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Optimization of Microwave-Assisted Extraction for the Recovery of Bioactive Compounds from the Chilean Superfruit (*Aristotelia chilensis* (Mol.) Stuntz)

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Abstract: Maqui berry (*Aristotelia chilensis*) is being explored in the food industry, and is considered to be one of the healthiest berries due to its bioactive components and high commercial value. Microwave-assisted extraction (MAE) was developed for the determination of total phenolic compounds and anthocyanins from maqui. A Box–Behnken experimental design was employed in conjunction with a response surface methodology to optimize the conditions based on 27 different experiments. The extractions were carried out with four factors (i.e., methanol percentage, pH, temperature, and solvent volume:sample mass ratio), and two responses—total phenolics and anthocyanins. Temperature and methanol percentage were found to be the most influential parameters for total phenolic compounds and anthocyanins, respectively. The optimum MAE conditions were: 65% MeOH in water at pH 2, temperature of 100 °C, and a ratio of 10:0.5 for total phenolics; and 60% MeOH in water at pH 2, temperature of 50 °C, and a ratio of 14:0.5 for anthocyanins. Kinetics assays were carried out and an optimum time of only 2 min was identified for the extractions. Repeatability and intermediate precision were also evaluated, and coefficients of variation below 5% were obtained. The new methods were successfully applied to a foodstuff made with maqui.

Keywords: anthocyanins; *Aristotelia chilensis* (Mol.) Stuntz; food analysis; maqui berry; microwave-assisted extraction; phenolic compounds; superfruit

1. Introduction

There is growing interest within the population in the consumption of foods that are rich in health-promoting compounds and in an increasing incorporation of vegetables and fruits into the diet to improve quality of life [1]. As a consequence, research effort in food science has focused on the intake of fruits such as berries, which are rich in nutrients and can prevent several diseases and disorders. In fact, food and pharmaceutical industries have shown increasing attention in recent years to the development of new formulations with integrated berry extracts as a source of bioactive compounds

with high antioxidant capacity [2–4]. Maqui (*Aristotelia chilensis* (Mol.) Stuntz) is a small tree belonging to the family Elaeocarpaceae and it is native to the center and south of Chile and southwestern Argentina. The fruit of this tree contains one of the highest concentrations of antioxidants in the world, far surpassing other highly recognized fruit species [5–7]. In December and January the tree produces purple/black berries that are around 6 mm in diameter. The collection of this fruit is attributed to the Mapuche Indians of South America, who have consumed and used it as a natural remedy for thousands of years. In traditional herbal medicine, infusions of maqui berries have been used to treat sore throats, kidney complaints, digestive ailments, ulcers, fevers, and scarred wounds [7]. At present, the fruit of the maqui is highly appreciated for its characteristic flavor and aroma, in addition to its innumerable beneficial properties such as the prevention of Alzheimer's disease, cardiovascular diseases, and the oxidation of low-density lipoproteins, analgesic, anti-inflammatory, anti-carcinogenic, anti-oxidant, and anti-diabetic properties, and also for the control of obesity [8–10]. It is now known that the excellent properties of this fruit are due to the fact that they are an extremely rich source of bioactive compounds such as phenolic acids, tannins, stilbenes, flavonoids, and anthocyanins [6,10,11]. It is therefore not surprising that maqui has recently been recognized as a "superfruit" [5,12]. Maqui berries are being explored in the pharmaceutical, food, and cosmetics industries as a potential source of bioactive compounds. Therefore, its consumption in both fresh and processed forms such as juice concentrates, liqueurs, pills, capsules, lyophilized, beverages, jams, and jellies, is highly recommended [2].

The main phenolic compounds that have been identified in this fruit are gallic acid, hexahydroxydiphenic acid, granatine B, punicacortein C, flavonols (myricetin, quercetin, kaempferol, and its derivatives) and eight anthocyanins, which are delphinidin 3-*O*-sambubioside-5-*O*-glucoside, delphinidin 3,5-*O*-diglycoside, cyanidin 3-*O*-sambubioside-5-*O*-glucoside, cyanidin 3,5-*O*-diglycoside, delphinidin 3-*O*-sambubioside, delphinidin 3-*O*-glucoside, cyanidin 3-*O*-glucoside, and cyanidin 3-*O*-sambubioside [6,13], and these are responsible for its intense color and high antioxidant potential [14].

Maqui is considered to be one of the healthiest fruits, but it is still an understudied and relatively unknown berry. Although it has recently been commercialized, only a few references can be found in the literature, so it is necessary to develop extraction and analysis methodologies for this fruit [13,15].

The development of such methodologies is essential for quality control, as food fraud is a serious and increasing problem in the food industry [16,17]. Due to the high price of this berry, the replacement of the fruit with cheaper similar berries or cheaper fruit juices as diluting agents should not be ignored [18,19]. The extraction technique applied is the key step for the appropriate recovery and to develop an efficient process. Thus, the availability of optimized extraction methods for this fruit could help in the detection of possible food fraud. In recent years, there has been an increase in the number of publications focused on developing new extraction methods for bioactives from plants [20,21]. Among the extraction methodologies employed, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are considered to be excellent green alternatives to conventional methods for the extraction of bioactive compounds due to their low instrument set-up costs on a laboratory scale [21–24].

