International Journal of Applied Engineering Research ISSN 0973-4562 Volume 10, Number 23 (2015) pp 43555-43559

Factorial Design Analysis of the Recovery of Flavonoids from Bilberry Fruit By-Products

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Abstract
A factorial design at two levels and four factors was used to investigate the recovery of flavonoids from bilberry fruit by-products. Experiments were conducted in batch with aqueous ethanol as extraction solvent. Temperature (T), extraction time (E), liquid-to-solid ratio (R) and solvent composition (C) were the factors considered. The extraction yield of flavonoids, expressed as the percentage amount of extracted flavonoids to their initial amount in the plant material, was taken as the response variable. The observed response ranged between 46.3 and 80.7%. Analysis of the data showed that three out of the four main factors, namely, T, E and C, were statistically significant, their contribution increasing in the order: E < T < C. A regression model was developed by assuming a polynomial equation for the response variable including all the statistically significant variables and their interactions. The model provided an accurate description of the effect of extraction conditions on the recovery of flavonoids, both inside and outside the design space.

Keywords: Bilberry, Flavonoids, Extraction, Aqueous ethanol, Factorial design.

Introduction

Bilberry (*Vaccinium myrtillus* L.) is a perennial shrub native to Northern Europe but now cultivated for fruit production in many parts of the world. Several studies have shown that bilberry fruits are a very rich source of polyphenols, particularly anthocyanins and flavanols [1]. These substances are believed to provide several health benefits, such as protection from UV radiation and decreased risk of cardiovascular, neurodegenerative and inflammatory diseases [2-4]. The amount of polyphenols in bilberry is closely related to the developmental stage of the plant. In addition, these compounds tend to accumulate in the outer part of the fruit, where they perform their biological functions. According to Riihinen et al. [5], the anthocyanin content in the fruit peel is over 20 times higher than in the pulp and a similar tissue-specific distribution occurs for hydroxycinnamic acids and quercetin.

Bilberries are usually sold as fresh whole berries or processed into juices, jams and purees. From the industrial processing of bilberries, a solid waste consisting mainly of the fruit peel and seeds is generated. This material has no commercial value and is currently disposed of or used for animal feeding. However, it is a very rich source of bioactive substances that could be recovered and used for a wide range of applications, particularly in the food, cosmetic and pharmaceutical fields.

In previous studies from our research group, we found that phenolic antioxidants can be easily recovered from bilberry processing waste by solvent extraction with aqueous ethanol [6] and that the resulting extracts can be used as ingredients for the production of functional foods with high antioxidant capacity [7].

The aim of this research was to investigate the influence of extraction conditions on the recovery of flavonoids, the most important and abundant phenolic compounds in bilberries, from the fruit by-products. To this end, a rigorous approach based on factorial design and influence factor analysis was used. From this approach, an empirical model was developed to describe the dependence of the extraction yield of flavonoids on process variables.

Experimental

Chemicals and plant material

Ethanol (CAS 64-17-5), methanol (CAS 67-56-1), sodium acetate (CAS 127-09-3) and aluminum chloride (CAS 7446-70-0) were purchased from Carlo Erba (Milano, Italy). Quercetin (CAS 117-39-5) was obtained from Sigma-Aldrich (Milano, Italy). All chemicals were reagent grade and used without further purification.

Bilberry press residues were provided from "Rigoni di Asiago SRL" (Asiago, VI, Italy). The material consisted mainly of the fruit peels and was stored in plastic bags at-20 °C until use. When needed, an aliquot of the frozen waste was thawed in air at room temperature.

Analytical Methods

Moisture content was determined by an electronic moisture analyzer (model MAC 50/1, Radwag, Poland).

The flavonoid content of bilberry residues was determined by a three-stage extraction procedure with aqueous ethanol (50% v/v) as solvent [8]. Briefly, 1 g of plant material and the appropriate amount of solvent (100, 50 and 20 mL in the first, second and third stage, respectively) were poured into glass flasks thermostated at 40 °C. After 90-min stirring, the resulting suspension was filtered and assayed for total flavonoids. The overall flavonoid content was calculated as the sum of the values obtained in each stage.

