Supercritical Carbon Dioxide Extraction of Lyophilized *Aristotelia chilensis* (Mol.) Stuntz Berries as Pre-treatment for Enhanced Anthocyanin Recovery

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doi: https://doi.org/10.15255/CABEQ.2022.2112

Original scientific paper
Received: June 23, 2022
Accepted: January 12, 2023

The supercritical carbon dioxide extraction of lyophilized berries of *Aristotelia chilensis* (Mol.) Stuntz was studied as possible pre-treatment for enhanced anthocyanin recovery. Effect of pressure, temperature, and process time on the extracted oil yields and on the anthocyanins recovery in the pre-treated fractions were considered. The operating parameters were optimized using the central composite design, and extractions were run in the pressure, temperature, and time ranges of 99.6 to 200.4 bar, 36.6 to 53.4 °C, and 0.7–2.3 h. The successive multiple regression analysis indicated pressure and time as major influencing parameters on the extraction yield. Those parameters induced no clear changes in the fatty acid composition of almost all the extracted oils, obtaining an average linoleic acid amount between 35–44 % weight in the lipophilic fractions. Standardized methanol extractions demonstrated the influence of the different conditions in the SCO_2 pre-treatment processes, resulting in extracted anthocyanin increments ranging from 9 to 26 %.

Keywords:

supercritical carbon dioxide extraction, *Aristotelia chilensis* (Mol.) Stuntz, fatty acids, anthocyanin, central composite design

Introduction

In the general classification system of the vegetable kingdom, the *Aristotelia chilensis* (Mol.) Stuntz is a 3–4 meters high evergreen bush, native to Chile as well as western Argentina (Patagonian region), commonly called Maqui, which belongs to the *Elaeocarpaceae* family. This dioecious plant produces small edible purple/black berries, with a diameter of 4–6 mm, that are eaten fresh or used for juice or jams: they ripen from December to January, the summer season in the southern hemisphere, and have a taste similar to blackberries. The *Aristotelia chilensis* was classified for the first time in 1844 by Claude Gay, a French botanist and naturalist, in his major work "Physical Atlas of History and Politics of Chile". Maqui is the Mapuche indigenous name of *Aristotelia chilensis*: the local Mapuche people have known for centuries the remarkable properties of the Maqui berry, which was used both for energy and as a remedy for dysentery, whereas the leaves were used to treat wounds. The Maqui berries contain more polyphenols, in particular the flavonoid subgroup called anthocyanins, than any other known berry. Anthocyanins are responsible for the very dark purple color of the Maqui berries, as they are for the color of red wine. Maqui cultivations exist also in Spain and in the more humid parts of Great Britain. An interesting study reports how the polyphenol and anthocyanin concentrations of the Maqui fruit are associated with different ripening stages[1].

In literature, many authors confirm the rich phenolic content and the high antioxidant capacity of the *Aristotelia chilensis* fruits. They indicate that

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Maqui consumption related to health benefits such as anti-diabetic, anti-inflammatory effects, and cardioprotective activities[2–8].

Generally, the isolation of natural compounds with functional properties from natural substrates is a very crucial step. Since these active compounds are present in low concentrations, in literature, several works have aimed toward the development of more effective and selective extraction methods from raw materials. Extraction processes are essential in the preparation of food additives, nutraceuticals, pharmaceuticals, cosmetic products, and so on. Supercritical fluid extraction (SFE) could be a "green" alternative to the conventional organic solvent extraction of these compounds, being the SFE fast, selective, and producing extracts free of residual solvents[9,10].

Supercritical fluids have solvating properties that are similar to organic solvents, but with lower viscosity and higher diffusivity rates. That is to say, supercritical fluids have better transport properties than conventional organic solvents, and can easily diffuse through materials, thereby improving the extraction efficiency and yield.

The main disadvantages of SFE are the high-energy cost and the complex configuration of the system, requiring high costs and the need for properly trained personnel to prepare and monitor the extraction[11].

It is well known that supercritical carbon dioxide (SCO_2) is the ideal solvent for the extraction of non-polar materials, such as lipids[10], because its low critical conditions and GRAS (Generally Recognized As Safe) status. For this reason, pure CO_2 is not usually employed for the extraction of hydrophilic phenolic compounds: several experimental articles are reported concerning the use of small amount of a polar co-solvent to increase the extraction power of the carbon dioxide[12–14].