Microwave-assisted extraction is considered to be an effective method for the extraction of compounds from solid samples [25]. Microwaves are non-ionizing electromagnetic (EM) waves. Microwaves consist of electric and magnetic fields which oscillate perpendicularly to each other in high frequency range from 0.3 to 300 GHz. Microwave power provides localized heating in the sample and acts as a driving force for the destruction of the plant matrix so the analyte of interest can diffuse easily and dissolve in the solvent. The transfer of energy occurs through two mechanisms: the inversion of dipoles and the displacement of charged ions [26]. One of the main differences from other techniques is that instead of acting through conventional heating, microwave irradiation is a "cold" technique in which the heating occurs at the core of the object and extends from the inside of the body. In recent years, microwave extraction has been developed greatly due to the reduction in the extraction time and the amount of solvents, the degree of automation, and its high performance [27].

This technique has been used to extract antioxidant substances from a wide variety of matrices, such as grapes [28], tomatoes [29], myrtle [30], or basil [31], amongst others [21].

The efficiency of the extraction process can be affected by various factors, such as the type and volume of solvent, pH, and temperature. Solvent plays an important role in the solubility of the phenolic compounds. In fact, hydroalcoholic mixtures with similar polarity of the phenolic compounds are normally employed in the literature [32,33]. Regarding pH and temperature, both must be controlled due to the degradation processes at certain conditions [34]. Therefore, it is necessary to optimize the different variables of the extraction method to achieve maximum production yield [35]. In the study described here, a Box–Behnken design was carried out. This is a type of response surface factorial design that allows the reduction of the number of experiments compared to other statistical designs, while ensuring maximum possible information on the system response, in addition to an adjustment with quadratic models [36]. The results were treated by the response surface methodology, which generates a mathematical model that is adjusted to the experimental responses obtained and this allows the effects of the independent variables to be analyzed. The optimum values of these variables were obtained by solving the mathematical equation and the evaluation of the response surface plots [37,38].

To the best of our knowledge, this is the first time that the microwave-assisted extraction of anthocyanins and phenolic compounds from maqui has been developed. Thus, the results described here offer efficient, green, and low instrument set-up methods to extract the bioactive compounds from maqui berries, which is of great interest in the food industry due to their high commercial value and the increasing demand related to the healthy properties of this fruit.

2. Materials and Methods

2.1. Biological Material

The extraction methods were developed using commercially available lyophilized maqui as a powder from organic farming (SuperAlimentos, Mundo Arcoíris, Besalú, Girona, Spain). The suitability of the final extraction method was evaluated by analyzing several real samples containing maqui commercialized in different formats, including capsules, pills, and lyophilized material. The lyophilized maqui sample and the commercial real samples were stored in a freezer at −20 °C prior to analysis.

2.2. Chemicals and Solvents

Methanol (Fisher Scientific, Loughborough, UK) and formic acid (Scharlab, S.L., Sentmenat, Barcelona, Spain) were HPLC grade. Ultra-pure water was supplied by a Milli-Q water purification system from EMD Millipore Corporation (Bedford, MA, USA). Hydrochloric acid (Panreac Química S.A.U., Castellar del Vallés, Barcelona, Spain) and sodium hydroxide (Panreac Química S.A.U., Castellar del Vallés, Barcelona, Spain) used for the adjustment of pH were of analytical grade. For the quantification of the total phenolic compounds, distilled water, Folin–Ciocalteu reagent (Merck KGaA, EMD Millipore Corporation, Darmstadt, Germany), and anhydrous sodium carbonate (Panreac Química S.A.U., Castellar del Vallés, Barcelona, Spain) were used. The phenolic compound standard and the anthocyanin standard, gallic acid and cyanidin chloride, respectively, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.3. Extraction Equipment and Procedure

The extraction of maqui by microwave-assisted extraction was carried out using a MARS 6 240/50 kit (One Touch Technology, CEM Corporation, Matthews, NC, USA). Approximately 0.5 g of lyophilized sample was weighed and placed in an extraction vessel. Based on the experimental design, a set volume and type of solvent was added, and the extraction was performed under controlled MAE conditions. The variables to be controlled in the different experiments were: percentage of methanol

(25–50–75%), pH (2–4.5–7), temperature (50–75–100 °C) and solvent volume (mL): sample mass (g) ratio 10:0.5–15:0.5–20:0.5. The program carried out consisted of a ramp of 3 min to reach the desired temperature. The temperature was then maintained for 5 min for the extraction of the compounds, and a time of 25 min was employed to allow the sample to cool to room temperature. In all experiments, the power was set at 800 W, as this was sufficient to reach the appropriate temperatures. At the end of the extraction, the extract was centrifuged twice for 5 min at 11,544 × *g*. In both cases, the supernatant was transferred to a 25 mL volumetric flask and the volume was completed with the same solvent. Finally, the extracts were stored in a freezer at −20 °C prior to analysis.

