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Recovery of phenolic antioxidants from the peel fraction of bilberry (*Vaccinium myrtillus* L.) processing waste

Antonio Zuorro, Roberto Lavecchia*

Department of Chemical Engineering, Materials & Environment, Sapienza University,
Via Eudossiana 18, 00184 Rome, (ITALY)
E-mail : roberto.lavecchia@uniroma1.it

ABSTRACT

The extraction of phenolic compounds from the peel fraction of bilberry processing waste was investigated. The phenolic content of bilberry peels was 25.3 ± 2.4 mg GAE per gram dry weight and the flavonoid content was 2.85 ± 0.42 mg QE per gram dry weight. Extraction experiments were carried out in batch mode, using ethanol–water mixtures as solvent. A central composite design was used to study the effects of liquid-to-solid ratio (R = 20–40 mL g^{-1}), aqueous ethanol concentration (C = 30–70 vol%), extraction time (E = 90–210 min) and temperature (T = 30–50 °C) on the recovery of phenolic compounds. Under the best conditions (R = 40 mL g^{-1}, C = 70%, E = 210 min and T = 50 °C) over 95% of the phenolics present in the waste were recovered. R, C and T were the most influential factors and all had a positive effect on the extraction efficiency. Based on the statistical analysis of the data, a simplified model was developed which provided an accurate estimation of the extraction yields both inside and outside the design space. Overall, the results of this study strongly support the potential of bilberry processing waste as a source of natural antioxidants and give useful directions on how to improve recovery by proper selection of extraction conditions.

KEYWORDS

Bilberry;
Phenolic compounds;
Antioxidant activity;
Extraction;
Waste valorization;
Influential factor analysis.

INTRODUCTION

In recent years, increasing awareness of the environmental impact of agricultural and food wastes has stimulated efforts to find possible ways of using them for energy production or other purposes[1]. The production of biofuels such as ethanol[2] and biodiesel[3], the recovery of functional compounds[4-6] and the use as low-cost adsorbents[7] are just a few examples of the approaches that have been proposed.

Bilberry (Vaccinium myrtillus L.) is a small perennial shrub native to northern Europe but now found in many parts of the world, including Australia, North America and Asia. Bilberry fruits have a diameter of 5 to 9 mm, are bluish black in color and possess a sweet and slightly acidic taste. Among wild berries, bilberries are the richest in phenolic compounds, particularly anthocyanins, flavanols, tannins and phenolic acids[8-10]. These substances are considered to be responsible for numerous health benefits such as protection from UV

radiation[11] and decreased risk of cardiovascular, neurodegenerative and inflammatory diseases[12,13]. The observed beneficial effects are generally attributed to the antioxidant and metal-chelating properties of phenolic compounds, but recent evidence suggests that modulating effects on cell signalling, gene expression and DNA repair could also be involved[14,15].

Bilberries are usually sold as fresh whole berries or processed into juice and juice concentrates that are subsequently used to produce beverages, syrups and other food products. Processing of bilberries generates a waste consisting mainly of the fruit seeds and peels. This waste has no commercial value and is currently disposed of in landfill or used for animal feeding. Nevertheless, it is an extremely rich source of bioactive substances that could be recovered and used for food or pharmaceutical applications. Studies on the distribution of these components in different parts of the fruit have shown, in fact, that they predominantly accumulate in the fruit peel. For example, the anthocyanin content in the peels of bilberries was found to be over 20 times higher than in the pulp and a similar organ-specific distribution was observed for quercetin and hydroxycinnamic acids[16]. However, despite these interesting findings, little attention has so far been given to the exploitation of bilberry or other berry wastes for recovery purposes[17,18].

The aim of this research was to evaluate the feasibility of recovering phenolic antioxidants from bilberry processing waste by an environmentally friendly procedure based on the use of aqueous ethanol as extraction solvent. In addition, we were interested in investigating the effect of the main process parameters (solvent composition, temperature, extraction time and liquid-to-solid ratio) on the extraction yield and the characteristics of the resulting products.