The most studied entrainers in the SFE extraction of phenolic compounds are methanol and ethanol, preferring the latter for applications in food and pharmaceuticals[15–19]. Nevertheless, pure SCO_2 can be used, in pre-treatment processes, for the removal of lipophilic compounds of natural raw materials, giving a higher accessibility to the hydrophilic polyphenol for further extraction steps[12,20].

In this work, the enhanced recovery of anthocyanins, after SCO_2 fatty acids extraction from lyophilized Maqui berries, is verified by standardized extraction with methanol solutions.

During supercritical CO_2 extraction, the effect of different extraction factors like pressure, temperature, and time on the yield and quality of the oil and raffinate fractions was also analyzed.

Operating parameters of SCO_2 were optimized using a central composite design.

Materials and methods

Materials

Lyophilized Chilean Maqui berries were provided in packages of 125 g by Nutrisslim (Vrhnika – Slovenia), CO_2 (99 % purity) was purchased from SIAD (Trieste – Italy). Methylpendatecanoate, dichloromethane, sulfuric acid, methanol, ethanol, n-hexane, diethyl ether, potassium chloride, hydrochloric acid, sodium acetate, sodium sulfate of analytical grade were provided by Sigma Aldrich.

Methods

Material preparation

Due to the non-homogeneity of the starting material, the eight packages of lyophilized Maqui berries (about 1000 g) were mixed. The material was then kept in the dark, in order to avoid any possible degradation.

Supercritical CO_2 extraction

The extraction process was operated using a continuous flow apparatus described previously[20] and reported in Fig. 1. The extraction of the samples with SCO_2 was conducted on a heated Separex SFE 20 system, equipped with a Lewa EKM210V1 high-pressure pump, and maintained at predetermined pressure by a Tescom 26–1000 valve (BPR1 of Fig. 1). In each experiment, about 15 g of lyophilized Maqui berries were loaded into a 100 cm^3 stainless steel extraction vessel. During the extraction process, every sample was soaked in SCO_2 for 30 min, and then extracted with a flow of 2 L min^{-1}, measured by a wet gas-meter at room conditions. The extract was recovered in a collecting chamber at 50 bar (by needle valve BPR2), to avoid the volatilization of the more volatile substances. At the end, each extract was accurately collected and weighed using a balance (Sartorius BP3100 S) after a gradual and slow CO_2 release. Maqui oil yields were then calculated using the following equation:

$$Y(\%) = \frac{\text{weight of the extracted oil}}{\text{starting material weight}} \cdot 100 \qquad (1)$$

Extraction optimization: RSM and DoE

Response surface methodology (RSM) is a group of mathematical and statistical techniques for empirical model building: the purpose is to optimize an output variable (response) affected by different input variables (independent variables). An experiment is a sequence of runs, in which independent variables changes are carried out to recognize the reasons for the variations in the output response.

