the linear coefficients, β_{ij} was the cross-product coefficients, and βii indicated the quadratic coefficients. After the response surface equation from the response over the BBD domain was established, a multi-response optimization (MRO) was used to simultaneously optimize the three responses (amount of benzoic acid derivatives, cinnamic acid derivatives, and flavonoids).

2.6. Kinetics Study

A kinetics study was performed to evaluate the total levels of benzoic acid derivatives, cinnamic acid derivatives, and flavonoids at different extraction times under the optimum conditions of pulse duty cycle (X_1), temperature (X_2), solvent-to-solid ratio (X_3), and solvent composition (X_4) as defined by the MRO. The extraction was conducted in triplicate at 5, 10, 15, 20, 25, and 30 min to confirm the optimum extraction time to recover the phenolic compounds from dried flower samples.

2.7. Method Validation

To validate the developed UAE, the precision and accuracy of the method were assessed. The precision was expressed as the coefficient of variation (CV, %) and evaluated at two levels: repeatability and intermediate precision. For the repeatability analysis, nine extractions were conducted on the same day. For the intermediate precision study, three extractions were completed on each of three consecutive days (a total of nine experiments). The extraction was repeated for up to three cycles to ensure a complete recovery and to measure the recovery (R, %). In the first extraction cycle, the extract (supernatant) was collected after the centrifugation, while the dried flower residue was re-extracted with fresh solvent for the second and so forth for the third extractions. The level of

phenolic compounds in the extract resulting from each extraction cycle was measured. The experiment was performed in triplicate.

3. Result and Discussion

3.1. Determination of Individual Phenolic Compounds

The analytical properties of the UPLC-PDA method used to determine individual phenolic compounds were assessed (Table 2). The chromatographic system was validated following the guideline by ICH guideline Q2 (R1) [17].

Table 2. Performance of the UPLC-PDA method for individual phenolic compounds.

Phenolic Compounds	Low Range (0.5–10 ppm)		High Range (10–50 ppm)		LOD (ppm)	LOQ (ppm)
	Linear Equation	R^2	Linear Equation	R^2		
2,4,6-Trihydroxybenzoic acid	$y = 9636x + 4632$	0.951	$y = 17928x - 95606$	0.985	1.35	4.10
Protocatechuic acid	$y = 18479x - 1531$	0.999	$y = 19551x - 12114$	0.999	0.15	0.46
Protocatechuic aldehyde	$y = 12871x - 1897$	0.999	$y = 13832x - 20349$	0.991	0.14	0.44
p-Hydroxybenzoic acid	$y = 38594x - 3740$	0.999	$y = 41359x - 36092$	0.999	0.16	0.50
Caffeic acid	$y = 30349x - 5105.6$	0.995	$y = 33955x - 88931$	0.997	0.24	0.74
Vanillic acid	$y = 26087x + 2031$	0.997	$y = 28323x - 38614$	0.998	0.22	0.66
Epicatechin	$y = 4115.8x - 830$	0.985	$y = 4698x - 5508$	0.995	0.53	1.61
p-Coumaric	$y = 64719x - 5985$	0.993	$y = 71255x - 58676$	0.998	0.36	1.10
Ferulic acid	$y = 42079x - 2380$	0.992	$y = 45430x - 19546$	0.995	0.38	1.15
Quercetin-3-rutinose	$y = 18675x - 4599$	0.999	$y = 20494x - 29827$	0.997	0.16	0.49
Iso-ferulic acid	$y = 33118x + 2623$	0.997	$y = 35497x - 11481$	0.992	0.26	0.78
Quercetin 3 glucose	$y = 8930x - 4066$	0.997	$y = 12290x - 17885$	0.992	0.22	0.66

The calibration curves were prepared to cover low (0.5–10 ppm) and high (10–50 ppm) concentration ranges of the analytes in the extract. By the regression analysis, the coefficients of determination (R^2) of the calibration curves were greater than 0.95, showing good linearity within the studied range to determine individual phenolic compounds in the extracts. The limits of detection (LOD) and quantification (LOQ) for the chromatographic determination were estimated based on the standard deviation at the origin from the regression analysis for the calibration curve. Protocatechuic aldehyde provided the lowest LOD (0.14 ppm) and LOQ (0.44 ppm). Meanwhile, all the limits for quantification were less than 4.10 ppm. This result demonstrates the usefulness of the chromatographic method for reliable determination within the studied concentration range, viz., starting from 0.5 to 50 ppm across two levels of calibration curves.

