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A PRELIMINARY STUDY ON IMPROVING THE EXTRACTION OF PHENOLIC COMPOUNDS FROM CORNELIAN CHERRY FRUITS

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Cornelian cherries (*Cornus mas*) are an excellent source of bioactive molecules that attracted the interest of many scientists activating in the field from medicine to food industry. The extraction of biologically active compounds is an important step in the development of new ingredients with increased functionality. This paper represents a preliminary study that aimed to explore the variables that have a significant effect on the recovery of total anthocyanins and total phenolic compounds from cornelian cherries during ultrasounds assisted extraction using Plackett–Burman design. Independent variables considered in the design were the number of extractions, solvent concentration, extraction temperature and extraction time. The mathematical model showed a satisfactory coefficient of determination (R^2=0.74) while the number of extractions as well as solvent concentration were found to influence significantly the recovery of anthocyanins from cornelian cherries. This study enabled us to identify the variables that influenced the recovery of phenolic compounds from cornelian cherries, parameters that will be further used to optimize and validate the extraction of phytochemicals from cornelian cherry fruits.

Keywords: cornelian cherries, anthocyanins, Placket Burman design

Introduction

Cornelian cherry (*Cornus mas L.*) trees are native in Southern Europe and South west Asia, being spread in many countries such as Turkey, Bulgaria, Romania, Italy as wild fruit trees and are considered a very profitable crop due to its resistance in harsh environmental conditions (Ochmian *et al.,* 2019; Klimenko, 2004). In recent years, the interest for cornelian cherry increased, as scientists found these fruits being a generous source of biological active compounds. Cornelian cherry is an excellent source of fructose and sucrose, organic acids,

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minerals, vitamins, pectins, tannins, phenolic compounds, anthocyanins, flavonoids, iridoids.

The unique composition of cornelian cherry extracts exhibits a wide range of biological and pharmacological properties, including antimicrobial, anti-inflammatory, anticancer, antidiabetic actions (De Biaggi *et al*., 2018). These fruits extracts act against pathogenic bacteria and therefore are considered useful as homeopathic remedy for gastrointestinal, urinary or dermatological disorders (Dinda *et al*., 2016; Tong et al., 2015). Other studies indicate that anthocyanins and ursolic acid purified from *Cornus mas* fruits have biological activities that improve certain metabolic parameters associated with diets high in saturated fats and obesity (Jayaprakasam *et al.*, 2006).

To maximize the use of biological active compounds from cornelian cherry, different extraction methods can be employed. Although reported to have a fair yield most of the conventional methods are time-consuming, expensive and, due to organic solvent employed have a negative impact on the environment. Ultrasound assisted extraction (UAE) is a modern technique that can be used to extract compounds from different plants material while preserving their structural and molecular properties (Amiri *et al.,* 2018). In the same time, UAE promotes extraction efficiency by increasing the mass transfer and possible rupture of cell wall due to its effect of acoustic cavitation (Xu *et al.,* 2017). UAE has been used to extract the anthocyanins from many plant materials, however, as far as we know, there are no reported data in the literature on screening the extraction conditions of phenolic compounds from cornelian cherry. Plackett–Burman design is widely used to set process conditions that allows the understanding of the effects of different variables using a small number of trials (Montgomory, 2009), and may provide information on two factors interactions and allow better evaluation of the factors. Therefore, our study aimed to identify which are the most important variables that influence the extraction of total anthocyanins and total polyphenols from cornelian cherry fruit using Placket Burman design.

Materials and methods

Materials

Cornelian cherry fruits, cultivar "Bordo" were purchased at full maturity stage from the local market (Roman, Romania) in August 2018 and stored at – 20 °C until analysis. The pulp was removed from the stones and freeze-dried (CHRIST Alpha 1-4 LD plus, Germany) at -42 °C under a constant pressure of 0.10 mBar. All the other needed reagents were of analytical grade.

Ultrasound-assisted extraction procedure (UAE)

The extraction of bioactive compounds was performed using an ultrasonic bath system (MRC Scientific Instruments). Freeze dried powder of cornelian cherry was mixed with the designated volume of ethanol of varying concentrations and placed in the ultrasonic bath. The ultrasound assisted extraction was performed at a constant frequency of 40 kHz, with a constant power of 100 W. Cold water was

added to maintain a constant temperature in the ultrasonic bath. Four variables were tested to evaluate their influence on the extraction of total anthocyanins and total phenolic compounds: number of extractions (1 to 3), extraction temperature (25 to 50 °C), solvent concentration (60% to 80% v/v), and extraction time (10 min to 40 min). The number of extractions was performed considering the following protocol: 10 mL of solvent was added to cornelian cherry powder and sonicated at a specific temperature and extraction time. Afterwards, the supernatant was separated by centrifugation at 9000 rpm for 10 minutes. Depending of the design, the collected precipitate was mixed with another 10 mL of solvent and so on, the extraction being repeated for maximum three times. For experiments where the extraction was repeated twice or three times, all the supernatant was collected in a single flask. At the end, the supernatant was dried at 40 °C using a vacuum rotary evaporator (AVC 2-18, Christ, UK) and further stored at 4 °C until analysis.