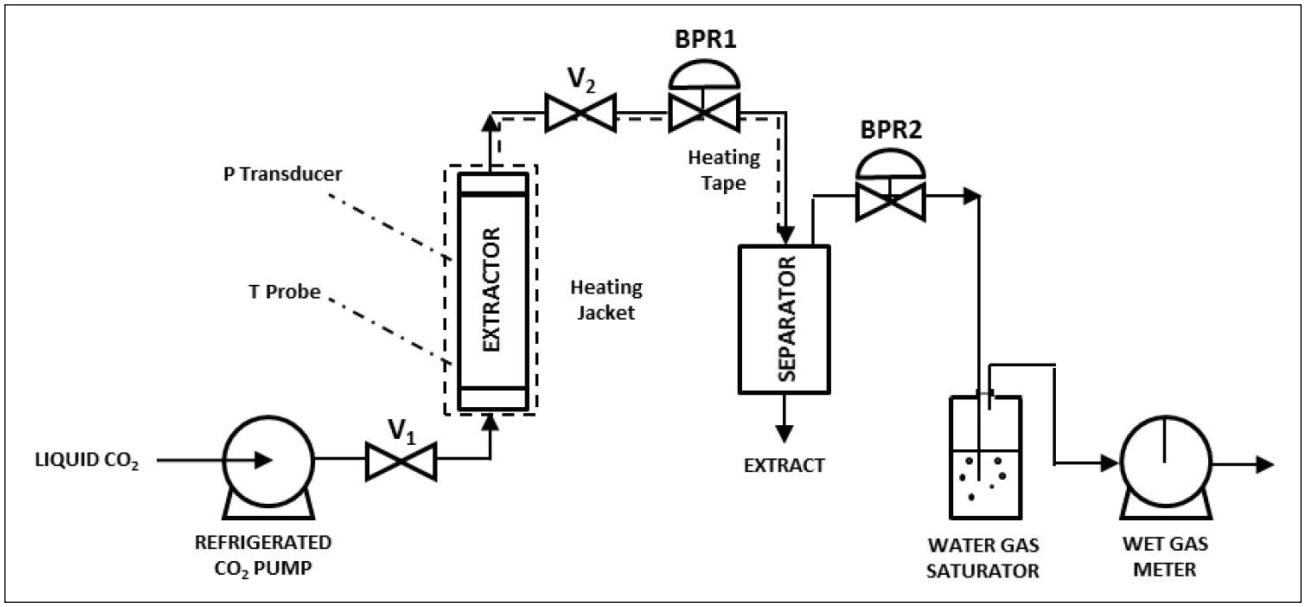

Fig. 1 – *SFE system: V_i = on-off valves; BPRi = back-pressure regulators*

The relationship between a response of interest, Y, and a number of input variables X_1, X_2, , , , , X_k, is usually unknown: the first step in RSM is to find an approximate explicit functional relation between input variables and output response through regression analysis for the range of expected variation in the input parameters[21]. A commonly used form is a second-order polynomial model[22,23].

$$Y = \alpha_0 + \sum_{i=1}^{k} \alpha_i x_i + \sum_{i=1}^{k} \alpha_{ii} x_i^2 + \sum\sum_{i<j} \alpha_{ij} x_i x_j + \varepsilon \quad (2)$$

where ε represents noise or error (such as measurement error) observed in the response Y. Eq. 2 is the polynomial equation used in this work to determine the Maqui oil content yield (Y) as a function of three independent variables, pressure (X_1), temperature (X_2), and time (X_3).

A very important aspect of RSM is the design of DoE experiments[24]. The DoE objective is the selection of the runs in which the response should be calculated: different experiment design can have a significant influence on the accuracy of the approximation of creating the response surface[22].

The most popular design for fitting a second-order model is the Central Composite Design (CCD).

In this study, a five-level central composite rotatable design, with k (= 3) factors (P, T and t), requiring 19 runs, was used.

The levels of independent variables P, T and t, used in this experiment, were determined based on pre-experiments: the operating conditions are reported in Table 1. The extractions were run in the pressure, temperature, and time ranges of 99.6 to 200.4 bar, 36.6 to 53.4 °C and 0.7 – 2.3 h, respectively.

The CCD was structured in 8 (2k) experimental points and 6 (2k) star points (extreme values), with an axial distance of 1.6818 ($\alpha = 2^{k/4}$) to allow the estimation of the curvature. The central point of the design, at 150 bar, 45 °C and 1.5 h, was then replicated 5 times for the determination of the experimental error[23].

By regression analysis of the experimental yields it is possible to evaluate the fitted model and ensure that it provides a good approximation of the true system, obtaining a probability plot along a straight line and a computed value of the correlation coefficient R^2 close to 1. Once more, it is possible to evaluate the linear effect of variables as well as their quadratic and interaction effects using the Student's 't' test and the p-value method. The regression analysis was performed with Matlab R2020a.