Total flavonoids were determined by the method of Chang et al. [9]. 300 μL of the sample to be tested were poured into an optical glass cuvette together with 900 μL of methanol, 60 μL of an aluminum chloride aqueous solution (10% w/v), 60 μL of 1 M sodium acetate and 1.7 mL of distilled water. The cuvette was shaken and kept in the dark at room temperature for 30 min. Then, the absorbance values at 415 and 700 nm

were determined. Measurements were made by a double-beam UV-VIS spectrophotometer (Lambda 25, Perkin Elmer, USA) against a blank of distilled water. The results were expressed as quercetin equivalents (QE) per unit weight of solid, or unit volume of liquid, using a calibration curve obtained with quercetin standards (Fig. 1).

Extraction Procedure

The extraction of flavonoids from bilberry residues was performed in batch mode [10] using ethanol-water mixtures as the solvent. Appropriate amounts of plant material and solvent were loaded into 50-mL screw-top flasks. The flasks were placed in a water bath thermostated at ±0.1 °C and were magnetically stirred. At the desired time, a sample of the liquid was taken, passed through a 45-μm nylon filter and assayed for total flavonoid content.

Factorial Design

A two-level factorial design was used to evaluate the effects of temperature (T), extraction time (E), liquid-to-solid ratio (R) and solvent composition (C) on the recovery of flavonoids. The extraction yield of flavonoids (y), expressed as the percentage amount of extracted flavonoids to their initial amount in the plant material, was used as the response variable.

The levels of each factor were chosen to cover a range of values of practical interest [11]. They are reported in Table 1 in both actual (X_i) and coded (x_i) values. The latter were obtained using the following equation:

$$x_i = \frac{X_i - X_{i,0}}{\Delta X_i} \qquad (1)$$

where $X_{i,0}$ is the actual value of the i-th factor at the center-point level and ΔX_i is the step change value for that factor.

Four replicates at the central point of the factorial domain, i.e., $x_1 = x_2 = x_3 = x_4 = 0$, were carried out to estimate the experimental error and check the statistical significance of the model, for a total of $2^4 + 4 = 20$ runs (Table 2). They were performed in random order to minimize the effects of uncontrolled factors. Five additional runs were made outside the experimental design region to validate the developed model.

TABLE 1. Actual and coded levels of the factors used in the experimental design.

Factor	Unit	Level		
		-1	0	+1
Temperature (T)	°C	30	40	50
Extraction time (E)	min	90	150	210
Liquid-to-solid ratio (R)	mL g^{-1}	20	30	40
Solvent composition (C)	vol%	30	50	70

TABLE 2. Experimental design layout and observed extraction yields (y). SO is the standard order and RO the run order of experiments.

SO	RO	x_1	x_2	x_3	x_4	y (%)
1	7	-1	-1	-1	-1	52.5
2	20	+1	-1	-1	-1	66.9
3	13	-1	+1	-1	-1	46.3
4	4	+1	+1	-1	-1	80.7
5	14	-1	-1	+1	-1	63.5
6	2	+1	-1	+1	-1	53.9
7	8	-1	+1	+1	-1	62.8
8	15	+1	+1	+1	-1	57.0
9	16	-1	-1	-1	+1	59.0
10	19	+1	-1	-1	+1	78.3
11	3	-1	+1	-1	+1	75.9
12	1	+1	+1	-1	+1	77.6
13	9	-1	-1	+1	+1	73.8
14	5	+1	-1	+1	+1	66.3
15	10	-1	+1	+1	+1	64.9
16	18	+1	+1	+1	+1	76.5
17	11	0	0	0	0	66.9
18	6	0	0	0	0	62.5
19	12	0	0	0	0	64.5
20	17	0	0	0	0	61.4