Placket–Burman (PB) design for screening of variables

The Placket–Burman experimental design was employed in this study to identify the variables that exert a significant influence on the extraction of bioactive compounds from cornelian cherry. As mentioned above, four variables were considered in this study, each variable being coded from X_1–X_4 and examined in two levels (Table 1). PB design is based on the assumption that the main effects of the variables have no interactions and can be expressed on a first-order polynomial model, as follows:

$$y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i \qquad (1)$$

where y is the response; β_0 is the constant, β_i is the linear regression coefficient, while x_i is the level of the independent variable.

Table 1. Input variables and levels chosen for screening

Factor level	Independent variable			
	Number of extractions X_1	Ethanol concentration (%) X_2	Temperature (◦C) X_3	Extraction time (min) X_4
-1	1	60	25	10
+1	3	80	50	40

The complete design matrix of PB with a total of 15 runs with three replicates in the central points is presented in Table 2. Minitab version 19 software (Minitab Inc., PA, USA) was used for the experimental design and statistical analysis. The main effect of each variable was calculated using Eq. (2) (Celli *et al*., 2015):

$$\text{Effect}_{Xi} = (2/N)\left[\sum x_i\,(+1) - \sum x_i\,(-1)\right] \qquad (2)$$

where N is the total number of experiments without center points, x_i (+1) and x_i (-1) terms are the responses for a given factor at low and high level, respectively.

Total anthocyanins content (TAC)

TAC was quantified using pH differential method. According to Eq. (3), the absorbance of cornelian cherry anthocyanins was measured at 520 nm (A_{520}) and 700 nm (A_{700}), using a 0.025 M potassium chloride buffer of pH 1.0 and 0.4 M sodium acetate buffer of pH 4.5:

$$A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH\ 4.5} \quad (3)$$

TAC was expressed in mg cyanidin-3-glucoside/100 g dry weight (d.w.), after applying Eq. (4):

$$TAC = \frac{A}{26900} \times MW \times DF \times \frac{V}{W_t} \quad (4)$$

where, MW is 449.1 and represents the molecular weight of cyanidin-3-glucoside (Cy3gl), 26900 is the extinction coefficient, *DF* represents the dilution factor, *V* is the total volume (mL), and Wt represents the sample weight (g).

Total phenolic content (TPC)

TPC content was measured following Folin-Ciocalteu's protocol. The diluted extract was mixed with distilled water and Folin-Ciocalteu's solution and after 10 minutes, sodium bicarbonate (20 % w/v) was added and allowed to stand in the dark for 60 min at room temperature. The absorbance was measured against the blank at 765 nm, results being expressed as gallic acid equivalent (GAE) mg/100 g d.w.

Table 2. Placket–Burman design matrix with coded variables, including three center points

Run order	X_1	X_2	X_3	X_4	TAC (mg Cy3gl/100g d.w.)	TPC (mg GAE/100g d.w.)
1	1	80	25.0	10	224.43	3719.66
2	1	80	50.0	10	251.15	3762.90
3	2	70	37.5	25	224.43	3000.44
4	1	60	25.0	40	229.78	4114.49
5	3	80	25.0	40	213.75	2688.39
6	2	70	37.5	25	189.70	3479.25
7	3	60	25.0	10	197.72	1740.77
8	3	60	50.0	40	200.39	1700.64
9	3	80	25.0	40	208.40	2873.13
10	2	70	37.5	25	203.06	2879.39
11	1	60	50.0	40	215.08	3496.23
12	3	60	50.0	10	193.70	2850.57
13	1	80	50.0	40	261.84	2578.17
14	1	60	25.0	10	235.12	4854.61
15	3	80	50.0	10	219.09	2214.55

Yield of TAC and TPC

The yield of TAC and TPC was determined using Eq. (5):

$$Yield\ (\%) = \frac{X}{X_0} \times 100 \qquad (5)$$

where: X is the content of TAC or TPC and X_0 is the mass of the concentrated extract resulted from the initial lyophilized powder.

Results and discussion

Factors selection

In this study, PB design was performed to assess the influence of four independent variables on the UAE yield of anthocyanins from cornelian cherry fruits. The selection of independent variables was based on previous studies dealing with screening and optimization of phenolic compounds from different cherries and berries fruits (Table 3). In most cases, the proportion between solvent and solid material, temperature and solvent type and solvent concentration were investigated for recovery of bioactive components. The results indicated that extraction is dependent on several factors that can be specific from one plant material to other.