3.2. Solvent Screening

Screening for the most suitable solvent type to extract phenolic compounds from dried edible flowers was carried out prior to developing the UAE method. Four solvents (water, ethyl acetate, methanol, and ethanol) were selected for the extraction at 40 °C using a pulse duty cycle of 0.5 s^{-1} and the solvent-to-sample ratio of 20:1 mL of solvent g^{-1} of sample in triplicate.

The results disclosed that the total compounds in increasing order of concentration were found in the extracts of water, ethanol, methanol, and ethyl acetate. However, the substances identified as phenolic compounds (phenolic acid and phenolic aldehyde) in the resulting extracts numbered seven compounds by water, six compounds by methanol and ethyl acetate, and four compounds by ethanol (Table S1, Supplementary Material).

Although methanol provided a lower phenolic concentration than ethanol, this solvent was able to recover several compounds that could not be extracted by ethanol. Therefore, the use of a mixture of water and methanol was selected. This solvent composition has been reported to be suitable for extracting phenolic compounds from *Chrysanthemum mori-*

folium [18], banana flowers [19], flowers of *Malus* Mill. species [20], and flowers of *Crataegus monogyna*, *Cytisus multiflorus*, *Malva sylvestris*, and *Sambucus nigra* from Portugal [21].

3.3. Optimization of UAE Method

The optimization of the UAE conditions of the pulse duty cycle, extraction temperature, solvent-to-solid ratio, and solvent composition was based on BBD-RSM. Once a total of 27 units of experiments of BBD was carried out, analysis of variance (ANOVA) was performed to calculate the main, interaction, and quadratic effects of the studied variables on the level of phenolic compounds extracted from dried edible flowers. After the BBD response, the phenolic compounds were divided into three groups of derivatives: flavonoids, benzoic acid, and cinnamic acid. The calculated effects on the response were graphically represented in a Pareto Chart (Figure 1).