Extracted oil analysis

GC-MS analyses of extracted oils were executed with a Varian 3800 gas chromatograph, equipped with auto-sampler (model 9800) and a Saturn 2100 Ion Trap Mass spectrometer. Mobile phase was helium with an ion trap temperature of 210 °C. Injector was set at 220 °C, and mass spectra were acquired in the range 40–650 Da. Fatty Acid Methyl

Table 1 – *Operating conditions in Maqui berries extractions*

Independent variables	Coded levels				
	–1.6818	–1	0	1	1.6818
X_1: P [bar]	99.6	120.0	150.0	180.0	200.4
X_2: T [°C]	36.6	40.0	45.0	50.0	53.4
X_3: t [h]	0.7	1.0	1.5	2.0	2.3

Esters (FAME) were prepared from extracted oils with the protocol of Indarti *et al.*[25] with a few modifications. Triplicate oil samples of 50 mg, placed in round bottomed flasks, were accurately weighed using a balance (Mettler Toledo Classic AB204-S). Methylpendatecanoate solution in hexane (10 mg mL^{-1}) was introduced as internal standard. Dichloromethane, methanol, and sulfuric acid (10:30:0.5 v/v) were then added, and the mixture was refluxed for 30 minutes. The resulting liquid was transferred to flasks kept in an ice bath containing water and diethyl ether. Flasks were vigorously shaken and the upper layer was collected, dried on anhydrous sodium sulfate, and used for GC-MS analysis. Agilent HP-88 (0.25 x 60 m) and HP-5 (0.25 x 30 m) columns were used for fatty acid assays. For quantitative purposes, calibration curves were obtained using methylpentadecanoate as internal standard. Identification of fatty acids was achieved by standard references compounds and by comparison of the MS spectra obtained with NIST 2012 library of the instrument.

Anthocyanin accessibility in original and pre-treated powders

Through a standard extraction method with methanol[26], the extracted amounts of anthocyanins in the original lyophilized Maqui (OLM) berries and in pre-treated powders with SCO_2 were compared.

An amount of 0.5 g of Maqui powder was extracted with 30 mL of acidified (0.1 % HCl) methanol (80 % aqueous solution) for 60 minutes under stirring. The mixture was then centrifuged for 10 minutes at 3000 rpm. The liquid fraction was collected and a second extraction was repeated on the solid residue. Subsequently, the two liquid fractions were combined and concentrated with the Rotavapor up to a final volume of 25 mL.

The solutions anthocyanin contents were then determined by the pH differential method[26–28] using a spectrophotometer UV/Vis (Agilent Technologies Cary 60 UV/Vis). The absorbance of the methanol solutions was measured at 520 and 700 nm in pH 1.0 and 4.5 buffers, respectively, using the following formula:

$$A = \left(A_{520} - A_{700}\right)\text{pH } 1.0 - \left(A_{520} - A_{700}\right)\text{pH } 4.5 \quad (3)$$

The results were expressed as mg of cyanidin-3-glucoside equivalent (Cyan-3-gluc) per gram of powder.

$$\text{Cyan-3-gluc} = \left(\text{A} \cdot \text{MW} \cdot \text{DF} \cdot 10^3\right) / \left(\varepsilon \cdot l\right) \quad (4)$$

with:

A = absorbance;

MW (Cyan-3-gluc molecular weight) = 449.2 g mol^{-1};

DF = dilution factor [L];

ε (cyan-3-gluc molar absorptivity) = 26900 L mol^{-1} cm^{-1};

l = optical path [cm].

Results and discussion

Supercritical extraction optimization: Maqui oil yields

In Table 2, the planned run conditions and the obtained *Aristotelia chilensis* (Mol.) Stuntz oil yields (see eq. 1) are reported. The runs were performed in random order.

Analysis by multiple regression was performed using the experimental yields of Table 2 to fit eq. 2, and to identify the variable coefficients and the linear effect of variables, as well as their quadratic and interaction effects using the Student's 't' test and p-values. A small p value (typically ≤ 0.05) indicates significant model terms.

All the independent operating conditions, pressure ($p = 1.48 \cdot 10^{-7}$), temperature ($p = 2.64 \cdot 10^{-3}$), and time ($p = 2.31 \cdot 10^{-4}$), has a significant linear effect on the yield, whereas both linear and quadratic effects were less important with p values greater than 0.05. In addition, with a calculated value of Student's 't' of 1.833, the variation of the yield seemed to be slightly temperature-dependent.