Table 3. Effect of different factors on extraction of phenolic compounds from different studies on cherries and berries using UAE

Plant material	Tested variables	Significant	References
Haskap berry	Solvent:solid ratio (5-25) Ethanol concentration (70-100 %); Extraction time (10-30 min) Formic acid concentration (0-1%); Extraction temperature (25-45°C);	Proportion solvent: solid; Ethanol concentration; Extraction time	Celli *et al*., 2015
Blue berry	Methanol (60-80%); Temperature (20-60°C); Extraction time (20 min); Ethanol (60-80%); Acetone (60-80%);	Methanol (70%); Temperature (30°C); Extraction time (20 min);	Wang *et al*., 2016
Cherry	Methanol (60-80%); Temperature (20-60°C); Extraction time (20 min); Ethanol (60-80%); Acetone (60-80%)	Methanol (80%); Temperature (30°C); Extraction time (20 min);	Wang *et al*., 2016
Blue berry	Solvent:solid ratio (15:1-25:1); Extraction power (1200-1600 W); Extraction time (20-40 min); Buffer time (2.0-3.0 s)	Extraction power; Extraction time;	Jiang *et al*., 2017
Mulberry	Methanol concentration (50–100%); Temperature (10–70 °C); Ultrasound amplitude (30–70%); Cycle (0.2–0.7 s); Solvent pH (3.0–7.0); Solvent:solid ratio (10:1.5–20:1.5)	Temperature (48°C); Methanol concentration (76%);	Espada Belido *et al*., 2017

Celli *et al., 2015* showed that the ratio between solvent and dried haskap berries, ethanol concentration, and extraction time were the most statistically significant variables that affected the UAE of anthocyanins, whereas Jiang *et al., 2017* found that extraction time and extraction power exert a significant influence on the recovery of anthocyanins from blueberry. On the other hand, Espada Belido *et al., 2017* tested six variables to extract TAC and TPC from mulberries and only extraction temperature and solvent composition were found to have a significant influence on TAC (48 °C and 76%) and TPC (64 °C and 61%) recovery. Wang *et al.*, (2016) optimized the ultrasound-assisted extraction for different anthocyanin rich fruit and their effects on anthocyanin compositions, and reported that among all the solvents tested, methanol recovered the maximum anthocyanins from cherries. In our study, we chose to use ethanol as being the most suitable solvent for TAC and TPC extraction from cornelian cherry as ethanol has the GRAS status, reduced toxicity compared with other solvents and it used widely for food applications.

The influence of extraction parameters on recovery of TAC

From Table 4 it can be seen that number of extractions (X_1) and solvent concentration (X_2), were significant for cornelian cherry TAC. The number of extractions exerted a negative while solvent concentration had a positive impact on anthocyanins recovery.

Table 4. Main effect of each input variable on TAC and TPC from cornelian cherries

Input variable	Main effect	
	TAC	TPC
Number of extraction	-0.30*	-1410*
Ethanol concentration (%)	0.17*	-153
Temperature (◦C)	0.05	-565
Extraction time (minutes)	0.01	-282

*significant at $p < 0.05$

As mentioned previously, it is important to select the appropriate variables that possess a major influence on the recovery of a target compound. Blackhall *et al*., (2018) showed that liquid/solid ratio exert a positive influence on the anthocyanins recovery from sweet cherries only when it is used in a ratio of maximum 10:1. On the other hand, increasing the ratio between liquid and solid decreased TAC, the authors explaining that when all the anthocyanins from the plant material have been extracted, the solvent doesn't change the yield of recovered anthocyanins. According to Ali *et al.,* 2018 solvent composition can improve or decrease the extraction of bioactive compounds because solvents solubilize on the principle "like dissolve like" therefore, compounds with similar properties to solvents are easily solubilized.

Based on ANOVA method presented in Table 5, it can be noticed that the model for TAC was significant ($p < 0.05$) and the lack of fit indicates that the model fits the experimental data well. The regression model showed a satisfactory

determination coefficient of R^2=0.74. The curvature was not significant and this is an indication that variables involved in the model could be described by a first order equation.