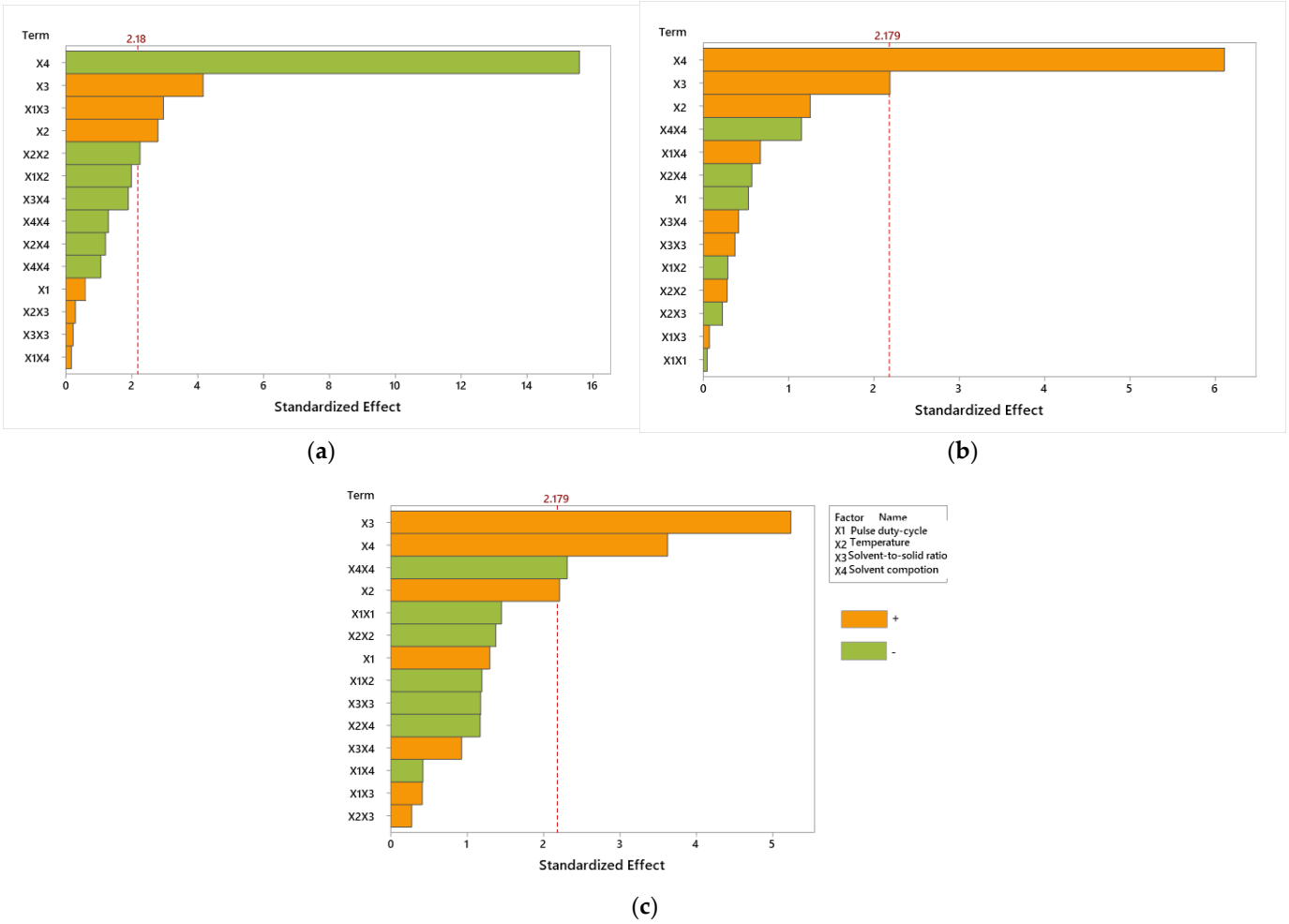

Figure 1. Pareto chart for the standardized effect of the UAE variables on the level of (**a**) benzoic derivatives, (**b**) cinnamic acid derivatives, and (**c**) flavonoids.

It can be observed that the most influential variable altering the three responses was the percentage of methanol in the extraction solvent (X_4). A similar result was shown in the optimization of phenolic compound extraction from cotton-lavender (*Santolina chamaecyparissus* L.) [12] and sunflower cake [22], disclosing that solvent composition was one of the factors significantly affected the extraction recovery.

The change of methanol percentage in the solvent composition used in the extraction significantly affected the recovery of the phenolic compounds. The affinity of the target compound to the extraction solvent defined the ratio of the solvent mixtures. The more polar phenolic compounds are, the more polar the solvent composition should be used over

non-polar solvents and vice versa [23]. The results showed that the percentage of methanol negatively affected the extraction of benzoic acid derivatives; however, it positively affected the other responses.

In addition to the extraction solvent, the solvent-to-solid ratio (X_3) significantly affected the three responses. A positive effect was observed as the level of the extracted phenolic compounds increased due to a greater ratio of solvent-to-solid. The higher the solvent-to-solid ratio, the larger the concentration gradient was, leading to increased diffusion of the compounds in the solvent [23,24].

The optimization of UAE for phenolic compounds in dried edible flowers, based on the coefficients for each variable, was considered employing only the significant main, interaction, and quadratic terms to build the second-order polynomial equations, thus avoiding high variability. The established equations to predict the amounts of benzoic derivatives (2), cinnamic acid derivatives (3), and flavonoids (4) under specific experimental conditions were as follows:

$$Y_{benzoic} = 64.35 - 0.11X_4 + 0.05X_3 + 1.32X_1X_3 + 0.62X_2 - 0.004{X_2}^2 \quad (2)$$

$$Y_{cinnamic} = 35.68 + 1.09X_4 + 0.15X_3 \quad (3)$$

$$Y_{flavonoid} = 26.07 + 1.51X_3 + 0.75X_4 + 0.009{X_4}^2 + 0.67X_2 \quad (4)$$

Table 1 compiles the experimental design run corresponding to the measured and predicted values for the responses. The differences between the measured and predicted values were, on average, 2.80% for benzoic derivatives, 8.82% for cinnamic acid derivatives, and 3.86% for flavonoids, while the R^2 of the prediction models were 0.9606, 0.7977, and 0.8268, respectively. The *p*-values for lack-of-fit in the ANOVA table of cinnamic acid derivatives (0.892) and flavonoids (0.178) were greater than 0.05, which means that the models were suitable for their intended purpose. However, the *p*-value for lack-of-fit of benzoic acid derivatives models (0.017) was lower than 0.05. The phenolic compounds included molecules with vast polarity and sizes; the lack-of-fit in these types of systems is typically due to the significant variety in the compound's structure [25].