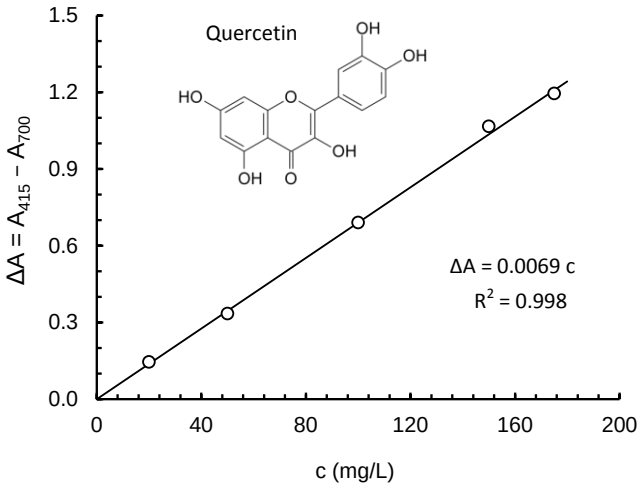

Fig.1. Calibration curve of quercetin. A_{415} and A_{700} are the absorbance values at 450 and 700 nm.

Results and Discussion

The initial moisture content of bilberry by-products was 52.4 ± 1.3 (% w/w) and the total flavonoid content was 291.3 ± 48.2 mg QE per 100 g dry weight (138.5 ± 22.9 mg QE per 100 g fresh weight).

In a recent review by Haminiuk et al. [12] on phenolic compounds in fruits, a total flavonoid content ranging from 0.4 to 58.7 mg QE per 100 g fresh bilberry is reported. On the other hand, onion skin, one of the richest source of phenolic components, was found to have a flavonoid content of about 20 mg QE per g dry weight [13]. Thus, it can be concluded

that amount of flavonoids present in bilberry by-products strongly supports their use as a source of these compounds.

Table 2 shows the results of the factorial design experiments, which were aimed at investigating the effects of extraction conditions on the recovery of flavonoids. As can be seen, the observed extraction yields ranged from 46.3 to 80.7%. The highest value was obtained under the following conditions: T = 50 °C, E = 210 min, R = 20 mL g^{-1} and C = 30%.

To evaluate the contribution of each factor and its interactions to the extraction yield, the following polynomial equation was used [14]:

$$y = \beta_0 + \sum_{i=1}^{4}\beta_i x_i + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij}x_i x_j + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\sum_{k=j+1}^{4}\beta_{ijk}x_i x_j x_k + \\ +\beta_{1234}x_1x_2x_3x_4 \quad (2)$$

where β_i are the coefficients associated with the four main effects, β_{ij} and β_{ijk} are those related to the two-way and three-way interactions, β_{1234} is the four-way interaction coefficient and x are the coded independent variables. The polynomial model contains 16 unknown coefficients, which represent the contributions of each factor, alone or in combination with the others, to y. Since the independent variables were made dimensionless and normalized between-1 and +1 (Eq. 1), all the coefficients can be compared directly with one another. Furthermore, a positive (negative) value of a coefficient indicates a direct (inverse) association between the corresponding term and the dependent variable. The 16 coefficients were determined from the data of runs 1-16 in Table 2, giving the results reported in Table 3.

To assess the statistical significance of each model coefficient, the standard deviation of the experimental response was estimated from the central points of the factorial design (runs 17-20 in Table 2). Then, the 95% confidence interval of each coefficient was determined by the Student's *t*-test and the coefficients with confidence intervals not spanning zero were considered statistically significant. Examination of Table 3 reveals that, in addition to the intercept, the statistically significant terms were those associated with the main factors T, E and C, the two-way interaction T×R, the three-way interaction T×R×C and the four-way interaction T×E×R×C.

The relative importance of significant factors and interactions can be readily appreciated from the Pareto chart shown in Figure 2.

TABLE 3. Values and *t*-statistics for the coefficients in Eq. (2). The values of the statistically significant coefficients (at the 95% confidence level) are marked in bold.