EXPERIMENTAL

Chemicals and reagents

Ethanol (CAS 64-17-5), methanol (CAS 67-56-1), sodium carbonate (CAS 497-19-8), hydrochloric acid (CAS 7647-01-0), sodium acetate (CAS 127-09-3) and aluminum chloride (CAS 7446-70-0) were obtained from Carlo Erba (Milano, Italy). Gallic acid (CAS 149-91-7), quercetin (CAS 117-39-5) and the Folin-Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (Milano, Italy). All chemicals were reagent grade and used without further purification. Aqueous solutions were prepared with distilled water.

Plant material

Bilberry processing waste was obtained from Rigoni di Asiago SPA (Asiago, VI, Italy). The material was previously passed through a steel screen to separate the peels from the seeds and other debris. Bilberry peels were packed in plastic bags and stored at –20 °C. Before performing a set of experiments, an appropriate amount of the frozen material was thawed in air at room temperature and assayed for moisture, total phenolic and total flavonoid contents.

Analytical methods

Moisture content was determined by an electronic moisture analyzer (model MAC 50/1, Radwag, Poland). A three-stage extraction procedure allowing complete exhaustion of the solid was used to evaluate the initial phenolic and flavonoid content of bilberry peels[19]. Briefly, 1 g of peels and appropriate amounts of solvent (100, 50 and 20 mL in the first, second and third stage, respectively) were poured into glass flasks thermostated at 40 °C and stirred for 90 min. After 90-min stirring, the resulting suspension was filtered at 0.45 μm and assayed for total phenolics and flavonoids. Aqueous ethanol (50% v/v) was used as extraction solvent and the total amount of phenolics or flavonoids was determined as the sum of the values obtained in each stage.

Total phenolics were determined by the Folin Ciocalteu's method. Five mL of 0.1 M HCl, 150 μL of Folin-Ciocalteu's reagent and 200 μL of the sample to be tested were poured into a graduated glass vial and an aqueous sodium carbonate solution (20% w/v) was added to a final volume of 10 mL. The vial was thoroughly shaken and kept in the dark at room temperature for 1 h. Then, the absorbance at 525 nm was measured with a colorimeter (HI83742, Hanna Instruments, Italy). The results were expressed as gallic acid equivalents (GAE), using a calibration curve obtained with gallic acid standards.

Total flavonoids were determined as described by Chang et al.[20] with some modifications. 300 μL of the sample to be tested were poured into an optical glass cuvette together with 900 μL methanol, 60 μL aluminum

chloride at 10% (w/v), 60 μL of 1 M sodium acetate and 1.7 mL distilled water. The cuvette was shaken and kept in the dark at room temperature for 30 min. Then, the absorbance at 415 nm was measured against a blank of distilled water by a double-beam UV-VIS spectrophotometer (Lambda 25, Perkin Elmer, USA). The results were expressed as quercetin equivalents (QE), using a calibration curve obtained with quercetin standards.

Extraction procedure

The extraction of phenolic antioxidants was performed in batch mode using ethanol-water mixtures as the solvent. Appropriate amounts of bilberry peels and the solvent were loaded into 50 mL screw-top pyrex 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 phenolic content.

Influential factor analysis

A central composite design was used to evaluate the effects of the four factors: liquid-to-solid ratio (R), aqueous ethanol concentration (C), extraction time (E) and temperature (T) on the recovery of phenolic antioxidants. The levels of each factor were chosen to cover a range of values of practical interest (TABLE 1) and the test variables were coded to vary between –1 and +1 using the following equations:

TABLE 1 : Natural and coded levels of the factors for the central composite design.