The developed models have suggested the optimum conditions of the studied factors for each response over the BBD domain. Subsequently, a multi-response optimization was applied to obtain the most compromised UAE setting to achieve satisfactory recoveries for each group of phenolic compounds. The suggested extraction condition was 0.98 s^{-1} of pulse duty of cycle, 42 °C of extraction temperature, 30:1 (mL of solvent g^{-1} of sample) of solvent-to-solid ratio, and 28% methanol in water as the extraction solvent. The ratio of solvent-to-solid should not be increased over 30:1, as the signal in the chromatographic system would be too low for a reliable determination.

3.4. Assessment of the Extraction Time

The effect of extraction time on the level of extracted phenolic compounds was assessed to define the most efficient time for the developed UAE. A longer time for extraction facilitated the cavitation effects of UAE to disrupt the permeability of plant tissues, allowing the analytes to be released and diffuse into the extraction solvent. However, extended extraction time could endorse the degradation of the compounds. Hence, this extraction factor should be assessed [26]. UAE was performed under the optimal conditions over a varied extraction time (5, 10, 15, 20, 25, and 30 min). The level of extracted phenolic compounds in different extraction times is presented in Figure 2.

There were no significant differences in the level of phenolic compounds extracted between 5 to 20 min. Therefore, 10 min was selected as the suitable extraction time because it was the shortest time with the lowest experimental error to recover phenolic compounds from dried edible flowers. From Figure 2, it can be seen that there was a decline in the level of phenolic compounds in the extract after 20 min, most likely as a result of phenolic degradation [27]. A previous study on UAE for phenolic compounds in the *Opuntia ficus-indica* flower revealed a lower recovery after 30 min of extraction time caused by

excessive cavitation from ultrasound [28]. There are two phases of sonication time: first, the increase of extraction rate (around 90% of phenolic compounds were recovered); second, the decrease of extraction rate [29]. The degradation of phenolic compounds may happen in the second phase.

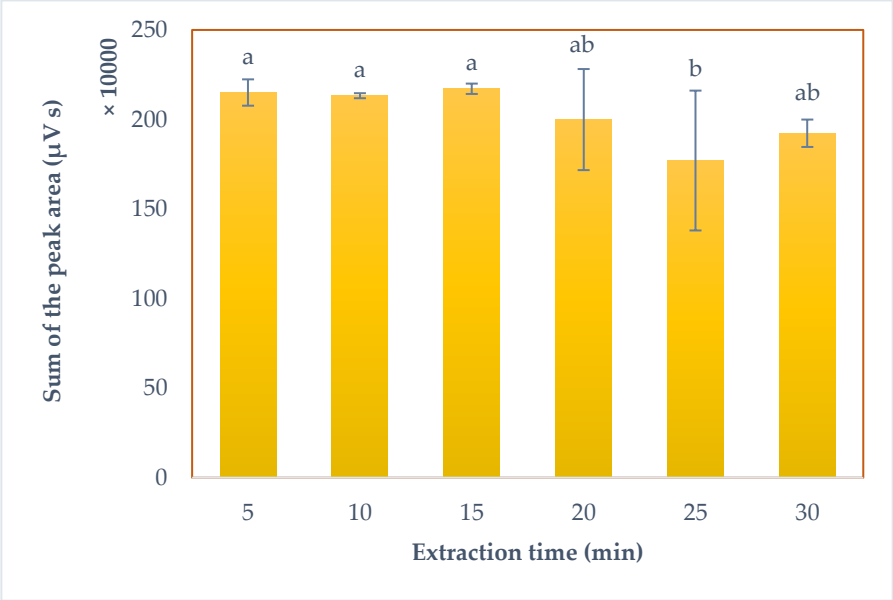

Figure 2. Average (n = 3) of the total area and standard deviations found for the phenolic compounds using the optimized extraction conditions and different extraction times. A different letter in different bars means significant differences based on Fisher LSD ($p < 0.05$).