Table 2 – *Planned runs and obtained Maqui oil yields*

Runs	Factor 1 A: P (bar)	Factor 2 B: T (°C)	Factor 3 C: t (h)	Y oil yield (%)
1	150.0	45.0	1.5	5.29
2	150.0	45.0	1.5	6.26
3	150.0	45.0	2.3	9.37
4	150.0	45.0	0.7	3.93
5	150.0	45.0	1.5	5.07
6	99.6	45.0	1.5	1.99
7	200.4	45.0	1.5	13.16
8	150.0	45.0	1.5	6.34
9	180.0	40.0	1.0	9.05
10	180.0	40.0	2.0	12.57
11	180.0	50.0	2.0	11.71
12	180.0	50.0	1.0	7.74
13	120.0	40.0	2.0	4.71
14	120.0	40.0	1.0	3.14
15	120.0	50.0	2.0	2.89
16	120.0	50.0	1.0	1.99
17	150.0	36.6	1.5	8.75
18	150.0	45.0	1.5	6.77
19	150.0	53.4	1.5	3.88

By fitting eq. 2, the following equation was obtained:

$$Y = \mathbf{6.43} + \mathbf{3.45} \cdot X_1 - \mathbf{0.98} \cdot X_2 + \mathbf{1.39} \cdot X_3 + 0.1 \cdot X_1 X_2 + \mathbf{0.63} \cdot X_1 X_3 - 0.027 \cdot X_2 X_3 + \mathbf{4.07 \times 10^{-5}} \cdot X_1^2 - 3.91 \cdot 10^{-6} \cdot X_2^2 + 7.96 \cdot 10^{-6} \cdot X_3^2 \quad (5)$$

where significant coefficients are reported in bold.

In Fig. 2, the goodness of fit (GOF) is reported: the R^2 value of 0.968 indicates that about the 97 % of the variability in oil extract yield can be satisfied by this equation.

In Fig. 3, the response surfaces of Maqui oil extract yields, generated by equation 5 are reported. The obtained surfaces indicate a yield extraction enhancement for pressure and time increases, while the yield variation seems to be only slightly temperature-dependent. This phenomenon is in accordance with the literature[29].

Composition of the Maqui supercritical oil extracts

In Table 3, the fatty acid compositions in SCO_2 extracted oils, obtained by GC-MS analysis, with Standard Deviations lower than ± 3 % are reported. The linoleic acid is the most quantitatively present component in almost all extracts (35 to 44 weight %), followed by the oleic acid (32 to 39 %). The results are in agreement with those reported by the study of Tahvonen *et al.*[30], which describes the linoleic acid (46.31 %) as the main component of the Maqui dried berries, followed by the oleic acid (33.28 %). The same study highlights also the presence of the tetradecanoic acid (myristic acid). The latter was found also in the SCO_2 extracted oils with an average concentration of 1.3 %: only in runs 15 and 16 (120 bar, 50 °C, 2 and 1 h) it reached a concentration over 2 % (2.1 and 4.5 %, respectively). The myristic acid is a saturated fatty acid and its occurrence is negative because it is potentially harmful, contributing to inflammation, elevated lipids, atherogenesis and vascular disease. Also,