2.4. Determination of Total Phenolic Compounds

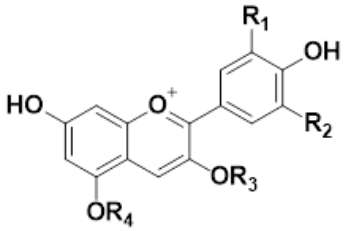
The quantification of the total phenolic compounds was carried out using a Helios Gamma (γ) Unicam UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by a modified Folin–Ciocalteu (FC) procedure [39,40]. This method has been employed previously by numerous researchers for the measurement of total phenolic compounds [41–43], and is based on the reactivity of the phenolic compounds present in the sample with the reagent (a mixture of sodium phosphomolybdate and sodium phosphotungstate). This reaction gives a complex with a blue color and this absorbs at a maximum wavelength of 765 nm. This absorbance is directly proportional to the concentration of polyphenols, as related by the Lambert–Beer law. The extracts were filtered through a 0.45 µm syringe filter (Nylon Syringe Filter, FILTER-LAB, Barcelona, Spain) prior to spectrophotometric analysis. The FC assay was performed by transferring 250 µL of extract, 12.5 mL of distilled water, 1.25 mL of Folin–Ciocalteu reagent, and 5 mL of 20% anhydrous sodium carbonate to a volumetric flask (25 mL). The solution was made up to the mark with water. Due to the kinetics of the reaction, it was necessary to measure the absorbances in the spectrophotometer at 765 nm after a time of 30 min. It was necessary to create a calibration curve under the same conditions and by the same procedure, with standards of gallic acid of known concentrations between 100 and 2000 mg L^{-1} and measuring the absorbance values ($y = 0.0010x + 0.0065$; $R^2 = 0.9998$). Results are expressed as mg of gallic acid equivalent per gram of lyophilized fruit.

2.5. Identification of Anthocyanins

The anthocyanins were identified by ultra-performance liquid chromatography (UHPLC), on a chromatograph coupled to a quadrupole-time-of-flight mass spectrometer (QToF-MS) (Xevo G2 QToF, Waters Corp., Milford, MA, USA). The chromatographic separation was performed on a reverse phase C-18 analytical column (Acquity UHPLC BEH C18, Waters Corporation, Milford Massachusetts, MA, USA) with dimensions of 100 mm × 2.1 mm and a particle size of 1.7 µm. The extracts were filtered through a 0.22 µm syringe filter (Nylon Syringe Filter, Filtros Anoia, S.A., FILTER-LAB, Barcelona, Spain) prior to chromatographic analysis. Milli-Q water acidified with 2% formic acid as solvent A and pure methanol as solvent B, both degassed and filtered, and a flow rate of 0.4 mL min^{-1} were used. The elution gradient employed was as follows: 0 min, 15% B; 3.30 min, 20% B; 3.86 min, 30% B; 5.05 min, 40% B; 5.35 min, 55% B; 5.64 min, 60% B, 5.94 min, 95% B; 7.50 min, 95% B. The total run time was 12 min, including 4 min for re-equilibration. The determination of the analytes was carried out using an electrospray source operating in positive ionization mode under the following conditions: desolvation gas flow = 700 L h^{-1}, desolvation temperature = 500 °C, cone gas flow = 10 L h^{-1}, source temperature = 150 °C, capillary voltage = 700 V, cone voltage = 20 V, and trap collision energy = 4 eV. Full-scan mode was used (m/z = 100–1200). Molecular ions [M]+ for the anthocyanins identified in the maqui showed the following m/z ratios: delphinidin 3-*O*-sambubioside-5-*O*-glucoside, 759; delphinidin 3,5-*O*-diglucoside, 627; cyanidin 3-*O*-sambubioside-5-*O*-glucoside, 743; cyanidin 3,5-*O*-diglucoside, 611; delphinidin 3-*O*-sambubioside, 597; delphinidin 3-*O*-glucoside, 465; cyanidin 3-*O*-glucoside, 449 and cyanidin 3-*O*-sambubioside, 581. These anthocyanins are shown in Figure 1.

2.6. Separation and Quantification of Anthocyanins

The separation of the anthocyanins was carried out by UHPLC on an Elite LaChrom Ultra System (VWR Hitachi, Tokyo, Japan) consisting of an L-2200 U autosampler, an L-2300 column oven set at 50 °C, two L-2160 U pumps, and a UV–Vis L-2420 U detector. The detector was set at 520 nm, as this corresponds to the maximum absorption of the anthocyanins. Anthocyanins were analyzed on a Fused-Core C-18 column (Phenomenex Kinetex, CoreShell Technology, Torrance, CA, USA), with dimensions of 100 × 2.1 mm and a particle size of 2.6 µm. The extracts were filtered through a 0.22 µm syringe filter (Nylon Syringe Filter, FILTER-LAB, Barcelona, Spain) and the injection volume used was 15 µL. The gradient method used 5% acidified Milli-Q water with formic acid as solvent A and pure methanol as solvent B, previously filtered using a 0.22 µm filter (Nylon Membrane Filter, FILTER-LAB, Barcelona, Spain) and degassed in an ultrasonic bath (Elma S300 Elmasonic, Singen, Germany). The flow rate was 0.7 mL min^{-1}, and the gradient was as follows: 0.0 min, 2% B; 2.0 min, 2% B; 3.5 min, 15% B; 5.5 min, 25% B; 6.5 min, 40% B; 7.0 min 100% B; 9.3 min, 100% B; 10.0 min, 2% B; 12.0 min, 2% B. The anthocyanins were quantified from a calibration curve, using cyanidin chloride as a reference standard. The standards of known concentrations were prepared between 0.05 and 30 mg L^{-1} ($y = 252{,}638.09x - 28{,}465.10$; $R^2 = 0.9999$). The limit of detection (0.179 mg L^{-1}) and quantitation (0.597 mg L^{-1}) were determined as the analyte concentration corresponding to the standard deviation of the signal of the blank values ($n = 10$) plus 3 or 10 times, respectively, divided by the slope of the linear regression. Assuming that the different anthocyanins have similar absorbances, and taking into account the molecular weights of each anthocyanin, a calibration curve was prepared for each anthocyanin present in maqui, which allowed the quantification of the compounds of interest.