3.5. Precision and Accuracy

The precision of the developed method was evaluated in terms of repeatability and intermediate precision. Repeatability was assessed by performing nine extractions under the optimum conditions on the same day. In contrast, intermediate precision was evaluated by performing three extractions daily for three consecutive days. The coefficient of variation (CV) of the two levels of precision is summarized in Table 3. The CV values were all satisfactorily below 7% for both repeatability and intermediate precision. Referring to AOAC, the acceptable limit for precision is ±10% [30]. Hence, the developed UAE is considered a precise extraction method.

Table 3. Precision and accuracy of UAE for phenolic compounds from dried edible flowers.

Phenolic Compounds	Precision CV (%)		Recovery (%)
	Repeatability	Intermediate Precision	
2,4,6-Trihydroxybenzoic acid	0.57	1.32	91.94 ± 1.04
Protocatechuic acid	2.50	1.15	100.00 ± 0.00
Protocatechuic aldehyde	2.95	2.42	84.9 ± 13.10
p-Hydroxybenzoic acid	5.00	3.08	93.61 ± 11.06
Caffeic acid	1.50	1.86	83.82 ± 0.61
Vanillic acid	1.45	2.23	93.23 ± 11.72
Epicatechin	4.63	6.66	94.12 ± 10.18
p-Coumaric	0.61	1.23	84.41 ± 0.21
Ferulic acid	0.95	3.96	87.04 ± 11.88
Quercetin-3-rutinose	1.12	3.94	82.07 ± 1.49
Iso-ferulic acid	5.82	5.22	99.34 ± 1.14
Quercetin 3-glucose	4.11	4.17	82.01 ± 1.58

The recovery of phenolic compounds from the dried edible flowers was measured by multi-cycle UAE to evaluate the accuracy of the method. The resulting recovery of each phenolic compound is presented in Table 3. The extraction was repeated for up to three cycles then using the total sum area in the three extractions as the total level in the sample. It must be noted that the recovery in the third extraction was always below 5% of the total area, so additional re-extractions were not used. The first extraction cycle can recover more than 82% of flavonoids, 83% of cinnamic acid derivatives, and 91% of benzoic acid derivatives. According to AOAC recommendations, these recovery levels reached the acceptable range (80–110%) [30]. Hence, one extraction cycle of UAE was adequate to recover phenolic compounds from dried edible flowers. In several cases, recoveries were very high, although below 90%. It must also be noted that the optimization of the extraction conditions using several different responses, i.e., 12 individual phenolic compounds, produced a common working condition allowing for high recoveries for all compounds; however, this was not as high as using 12 different extraction methods optimized individually.

3.6. Real Sample Application

The validated UAE method was used to extract the phenolic compounds from 15 different commercial dried edible flowers to evaluate its applicability. The extractions were carried out in triplicate using the optimum UAE condition. The content of individual phenolic compounds in 15 types of dried edible flowers is shown in Tables 4–6.

Table 4. Individual phenolic compounds from different dried edible flowers.