Coefficient	Effect	Value	*t*-Value
β_0	-	**65.995**	108.749
β_1	T	**3.645**	6.007
β_2	E	**1.714**	2.825
β_3	R	-1.161	1.913
β_4	C	**5.533**	9.118
β_{12}	T×E	1.586	2.613
β_{13}	T×R	**-5.066**	8.347
β_{14}	T×C	-0.517	0.852
β_{23}	E×R	-1.247	2.054
β_{24}	E×C	0.470	0.774
β_{34}	R×C	-0.002	0.004
β_{123}	T×E×R	1.285	2.118
β_{124}	T×E×C	-1.375	2.266
β_{134}	T×R×C	**2.959**	4.876
β_{234}	E×R×C	-0.603	0.993
β_{1234}	T×E×R×C	**3.302**	5.441

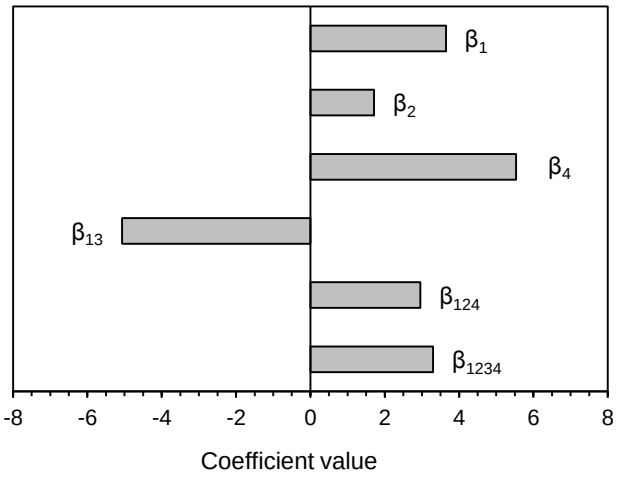

Fig.2. Pareto chart showing the effects of the significant model coefficients on the extraction yield of flavonoids.

We see that: (a) the contribution of the three main factors increases in the order: E < T < C; (b) there is a negative interaction between C and T; (c) the three-way interaction and the four-way interaction coefficients are both positive. Observation (b) implies that solvent concentration has a more pronounced effect on the recovery of flavonoids at lower temperature. It is also interesting to note that the contributions of temperature, extraction time and solvent composition were all positive and that the liquid-to-solid ratio was not a significant factor. This indicates that an increase in temperature, extraction time or solvent composition facilitates the release of flavonoids from the plant tissue and that mass-transfer effects are not significant, at least under the experimental conditions tested [11, 15]. The enhancement in flavonoid recovery at higher ethanol concentration is in agreement with the results of other studies [16-19] and suggests that these compounds have averagely higher affinity for ethanol than for water.

Removing the non-significant terms from Eq. 2 led to the following simplified model:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_4 x_4 + \beta_{13}x_1x_3 + \beta_{134}x_1x_3x_4 + \\ +\beta_{1234}x_1x_2x_3x_4 \quad (3)$$

This model provided a very good description of the experimental data, with an average percentage error of about 4% (Fig. 3). A normal-probability plot was also constructed by plotting the model residuals:

$$r_i = y_{i,\exp} - y_{i,calc} \quad (4)$$

against the corresponding normal-order statistic medians:

$$M_i = \Phi^{-1}\left(\frac{i}{N+1}\right) \quad (5)$$

where Φ is the standard normal cumulative distribution function and *N* is the number of experimental points. It is known that data plotted in this way should lie on a straight line, with the intercept and slope being, respectively, equal to the location and scale parameters of the normal distribution [20]. The results yielded a highly linear plot, with $R^2 = 0.963$ (Fig. 4).

Finally, to further validate the model, the results of the experiments performed outside the factorial design space ($x_i > +1$ or $x_i < -1$) were compared with the values predicted by Eq. (3). The experimental conditions of these runs and the results are summarized in Table 4. It can be seen that the predicted extraction yields were in fairly good agreement with the experimental values, the average percent error being about 6%.