Anthocyanins Present in the Maqui Berry	R1	R2	R3	R4
Delphinidin 3-*O*-sambubioside-5-*O*-glucoside	OH	OH	$C_{11}H_{19}O_9$	$C_6H_{11}O_5$
Delphinidin 3,5-*O*-diglucoside	OH	OH	$C_6H_{11}O_5$	$C_6H_{11}O_5$
Cyanidin 3-*O*-sambubioside-5-*O*-glucoside	OH	H	$C_{11}H_{19}O_9$	$C_6H_{11}O_5$
Cyanidin 3,5-*O*-diglucoside	OH	H	$C_6H_{11}O_5$	$C_6H_{11}O_5$
Delphinidin 3-*O*-sambubioside	OH	OH	$C_{11}H_{19}O_9$	H
Delphinidin 3-*O*-glucoside	OH	OH	$C_6H_{11}O_5$	H
Cyanidin 3-*O*-glucoside	OH	H	$C_6H_{11}O_5$	H
Cyanidin 3-*O*-sambubioside	OH	H	$C_{11}H_{19}O_9$	H

Figure 1. Radicals of the different anthocyanins present in the maqui berry.

2.7. Box–Behnken Design Experiments (BBD)

BBD is a spherical response surface methodology in which all the limit points are at a distance of the square root of 2 from the center of the design. This specific design avoids the need for experiments under extreme conditions and allows optimal results to be obtained quickly [44,45]. During the optimization procedure, two types of variables can be distinguished: independent variables or factors (i.e., percentage of methanol, pH, temperature, and solvent volume:sample mass ratio) and dependent variables or responses (i.e., concentration of total phenolic compounds and total anthocyanins). Each independent variable can take three possible values, coded as −1, 0, and 1, which are minimum, average, or maximum values, respectively. Therefore, the design indicates the need to carry out 27 experiments, which were performed in duplicate, to identify the optimal MAE conditions to obtain the highest concentration of phenolic compounds and anthocyanins.

The response of the total phenolic compounds and anthocyanins obtained in each of the experiments was introduced into a second-order polynomial equation in order to correlate the relationship between the independent variables and the response [38]:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot x_i + \beta_{ii} \cdot x_i^2 + \sum_{i} \sum_{i=1}^{k} \beta_{ij} \cdot x_i x_j + r, \quad (1)$$

where y is the response; β_i is the coefficient for each main effect; β_{ij} is the coefficient corresponding to the interactions i, j; β_{ii} is the coefficient of the quadratic factors that represents the curvature of the surface; x represents each factor; and r is the residual value.

The statistical significances of the model, lack of fit, and regression terms were evaluated from the analysis of variance (ANOVA). The fitting quality of the polynomial model was evaluated by the use of the determination coefficient (R^2). The Design Expert software 11 (Trial Version, Stat-Ease Inc. statistic made easy, Minneapolis, MN, USA) was employed for experimental design, data analysis, and model building.

3. Results and Discussion

3.1. Development of the MAE Method

In order to identify the optimum extraction conditions for phenolic compounds and anthocyanins, a three-level Box–Behnken design was used. The measured and predicted results for each trial for both responses are shown in Table 1. The four independent variables and their corresponding levels were: percentage of methanol in water (%): 25–50–75; pH: 2–4.5–7; temperature (°C): 50–75–100; and solvent volume (mL): sample mass (g) ratio 10:0.5–15:0.5–20:0.5. The concentrations of total phenolic compounds and anthocyanins extracted in each of the experiments were considered as the response variables.

Table 1. Box–Behnken design matrix with coded variables and measured and predicted responses.

Run	Factors				Responses			
	Solvent	pH	Temp.	Ratio	Phenolic Compounds (mg g^{-1})		Anthocyanins (mg g^{-1})	
	X_1	X_2	X_3	X_4	Measured	Predicted	Measured	Predicted
1	−1	−1	0	0	46.32	45.49	27.92	28.85
2	1	−1	0	0	49.95	50.81	37.70	39.48
3	−1	1	0	0	38.06	34.63	28.72	28.04
4	1	1	0	0	46.88	45.12	38.22	38.39
5	0	0	−1	−1	46.72	47.69	42.75	39.92
6	0	0	1	−1	62.03	60.15	20.20	26.20
7	0	0	−1	1	51.13	50.44	43.04	38.14
8	0	0	1	1	60.60	57.06	30.15	34.07
9	0	0	0	0	45.93	43.87	43.68	41.25
10	−1	0	0	−1	39.60	36.36	26.63	27.65
11	1	0	0	−1	57.18	49.82	37.57	37.61
12	−1	0	0	1	39.20	41.75	29.01	30.17
13	1	0	0	1	45.67	44.09	41.03	41.19
14	0	−1	−1	0	56.85	50.67	39.07	43.71
15	0	1	−1	0	49.43	49.47	25.63	33.43
16	0	−1	1	0	72.13	67.28	32.09	25.48
17	0	1	1	0	50.57	51.94	37.31	33.86
18	0	0	0	0	43.30	43.87	40.14	41.25
19	0	−1	0	−1	46.86	54.42	38.26	36.91
20	0	1	0	−1	43.48	47.43	38.86	35.98
21	0	−1	0	1	52.10	55.54	39.38	39.98
22	0	1	0	1	46.15	45.98	39.96	39.01
23	−1	0	−1	0	35.61	37.32	32.56	30.13
24	1	0	−1	0	43.00	47.15	41.67	39.38
25	−1	0	1	0	45.55	48.78	20.00	19.99
26	1	0	1	0	49.08	54.76	31.59	31.72
27	0	0	0	0	42.39	43.87	39.94	41.25