Phenolic Compounds	Phenolic Compounds from Extracted Edible Flowers (μg g^{-1})					
	 *Calendula officinalis*	 *Dianthus caryophyllus*	 *Lilium bulbiferum*	 *Chrysanthemum morifolium*	 *Osmanthus fragrans*	 *Prunus persica*
2,4,6-Trihydroxybenzoic acid	265.69 ± 2.86	<LOD	<LOD	317.51 ± 3.93	765.58 ± 13.17	700.66 ± 14.36
Protocatechuic acid	<LOD	<LOD	52.39 ± 2.56	30.63 ± 0.36	19.06 ± 1.90 *	64.95 ± 2.14
Protocatechuic aldehyde	<LOD	<LOD	<LOD	<LOD	38.66 ± 2.95	32.81 ± 1.79
p-Hydroxybenzoic acid	36.42 ± 3.56	73.09 ± 0.34	61.4 ± 0.86	<LOD	<LOD	27.28 ± 1.95 *
Caffeic acid	102.17 ± 2.81	148.47 ± 1.50	570.15 ± 11.47	73.05 ± 1.98	86.77 ± 1.36	70.74 ± 1.46
Vanillic acid	9.08 ± 1.26 *	228.95 ± 2.17	29.64 ± 0.77 *	4.77 ± 1.23 *	<LOD	30.83 ± 1.55 *
Epicatechin	302.72 ± 1.81	<LOD	<LOD	<LOD	409.78 ± 4.07	<LOD
p-Coumaric	8.78 ± 0.10 *	300.52 ± 2.56	138.43 ± 3.76	28.82 ± 0.50 *	291.68 ± 9.76	130.17 ± 15.01
Ferulic acid	11.06 ± 0.41 *	10.13 ± 0.87 *	95.49 ± 2.70	<LOD	376.01 ± 23.25	265.34 ± 21.61
Quercetin-3-rutinose	244.06 ± 0.48	<LOD	94.79 ± 2.29	485.21 ± 3.87	<LOD	<LOD
Iso-ferulic acid	<LOD	<LOD	15.77 ± 0.70 *	519.58 ± 6.64	53.8 ±1.99	<LOD
Quercetin-3-glucose	2377.99 ± 139.00	<LOD	<LOD	951.39 ± 15.65	<LOD	1215.06 ± 118.79
Total phenolic compounds	3357.97 ± 148.89	761.16 ± 3.09	1058.06 ± 18.21	2410.97 ± 21.63	2041.33 ± 22.50	2537.84 ± 173.68

* The value is between LOD and LOQ; <LOD in which the concentration was lower than the LOD value.

Table 5. Individual phenolic compounds from different dried edible flowers.

Phenolic Compounds	Phenolic Compounds from Extracted Edible Flowers (μg g^{-1})					
	 *Jasminum sambac*	 *Clitoria ternatea*	 *Rosa gallica* (bud)	 *Rose mengyin* (bud)	 *Malva sylvestris*	 *Hibiscus sabdariffa*
2,4,6-Trihydroxybenzoic acid	880.23 ± 8.33	641.88 ± 12.47	110.16 ± 10.49 *	151.48 ± 11.66 *	181.77 ± 6.68 *	<LOD
Protocatechuic acid	<LOD	39.45 ± 1.75	46.97 ± 7.15	195.8 ± 4.59	31.72 ± 0.53	<LOD
Protocatechuic aldehyde	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
p-Hydroxybenzoic acid	<LOD	<LOD	12.51 ± 0.6 *	<LOD	33.74 ± 2.11	<LOD
Caffeic acid	<LOD	23.92 ± 0.81 *	<LOD	<LOD	<LOD	72.88 ± 1.11
Vanillic acid	5.19 ± 0.92 *	22.62 ± 0.51 *	<LOD	<LOD	<LOD	13.25 ± 1.27 *
Epicatechin	63.69 ± 1.53	<LOD	182.49 ± 11	<LOD	89.69 ± 12.31	263.24 ± 11.02
p-Coumaric	17.07 ± 0.42 *	16.82 ± 1.20 *	<LOD	<LOD	460.57 ± 12.27	33.04 ± 0.59 *
Ferulic acid	<LOD	<LOD	<LOD	41.47 ± 2.88 *	445.62 ± 16.5	<LOD
Quercetin-3-rutinose	472.54 ± 14.76	492.29 ± 22.19	791.78 ± 30.82	1193.39 ± 125.49	396.41 ± 10.67	24.57 ± 0.95 *
Iso-ferulic acid	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Quercetin-3-glucose	639.9 ± 18.07	174.21 ± 3.61	1158.57 ± 41.22	2154.83 ± 160.92	381.36 ± 7.45	<LOD
Total phenolic compounds	2078.62 ± 35.95	1411.19 ± 37.14	2302.48 ± 64.81	3736.97 ± 265.48	2020.87 ± 47.12	406.98 ± 13.17

* The value is between LOD and LOQ; <LOD in which the concentration was lower than the LOD value.

Table 6. Individual phenolic compounds from different dried edible flowers.