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

$$x_1 = \frac{R - 30}{10}$$
$$x_2 = \frac{C - 50}{20}$$
$$x_3 = \frac{E - 150}{60} \quad (1)$$
$$x_4 = \frac{T - 40}{10}$$

Four replicates at the central point of the experimental domain ($x_1 = x_2 = x_3 = x_4 = 0$) were carried out to estimate the experimental error and check the adequacy of the models. Overall, the design consisted of $2^4 + 4 = 20$ runs, which were performed in random order to eliminate possible bias (TABLE 2). Additional runs were made outside the experimental design region to validate the developed model. The extraction yield of phenolic compounds (y), expressed as mg GAE per gram dry weight, was used as the response variable.

TABLE 2 : Experimental design layout and observed response. y is the extraction yield of phenolic compounds and x_i's are the coded levels of factors.

Trial	Factor level				Response
	x_1	x_2	x_3	x_4	y (mgGAE g^{-1})
1	–1	–1	–1	–1	14.32
2	+1	–1	–1	–1	15.98
3	–1	+1	–1	–1	17.18
4	+1	+1	–1	–1	20.00
5	–1	–1	+1	–1	15.26
6	+1	–1	+1	–1	16.14
7	–1	+1	+1	–1	17.48
8	+1	+1	+1	–1	19.04
9	–1	–1	–1	+1	20.56
10	+1	–1	–1	+1	22.17
11	–1	+1	–1	+1	20.07
12	+1	+1	–1	+1	23.94
13	–1	–1	+1	+1	20.94
14	+1	–1	+1	+1	22.46
15	–1	+1	+1	+1	21.96
16	+1	+1	+1	+1	24.10
17	0	0	0	0	22.52
18	0	0	0	0	22.47
19	0	0	0	0	21.17
20	0	0	0	0	22.41

Statistical analysis was performed by Minitab® (version 15, Minitab Inc, PA, USA).

RESULTS AND DISCUSSION

Characterization of bilberry peels

The initial moisture content of bilberry peels was 52.4 ± 1.3 (% w/w). The total phenolic content was 25.3 ± 2.4 mg GAE per g dry weight (1204 ± 129 mg

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GAE per 100 gram fresh weight) and the total flavonoid content was 2.85 ± 0.42 mg QE per g dry weight (135.7 ± 24.3 mg QE per 100 gram fresh weight). The percentages of phenolics and flavonoids extracted in each stage are shown in Figure 1.

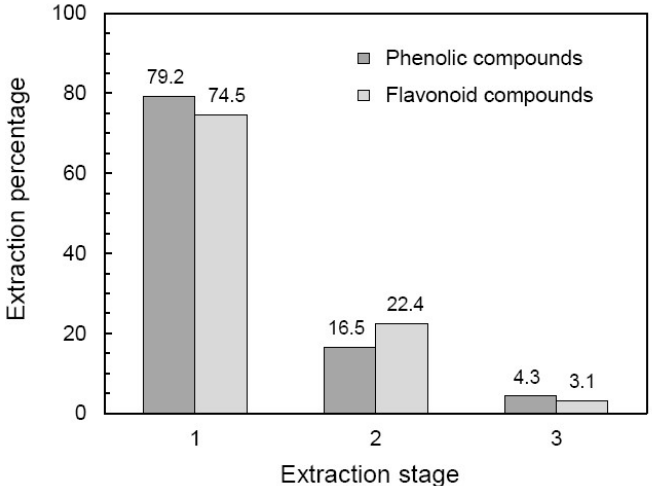

Figure 1 : Percentages of phenolic and flavonoid compounds recovered from bilberry peels in the three stages of extraction.

A review of the literature reveals that the phenolic content found in bilberry peels is generally higher than that reported for similar agro-industrial wastes. For example, values close to 14 mg GAE/g dry matter were determined for grape pomace[21] and carrot peel waste[22]. A total phenolic content of 8.2 and 11.4 mg GAE/g dry matter was measured, respectively, for kiwi and apple peel wastes[23]. Finally, values ranging from 17.7 to 35.5 mg/g are reported for spent coffee grounds[6,19]. Therefore, based on the phenolic content, we can conclude that bilberry processing waste can be regarded as a potentially valuable source of phenolic antioxidants.