Firstly, an analysis of variance (ANOVA) was performed to evaluate the effect of the variables, to identify possible interactions between them, and to assess the statistical significance of the model (Table 2). From the results obtained, it was determined that the analysis explained 80.44% of total variability in the case of total phenolic compounds and 78.78% in the case of anthocyanins. These values are consistent with a statistically significant agreement between the measured and predicted responses. This analysis provides information on the mathematical model that is generated from the experimental data. The extraction of both types of compounds is related to the experimental conditions by a second-order polynomial equation.

Table 2. Analysis of variance (ANOVA) for the response surface quadratic model of the recovery. (**A**). Total phenolic compounds; (**B**). Total anthocyanins.

(A)						
Source	**Degrees of Freedom**	**Sum of Squares**	**Mean Square**	***F*-Value**	***p*-Value**	**Coefficient**
Model	14	1337.37	95.53	3.52	0.0174	43.87
A-Solvent	1	187.39	187.39	6.91	0.0219	3.95
B-pH	1	205.34	205.34	7.58	0.0175	−4.14
C-Temp.	1	272.84	272.84	10.07	0.0080	4.76
D-Ratio	1	0.0867	0.0867	0.0032	0.9584	−0.08
AB	1	6.73	6.73	0.2485	0.6274	1.29
AC	1	3.72	3.72	0.1374	0.7177	−0.96
AD	1	30.86	30.86	1.14	0.3065	−2.78
BC	1	49.98	49.98	1.84	0.1993	−3.53
BD	1	1.65	1.65	0.0609	0.8093	−0.64
CD	1	8.53	8.53	0.3146	0.5894	−1.44
A^2	1	78.93	78.93	2.91	0.1135	−3.85
B^2	1	84.82	84.82	3.13	0.1019	3.99
C^2	1	259.69	259.69	9.58	0.0093	6.97
D^2	1	47.38	47.38	1.75	0.2112	2.98
Residual	12	325.23	27.10			
Lack of Fit	10	318.47	31.85	9.42	0.0997	
Pure Error	2	6.76	3.38			
Total	26	1662.60				
(B)						
Model	14	964.38	68.88	3.18	0.0256	41.23
A-Solvent	1	330.12	330.12	15.26	0.0021	5.24
B-pH	1	2.73	2.73	0.1260	0.7287	−0.47
C-Temp.	1	237.45	237.45	10.98	0.0062	−4.45
D-Ratio	1	27.91	27.91	1.29	0.2782	1.52
AB	1	0.0196	0.0196	0.0009	0.9765	−0.07
AC	1	1.54	1.54	0.0711	0.7943	0.62
AD	1	0.2916	0.2916	0.0135	0.9095	0.27
BC	1	87.05	87.05	4.02	0.0679	4.66
BD	1	0.0001	0.0001	4.623E−06	0.9983	−0.01
CD	1	23.33	23.33	1.08	0.3195	2.41
A^2	1	172.52	172.52	7.98	0.0153	−5.69
B^2	1	18.75	18.75	0.8668	0.3702	1.87
C^2	1	147.42	147.42	6.81	0.0228	−5.26
D^2	1	10.60	10.60	0.4902	0.4972	−1.41
Residual	12	259.59	21.63			
Lack of Fit	10	250.73	25.07	5.66	0.1593	
Pure Error	2	8.85				
Total	26	1223.97				

As far as total phenolic compounds are concerned, it can be seen that the variables that influenced the process were the percentage of methanol in the extraction solvent, temperature, pH, and the quadratic temperature interaction, as the *p*-values were less than 0.05 (95% confidence level). Once the influence of the different factors was known, the second-order polynomial equation was obtained by taking into account only the four significant variables mentioned above. Thus, the non-significant terms were removed from the mathematical model and this gave similar results to those obtained with the full equation. The reduced equation that could reliably predict the experimental results was: y_{TP} = 43.87 + 3.95 Solvent − 4.14 pH + 4.76 Temperature + 6.97 Temperature^2.

In the case of anthocyanins, the factors that influenced the process in a statistically significant way (i.e., a confidence level of 95%) were the percentage of methanol in the extraction solvent, temperature,

the quadratic interaction of temperature, and the quadratic interaction of the percentage of methanol. As mentioned above, the reduced polynomial equation only contained the variables and interactions between them that had a significant effect on the response, and it was: $y_{TA} = 41.23 + 5.24$ Solvent $- 4.45$ Temperature $- 5.69$ Solvent^2 $- 5.26$ Temperature^2.