Table 5. ANOVA results of screening using TAC and TPC as response

Source	dF	Adj SS	Adj MS	*F*-Value	*p*-Value
TAC					
Model	5	4425.24	885.05	5.09	0.017
Linear	4	3875.09	968.77	5.57	0.015
X_1	1	2832.25	2832.25	16.28	0.003
X_2	1	951.82	951.82	5.47	0.044
X_3	1	85.66	85.66	0.49	0.501
X_4	1	5.35	5.35	0.03	0.865
Curvature	1	550.15	550.15	3.16	0.109
Error	9	1565.74	173.97		
Lack-of-Fit	6	937.54	156.26	0.75	0.653
Pure Error	3	628.20	209.40		
Total	14	5990.98			
R^2	0.74				
TPC					
Model	5	7239047	1447809	4.10	0.032
Linear	4	7227224	1806806	5.12	0.020
X_1	1	5961493	5961493	16.89	0.003
X_2	1	70614	70614	0.20	0.665
X_3	1	956544	956544	2.71	0.134
X_4	1	238573	238573	0.68	0.432
Curvature	1	11823	11823	0.03	0.859
Error	9	3176832	352981		
Lack-of-Fit	6	2958516	493086	6.78	0.072
Pure Error	3	218316	72772		
Total	14	10415878			
R^2	0.69				

After excluding the nonsignificant terms (p>0.05), the final developed model obtained for PB design of TAC is described by Eq. (6):

$$Y_{TAC} = 1.801 - 0.02\,X_1 + 0.009X_2 \qquad (6)$$

In this study, TAC ranged between 189.70 and 261.84 mg Cy3gl/100 g d.w (Table 2). Previously, it has been reported that the TAC of cornelian cherry can range between 15.5 mg and 1450 mg Cy3gl/100 g fresh weight (Tepić Horecki et al., 2018; Milenković-Andjelković *et al.,* 2015; Kucharska *et al., 2015*). Similar to other plant materials, the composition of cornelian cherry in anthocyanins is affected by cultivar, soil properties, maturity, places of harvest, extraction conditions, factors that can explain the large variability of anthocyanins. For example, Pantelidis *et al.,* (2007) reported a mean value of 233 ± 4.2 mg Cy3gl/100 g fresh weight, Tural and Koca, 2008 reported TAC values that ranged between 112 and 292 mg Cy3gl/100 g, Yilmaz *et al*., 2009 calculated in various genotypes

from Western Black Sea and Inner Anatolia regions in Turkey a mean value of 115 mg Cy3gl/100g fresh weight, whereas Hassanpour *et al*., 2011 indicated 442.11mg Cy3gl/100g fresh weight.

The influence of extraction parameters on recovery of TPC

Our TPC results, depending on the extraction conditions varied in the range of 1.70 – 4.85 g GAE/100 g d.w. (Table 2). The lowest value was calculated when applying three extractions in 60 % ethanol at 50°C for 40 minutes. On the other hand, the highest value calculated for TPC was obtained when using a single extraction with 10 mL of 60% ethanol. Gastol *et al.*, 2013 evaluated the phytochemical content of cornelian cherry juice and reported a TPC value of 45.6 g GAE/100 g. In other studies, TPC was found to be in the range 1.42 - 8.11g GAE/100 g fresh weight (Rop *et al.*, 2010, Pantelidis *et al.,* 2007, Antolak *et al.,* 2017, Behrangi *et al.*, 2015). Tepic Horecki *et al.,* 2018 investigated the changes of physico-chemical properties of cornelian cherries after three drying techniques and reported in methanol extracts derived from freeze dried samples, TPC values ranging from 2.5 g to 4.5 g GAE/100 g d.w. These large variations of TPC values can be attributed to factors presented previously for TAC, however, method of determination can lead also to different results. If Folin-Ciocalteu method is employed, method that is considered to be not specific, higher values in TPC can result due to interference of reducing substances, such as ascorbic acid, which is found in high concentrations in different fruits including cornelian cherry fruits.

From Table 4 it can be seen that only X_1 was found significant for the extraction of TPC from cornelian cherry and exerted a negative influence on their recovery.

The regression model showed a determination coefficient of R^2=0.69 which can be considered satisfactory, the model was significant and the lack of fit and curvature were not found to be significant (Table 5).

The final model obtained for PB design of TPC is presented by Eq. (7):

$$Y_{TPC} = 6078 - 70.5\, X_1 \qquad (7)$$

Conclusions

Ultrasound assisted extraction was employed to enhance the recovery of total anthocyanins and total phenolic compounds from cornelian cherries using the screening of Placket Burman design. Ethanol concentration, number of extractions, temperature and extraction time were considered as independent variables. The number of extractions was found to influence negatively the recovery of both total anthocyanins and phenolic compounds, whereas ethanol concentration exerted a positive effect only on the extraction of total anthocyanins. The maximum recovery of total anthocyanins and phenolic compounds was obtained when using a single extraction, however, the maximum recovery for total anthocyanins and phenolic content was calculated at 80% and 60% ethanol concentration, respectively. Although in both cases, the determination coefficient was satisfactory, further studies are needed to optimize and validate the extraction conditions of these biological compounds from cornelian cherries.

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