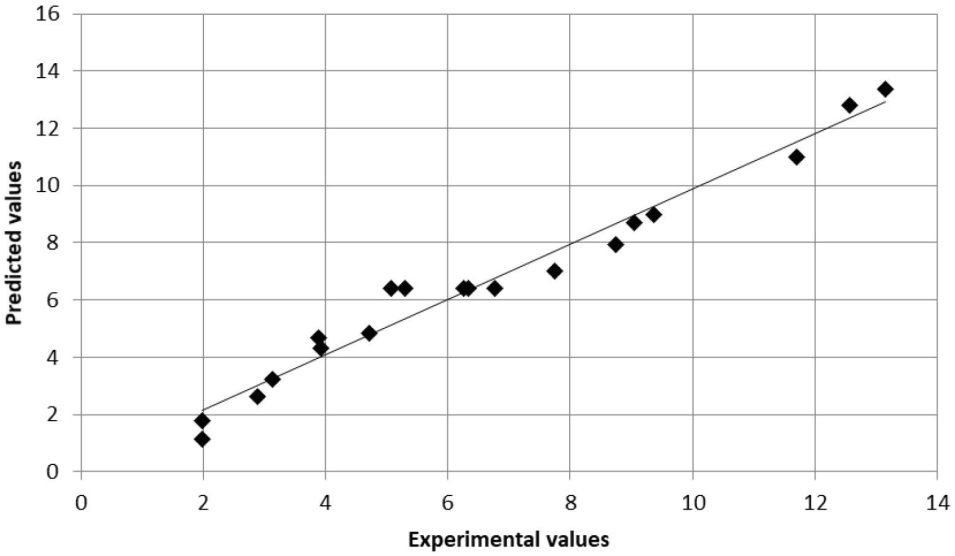

Fig. 2 – *Predicted vs observed values (Yield %)*

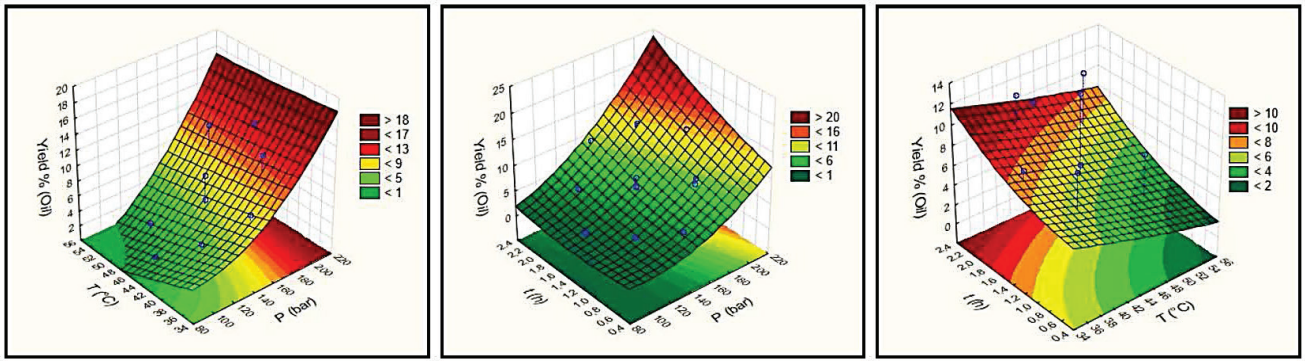

Fig. 3 – *Extracted mass (Y% exp) surfaces response area*

Table 3 – *Fatty acid amounts (weight %) in SCO_2 extracted oils*

Run	Myristic acid	Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid
1	1.73	14.31	1.40	1.61	36.14	42.94	1.88
2	1.55	13.68	0.65	3.03	37.76	40.72	2.61
3	1.24	12.60	0.56	2.96	37.13	43.20	2.30
4	1.36	13.80	0.65	2.85	36.35	42.86	2.14
5	1.22	12.78	ND	2.61	37.76	43.83	1.79
6	4.34	13.77	0.45	2.89	32.80	43.43	2.32
7	1.18	12.48	0.62	2.85	37.16	44.01	1.70
8	1.14	11.86	0.49	2.94	38.02	43.62	1.92
9	1.06	11.85	0.44	2.72	37.50	44.62	1.81
10	0.89	11.40	0.67	2.88	38.67	44.13	1.35
11	0.84	10.85	0.69	3.05	38.53	44.45	1.59
12	0.85	11.35	0.71	1.88	39.70	43.82	1.68
13	1.37	13.39	0.76	2.74	37.18	42.50	2.07
14	1.86	16.87	5.32	4.18	26.05	34.20	11.52
15	2.30	16.93	2.90	3.00	27.71	37.91	9.25
16	8.84	17.99	8.70	0.00	19.73	26.05	18.70
17	1.25	12.63	0.52	2.77	36.27	44.39	2.17
18	1.37	12.99	0.59	2.80	36.81	43.54	1.90
19	1.16	12.43	0.81	2.65	36.84	44.48	1.62

Table 4 – *Effect of SCO_2 pre-treating extraction on anthocyanin content in methanol solutions*