In both cases, phenolic compounds and total anthocyanins, lack of fit test showed a *p*-value higher than 0.05 (not significant), which means that the model fit well.

These results can be graphically represented in standardized Pareto charts (Figure 2), where the significance of each of the variables analyzed in decreasing order can be easily seen. The sign of each effect is also observed due to the two different colors of the bars. Positive signs indicate a direct relationship between the effect and the response variable, while negative signs refer to an inverse relationship.

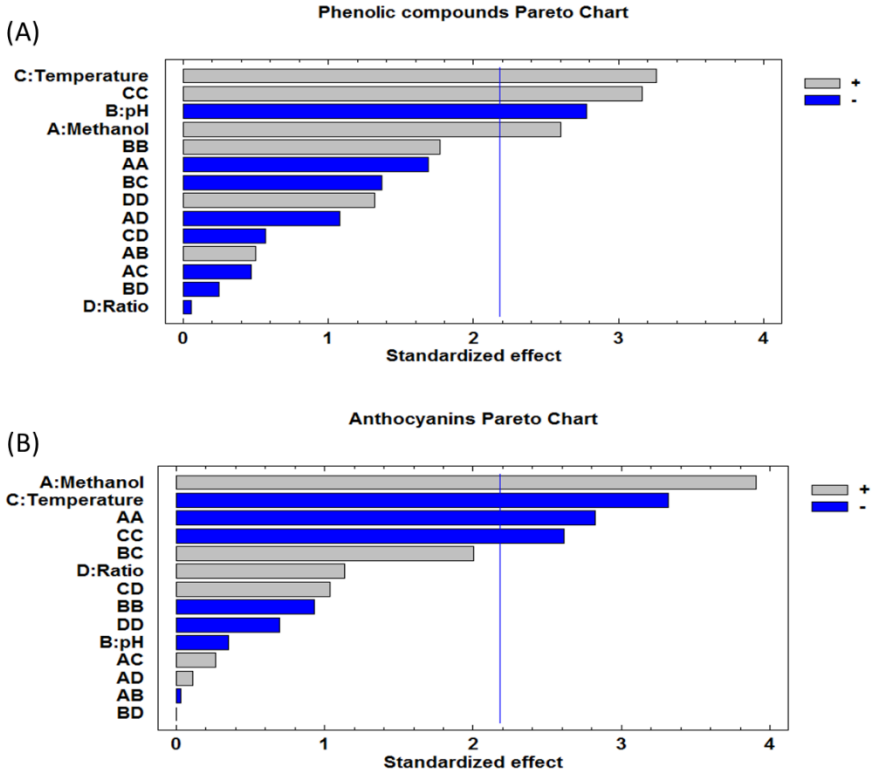


Figure 2. Pareto charts for the standardized effects: (**A**) total phenolic compounds and (**B**) total anthocyanins.

For phenolic compounds, it was observed that, of the four variables analyzed, temperature had the most marked effect on the response variable. However, pH and percentage of methanol also had an effect, thus confirming the information discussed above. The variable that had the smallest effect on the response variable was the solvent volume:sample mass ratio. It was also observed that the percentage of methanol in the extraction solvent and the temperature had a positive effect, which indicates that the use of higher temperatures and higher percentages of methanol led to higher levels of these compounds in the extract. In contrast, pH had a negative effect on the extraction. On the other hand, for anthocyanins it was clearly observed that, of the four variables analyzed, the ones that had the greatest influence were the temperature and the percentage of methanol, while the pH and the ratio hardly had any influence. In this case, the percentage of methanol had a positive effect, which means that an increase in this variable favored the recovery of anthocyanins in the extract. Temperature had a negative influence on the extraction, and an increase in this factor would decrease the recovery of anthocyanins. The results mentioned above were recorded in three-dimensional (3D) response surface plots obtained using the fitted model. These surface plots help to improve the understanding of the

main and interactive effects of the most influential parameters [46,47]. 3D plots are represented in Figure 3 which show the influences of two selected factors on each response (anthocyanins and total phenolic compounds) keeping the rest of the variables constant. Methanol and temperature were selected as the most significant factors for anthocyanins. These variables as well as the effect of pH were selected for phenolic compounds. The combined effects of methanol–pH, methanol–temperature, and pH–temperature on the total phenolic compounds recovery are represented in Figure 3A–C, respectively. The interactive effect of methanol and temperature on the anthocyanins extraction is represented in Figure 3D. Thus, temperature and solvent composition were found to be the most influential factors for both anthocyanins and total phenolics, and these had a marked effect on the extraction. Similar findings were reported by other authors [48,49]. The largest difference in behavior between total phenolics and anthocyanins was observed on considering temperature. For total phenolics, the use of higher extraction temperatures led to a more efficient extraction [50], but high temperatures can lead to the degradation of individual anthocyanins [51].