From the above results, it can be concluded that the model described by Eq. (3) is statistically sound and can be used to evaluate the influence of process conditions on the recovery of flavonoids with good descriptive and predictive capabilities.

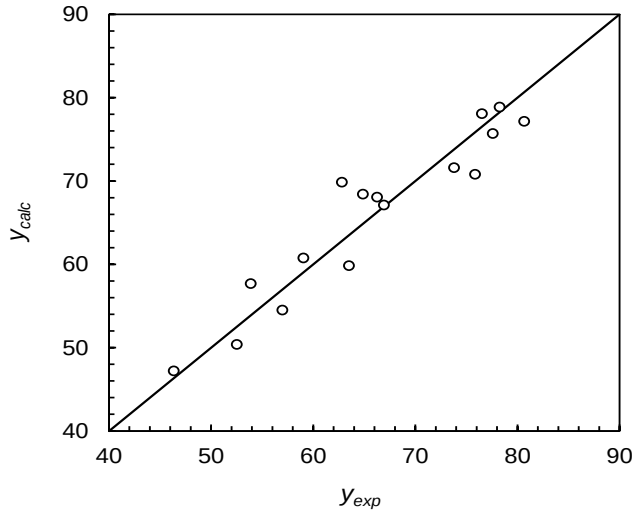

Fig.3. Experimental and calculated (by Eq. 3) extraction yields.

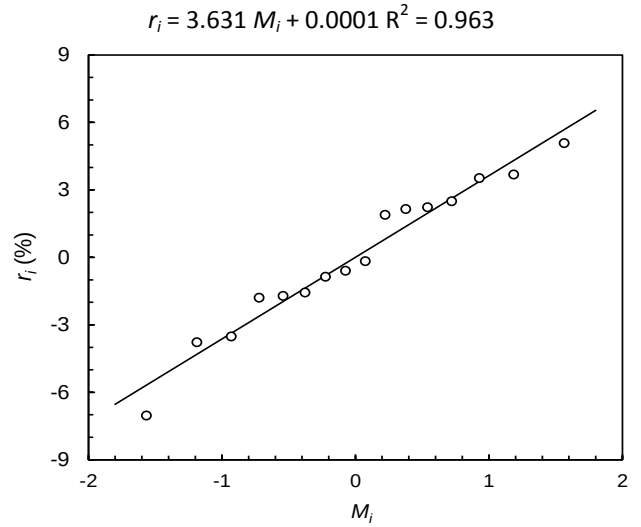

Fig. 4. Normal probability plot of ordered residuals (r_i) against normal-order statistic medians (M_i).

TABLE 4. Experimental conditions and results of the runs performed outside the factorial design space.

Run	T (°C)	E (min)	R (mL g^{-1})	C (vol%)	y_{exp} (%)	y_{pred} (%)
1	60	150	30	50	74.9	73.3
2	40	270	30	50	72.5	69.4
3	40	150	10	50	66.3	66.0
4	20	150	30	50	76.4	58.7
5	40	150	30	90	76.7	77.1

Conclusion

The results of this study demonstrate that bilberry by-products are a rich source of flavonoids and that they can be easily recovered from this plant material by an environmentally friendly extraction procedure using aqueous ethanol as solvent. At present, considerable amounts of bilberry or other wild berry processing waste are produced in many parts of the world. The possibility of recovering valuable bioactive compounds from this waste could not only provide significant economic benefits to the producers but also contribute to reduce its environmental impact.

The statistical approach followed proved to be very effective for evaluating the contribution of the most important process variables, alone or in combination, to the extraction yields. Future research should be directed at investigating and optimizing the process on a larger scale as well as at characterizing the hydroalcoholic extracts obtained in order to identify the major flavonoids recovered.

Acknowledgements

The authors gratefully thank "Rigoni di Asiago SRL" (Asiago, VI, Italy) for the kind gift of bilberry residues.

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