Runs	*P* (bar)	*T* (°C)	*t* (h)	mg g^{-1}	Anthocyanin increase (%)
OLM				9.70 ± 0.22	
1	150.0	45.0	1.5	11.54 ± 0.30	18.97
2	150.0	45.0	1.5	11.52 ± 0.11	18.76
3	150.0	45.0	2.3	12.23 ± 0.23	26.08
4	150.0	45.0	0.7	11.18 ± 0.17	15.26
5	150.0	45.0	1.5	11.35 ± 0.20	17.01
6	99.6	45.0	1.5	10.57 ± 0.31	8.97
7	200.4	45.0	1.5	12.01 ± 0.13	23.81
8	150.0	45.0	1.5	11.51 ± 0.18	18.66
9	180.0	40.0	1.0	11.04 ± 0.17	14.12
10	180.0	40.0	2.0	11.40 ± 0.19	17.53
11	180.0	50.0	2.0	11.48 ± 0.21	18.35
12	180.0	50.0	1.0	11.21 ± 0.31	15.57
13	120.0	40.0	2.0	11.15 ± 0.19	14.95
14	120.0	40.0	1.0	10.71 ± 0.35	10.41
15	120.0	50.0	2.0	11.83 ± 0.21	21.96
16	120.0	50.0	1.0	10.82 ± 0.30	11.55
17	150.0	36.6	1.5	10.73 ± 0.27	10.62
18	150.0	45.0	1.5	11.41 ± 0.10	17.63
19	150.0	53.4	1.5	11.69 ± 0.19	20.52

given that more experimental data would be needed to give a final conclusion, to avoid massive extraction of this component, it would seem appropriate to operate at pressures above 120 bar and temperatures below 50 °C.

Enhanced anthocyanin recovery in pre-treated Maqui powders

Table 4 illustrates the effect of the SCO_2 pre-treating conditions on the anthocyanin content in the methanol solutions. The results represent the average of five separate measurements and are reported with own standard deviation.

The data, in mg g^{-1}, were compared with those obtained from the methanol extraction of the original lyophilized Maqui powder, having the value of 9.27 (± 0.22) mg g^{-1}.

In all the solutions of pre-treated powders there was an enhanced anthocyanin content respect OLM: this amount is also related to the different values of *P*, *T*, and time of the SCO_2 pre-treatments.

The replicate CCD central point (run 1, 2, 5, 8, 18), at 150 bar, 45 °C, and 1.5 h, shows an average increase in the anthocyanin content of over 18 %.

At the same, *T* and *t*, at lower pressure of 99.6 bar (run 6), the anthocyanin content decreased to a positive 8.97 % respect the OLM value, whereas at 200.4 bar (run 7), the content increased up to about 24 %.

Similar increasing functions are reported by *T* and *t* also if the temperature effect seemed to be less important, as shown by runs (9–12), (10–11), and (14–16), in which differences of 10 °C (from 40 to 50 °C), at the same *P* and *t* values, give an anthocyanin improvement of maximum 1 %.

Comparable behavior also at 53.4 °C, run (19) at 150 bar, and 1.5 h, with an increase of about 2 % respect the average value at 45 °C. Only run (17), again at 150 bar and 1.5 h, with a temperature of 36.6 °C showed an anthocyanin decrease of over 8 % respect the replicate central point.

Despite the small number of experimental data, it is thus possible to certify the influence of the pre-treatment with SCO_2 in obtaining greater accessibility of the anthocyanins for a subsequent extraction process.

Conclusions

This paper reports the supercritical carbon dioxide extraction of *Aristotelia chilensis* (Mol.) Stuntz berries as possible pre-treatment for enhanced anthocyanin recovery.

The experimental parameters, pressure, temperature, and process time of the supercritical extraction were optimized using a central composite design. The 19 runs of the design indicated the best yield of over 13 %, at 200 bar, 45 °C, and 1.5 h, and pressure and process time as the parameters with the greatest effect on the yield variation.

Quantitative analysis of the fatty acids in almost all the extracted oils showed the prevalence of linoleic acid, from 35 to 44 %, and of oleic acid from 32 to 39 % in the lipophilic fractions.

Anthocyanin extractions with methanol attest the influence of the different *P*, *T*, and *t* conditions of the pre-treatment processes with SCO_2. Again, pressure and process time are the parameters with the greatest effect on the extracted anthocyanins, obtaining their maximum increase, respect the amount extracted from the original lyophilized Maqui powder, of about 24 % at 200.4 bar, 45 °C and 2.3 h.

ACKNOWLEDGEMENTS

The authors wish to remember Mr. Dario Solinas, skilled technician of the Prof. Paolo Alessi laboratory, and very good long-time friend.

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