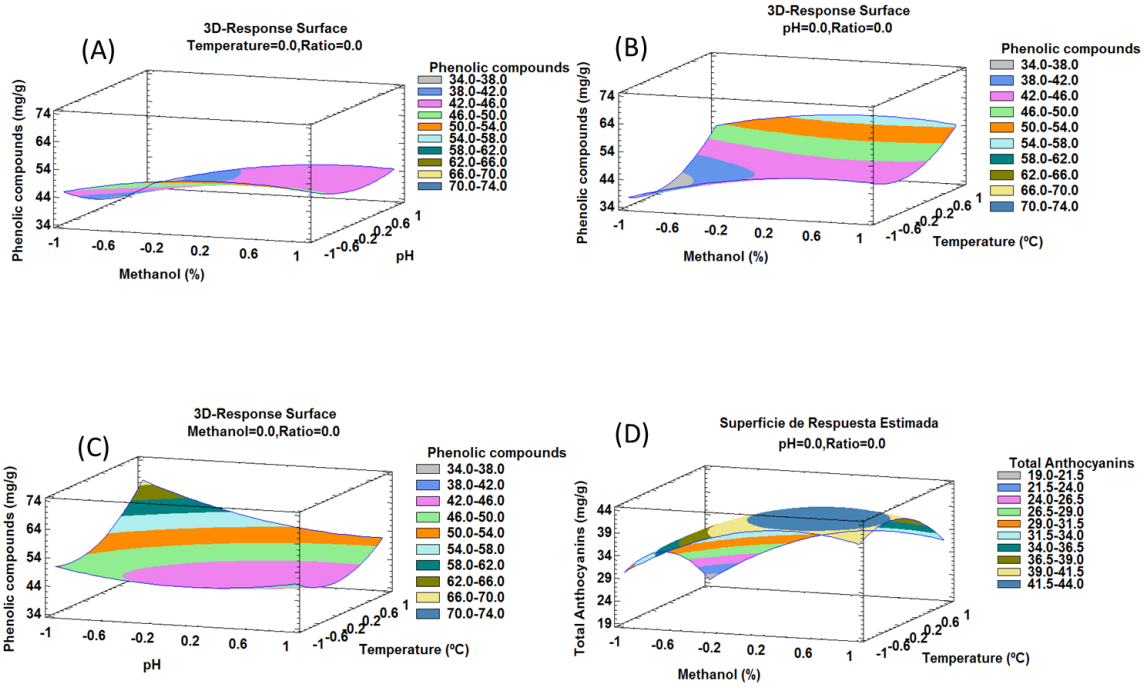


Figure 3. Three-dimensional response surface plots for the graphical representation of the influence of (**A**) methanol–pH, (**B**) methanol–temperature, and (**C**) pH–temperature on total phenolic compounds, and the influence of (**D**) methanol–temperature on total anthocyanins extraction.

3.2. Optimized Conditions

Having carried out the detailed experimental design for both total phenolic compounds and anthocyanins and having performed the statistical treatment of the data, it was concluded that the optimum MAE conditions for total phenolic compounds were as follows: extraction solvent with 65% of MeOH in water at pH 2, extraction temperature of 100 °C and a solvent volume:sample mass ratio of 10:0.5. The optimum MAE conditions for anthocyanins were as follows: extraction solvent with 60% of MeOH in water at pH 2, extraction temperature of 50 °C, and a solvent volume:sample mass ratio of 14:0.5.

It was observed in both cases that the optimum percentage of methanol was an intermediate value, which was determined by the polarity of both the solvent and the set of phenolic compounds present in maqui.

Regarding temperature, although it appears to play a positive role by increasing the extraction kinetics, it must be borne in mind that degradation and fragmentation of the phenolic compounds

can occur at high temperatures [51,52]. As a consequence, other phenolic compounds can be produced, and their hydroxyl groups will also react with the Folin–Ciocalteu reagent, thus causing a significant increase in the absorbance. For this reason, the use of temperatures above 100 °C was not considered. As far as anthocyanins are concerned, the opposite behavior was observed for this factor. High temperatures lead to fragmentation and degradation of anthocyanins and, as a consequence, the optimum temperature was the minimum value used. Lower values were not tested, because this was the minimum temperature that could be used in the equipment. Finally, pH values less than 2 were not considered because this may cause acid hydrolysis of these compounds [53,54]. The total average concentrations in maqui were found to be 54.27 and 34.94 mg g^{-1} for phenolic compounds and anthocyanins, respectively. Similar results were reported by other authors [2,4,13,55]. The outstandingly high total phenolic levels in this fruit are due to the remarkably high anthocyanins content, which contribute the most to the antioxidant capacity [56].

3.3. Extraction Time

An evaluation of the extraction kinetics was performed under the optimum conditions with times between 2 and 25 min (extractions for each time were carried out in triplicate) in order to identify the optimal extraction time (see Figure 4). For the extraction of both phenolic compounds and anthocyanins, an optimal extraction time of only 2 min was identified. Regarding the phenolic compounds, almost the same amount was extracted regardless of the extraction time employed (statistically significant differences were not observed). In relation to anthocyanins, it was observed that there was a slight decrease in the amount of extracted anthocyanins as the extraction time increased. This finding may be due to the long exposure time to microwave radiation, which could lead to product degradation. An optimum time of 2 min was used for both compounds in further experiments to save both time and money. A reasonably rapid method was proposed since most of the compounds of high biological interest present in the maqui samples could be extracted in such a short period of time.