Phenolic Compounds	Phenolic Compounds from Extracted Edible Flowers (μg g^{-1})		
	 *Chrysanthemum morifolium* (bud)	 *Malus* sp.	 *Paeonia suffruticosa* (bud)
2,4,6-Trihydroxybenzoic acid	1208.72 ± 7.6	<LOD	852.61 ± 38.92
Protocatechuic acid	37.18 ± 1.23	<LOD	65.64 ± 2.77
Protocatechuic aldehyde	<LOD	<LOD	<LOD
p-Hydroxybenzoic acid	<LOD	30.97 ± 2.72	161.74 ± 3.13
Caffeic acid	<LOD	111.26 ± 2.4	<LOD
Vanillic acid	<LOD	<LOD	<LOD
Epicatechin	<LOD	<LOD	1101.82 ± 18.81
p-Coumaric	24.27 ± 1.29 *	155.89 ± 1.97	31.86 ± 0.97 *
Ferulic acid	43.64 ± 1.57 *	51.55 ± 2.09 *	54.54 ± 0.55
Quercetin-3-rutinose	1035.07 ± 36.45	409.24 ± 20.7	<LOD
Iso-ferulic acid	1272.76 ± 33.89	<LOD	<LOD
Quercetin-3-glucose	2277.83 ± 50.29	189.22 ± 2.94	4261.23 ± 222.58
Total phenolic compounds	5899.48 ± 99.26	948.16 ± 26.42	6529.45 ± 191.95

* The value is between LOD and LOQ; <LOD in which the concentration was lower than the LOD value.

Twelve phenolic compounds were detected from the dried edible flower samples: 2,4,6-trihydroxy benzoic acid, protocatechuic acid, protocatechuic aldehyde, *p*-hydroxybenzoic acid, caffeic acid, vanillic acid, epicatechin, *p*-coumaric, ferulic acid, quercetin-3-rutinose, iso-ferulic acid, and quercetin-3-glucoside. The composition and concentration of phenolic

compounds varied in different types of flowers. A former study reported that *p*-coumaric, quercetin-3-rutinose, quercetin-3-glucoside, and 2,4,6-trihydroxy benzoic acid were widely found in edible flowers [31]. The highest total number of phenolic compounds found in the studied edible flower samples was 6529.45 ± 191.95 μg g^{-1} (*Paeonia suffruticosa*), while the lowest was 406.98 ± 13.17 μg g^{-1} (*Hibiscus sabdariffa*).

Prunus persica (peach blossom) comprised the most identified phenolic compounds (9 out of 12). This fact is relevant to the result previously reported that *Malus* sp. (apple blossom) had several phenolic compounds: caffeic acid, *p*-hydroxybenzoic acid, *p*-coumaric, quercetin-3-rutinose, and ferulic acid. Those phenolic compounds were identified and quantified in five varieties of apple blossoms in Korea [32]. In addition, the real sample application also disclosed that the bud of *Chrysanthemum morifolium* contained higher total phenolic compounds (5899.48 ± 99.26 μg g^{-1}) than the blossom (2410.97 ± 21.63 μg g^{-1}). Other samples of flower buds (*Paeonia suffruticosa, Rose mengyin*, and *Rosa gallica*) contained relatively higher total phenolic compounds than the blossom flowers. This finding corresponds with a former study on the phenolic compound in *Rosa xhybrida* (groundcover rose) during flower development. The highest phenolic compound found in bud or partially open flowers [33] indicated that blossom flowers are more susceptible to oxidation.

4. Conclusions

In the present study, the extraction method for phenolic compounds from dried edible flowers using ultrasound-assisted extraction was optimized employing a Box–Behnken design in conjunction with multi-response optimization. The optimum extraction condition was set by a pulse duty cycle of 1.0 s^{-1} at 42 °C and 28% methanol in water as an extraction solvent with the solvent-to-solid ratio of 30:1 (mL of solvent g^{-1} of sample). Based on the kinetics study, the optimal extraction time was 10 min. The developed method was validated with high precision (CV less than 7%) and accuracy (82% of flavonoids, 83% of cinnamic acid derivatives, and 91% of benzoic acid derivatives). Henceforth, the optimized and validated analytical method of the UAE approach is effective for determining individual phenolic compounds from dried edible flowers. Among the 15 dried edible flowers evaluated with the new method, the highest level of phenolic compounds was found in *Paeonia suffruticosa*, which was twelve times higher than the levels found in *Hibiscus sabdariffa*.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae8121216/s1, Table S1: Identified phenolic compounds in different solvents.

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