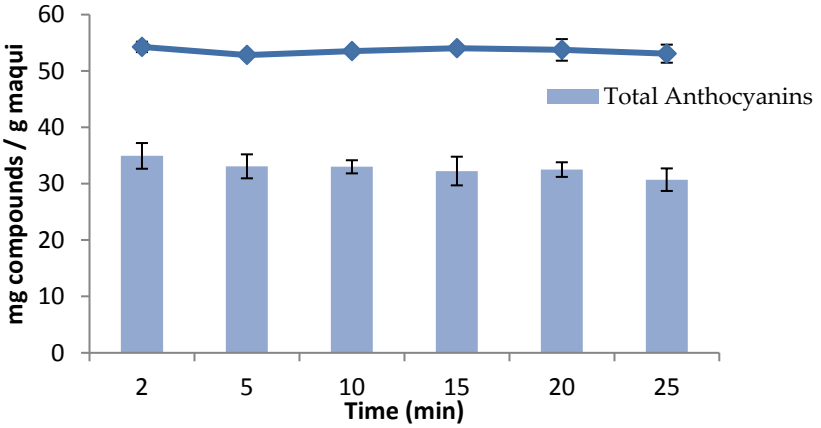


Figure 4. Recovery of total phenolic compounds (mg g^{-1}) and total anthocyanins (mg g^{-1}) using different extraction times (n = 3).

3.4. Analytical Precision of the MAE Method

In order to study the precision of the MAE of maqui samples on the same day and on different days, the repeatability and intermediate precision were studied under the optimum conditions described above. A total of 36 extractions were performed. For repeatability, 12 extractions were carried out on the same day, and for intermediate precision, 12 extractions were performed every day on three different consecutive days. Repeatability results were 3.91% for phenolic compounds and 3.62% for anthocyanins. In the case of intermediate precision, the values were 4.24% for phenolic compounds and 3.78% for anthocyanins. In both cases, the coefficients of variation were less than 5%, which is usually considered to be the limit to confirm that a method is accurate in this type of

test [57]. Therefore, the extraction methods had good precision. Similar repeatability and intermediate precision were obtained for phenolic compounds in other matrices, such as mulberries [48], grapes [28], and peppers [58].

3.5. Application to Real Samples

The developed methods were applied to the extraction of total phenolic compounds and total anthocyanins from seven real samples made from maqui (capsules, pills, and lyophilized material) using the optimum conditions. The samples were analyzed in triplicate, and the results obtained are shown in Table 3. Firstly, the large difference between the two types of capsules analyzed (M-1 and M-2) is remarkable, with a far higher concentration of compounds of biological interest found in the second sample. It can also be seen that the amounts extracted from the various lyophilized samples (M-4–7) were very similar, despite the fact that they were obtained from different lots or even from different brands. In addition, relatively high concentrations of active compounds were obtained because lyophilization allows the preservation of beneficial properties and characteristics during transport [59]. Finally, it should be noted that in the case of M-3, the extraction of the eight specific anthocyanins from maqui was not observed. In contrast, only two anthocyanins (cyanidin 3-*O*-glucoside and delphinidin 3-*O*-glucoside) were detected in this sample. Therefore, it can be concluded that the composition of the pill is not maqui, since the eight characteristic anthocyanins were not detected. These results confirm the great importance of having appropriate extraction methods to guarantee the quality of products consumed and to avoid possible food frauds. Such fraud mostly occurs when the fruit is squeezed and processed to obtain a concentrate or a dried food [19].

Table 3. Concentrations of total phenolic compounds (mg g^{-1}) and total anthocyanins (mg g^{-1}) in different real samples made with maqui (n = 3).

Foodstuff made with Maqui	Type of Sample Analyzed	Sample Preparation	Total Phenolic Compounds (mg g^{-1})	Total Anthocyanins (mg g^{-1})
M-1	Capsules	Opening each of the capsules and using the powder inside	8.22 ± 0.34	1.73 ± 0.16
M-2			103.30 ± 0.30	74.55 ± 3.80
M-3	Pills	Crushing with a conventional electric grinder to get a fine powder	11.45 ± 0.45	– *
M-4	Lyophilized	Does not need any previous preparation	53.06 ± 1.53	30.35 ± 3.25
M-5			49.29 ± 2.17	27.66 ± 1.02
M-6			59.57 ± 0.70	34.51 ± 1.40
M-7			52.13 ± 2.53	19.89 ± 1.44

* The extraction of the eight specific anthocyanins from maqui was not observed.

4. Conclusions

Based on the results obtained in this work, it can be stated that microwave-assisted extraction (MAE) was proven to be a suitable and rapid method for the extraction of total phenolic compounds and total anthocyanins from maqui. The most influential variable for total phenolic compounds was the temperature, whereas for anthocyanins the most significant parameter was the percentage of methanol in the extraction solvent. The extraction methods developed had a high repeatability and intermediate precision, with a coefficient of variation of less than 5%. A short period of time (2 min) was sufficient for the extraction of the bioactive compounds. In addition, the applicability of these methods has been demonstrated by the successful extraction of both total phenolic compounds and anthocyanins in real samples. Moreover, the developed methods proved to be useful for the detection of possible fraud in food made from maqui. It can be concluded from all the results that MAE under the optimum conditions can be considered as an easy, rapid, and economical tool for the extraction of both total phenolic compounds and anthocyanins in maqui, and that this approach is an excellent an affordable alternative for industry [21].

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writing—review and editing, G.F.B. and E.E.-B.; supervision, E.E.-B. and G.F.B.; project administration, G.F.B. and E.E.-B.

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