Phenolic extraction and influential factor analysis

TABLE 2 shows the results of the experimental design, which was aimed at investigating the effects of liquid-to-solid ratio (R), aqueous ethanol concentration (C), extraction time (E) and temperature (T) on the extraction yield (y) of phenolic compounds from bilberry peels. The observed yields ranged from 14.32 to 24.1 mg/g and the maximum value, corresponding to 95.2% of the phenolic compounds contained in the starting material, was achieved under the following conditions: R = 40 mL/g and C = 70%, E = 210 min and T = 50 °C.

To evaluate the contribution of the four factors and their interactions to the extraction yield we used the following polynomial equation, referred to as the full model:

$$y = \beta_0 + \sum_{i=1}^{4}\beta_i x_i + \sum_{i=1}^{4}\sum_{j=i+1}^{4}\beta_{ij} x_i x_j + \sum_{i=1}^{4}\sum_{j=i+1}^{4}\sum_{k=j+2}^{4}\beta_{ijk} x_i x_j x_k + \beta_{1234} x_1 x_2 x \quad (2)$$

where β_i are the coefficients associated with the four main effects, β_{ij} and β_{ijk} are those related to the binary and ternary interactions, β_{1234} is the quaternary interaction coefficient and the x's are the coded independent variables. The polynomial model contains 16 unknown coefficients, representing the contribution 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, 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.

TABLE 3 : Values and t-statistics for the coefficients in Eq. (2). Statistically significant coefficients (at the 95% confidence level) are represented in bold.

Coefficient	Effect	Value	t-value	Coefficient	Effect	Value	t-value
β_0	–	**19.473**	199.464	β_{23}	C–E	–0.025	0.252
β_1	R	**1.005**	10.293	β_{24}	C–T	**–0.503**	5.152
β_2	C	**0.996**	10.202	β_{34}	E–T	0.143	1.460
β_3	E	0.198	2.030	β_{123}	R–C–E	–0.133	1.364
β_4	T	**2.551**	26.126	β_{124}	R–C–E	0.065	0.663
β_{12}	R–C	**0.295**	3.025	β_{134}	R–C–E	0.014	0.143
β_{13}	R–E	–0.242	2.476	β_{234}	R–C–E	0.197	2.013
β_{14}	R–T	0.139	1.428	β_{1234}	R–C–E–T	–0.073	0.751

To assess the statistical significance of the model coefficients, we followed the procedure described in a previous paper[24]. In particular, the standard deviation of the experimental response was first 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 ($p < 0.05$).

As shown in TABLE 3, six out of the 16 coefficients were statistically significant at the confidence level considered. In addition to the intercept, a_0, they included three of the four coefficients associated with the main effects (R, C, T) and two interaction coefficients (R–C, C–T). From the Pareto chart presented in Figure 2, the following considerations can be made: (a) all the three main factors, temperature, ethanol concentration and liquid-to-solid ratio, have a positive effect on phenolic extraction and their influence increases in the order T > R > C; (b) the interaction between C and T is stronger than that between R and C; (c) there is a negative interaction between C and T, and a weak positive interaction between R and C. Thus, an increase in solvent concentration has a more pronounced effect on the recovery of phenolics at lower temperature, while the opposite is true for R and C.

It is interesting to note that, under the experimental conditions examined, the extraction time was not a significant factor. This may indicate that most of the phenolic compounds present in bilberry peels are extracted within the first 90 min, which is the lower level of the time factor studied. Similar results were obtained in other studies on the recovery of phenolic or carotenoid compounds from agricultural wastes and attributed to their high affinity for the extraction solvent, which would allow their almost complete recovery in a short time[25,26]. From a practical viewpoint, this means that increasing time to get a quantitative extraction is neither technically appropriate nor economically justified.

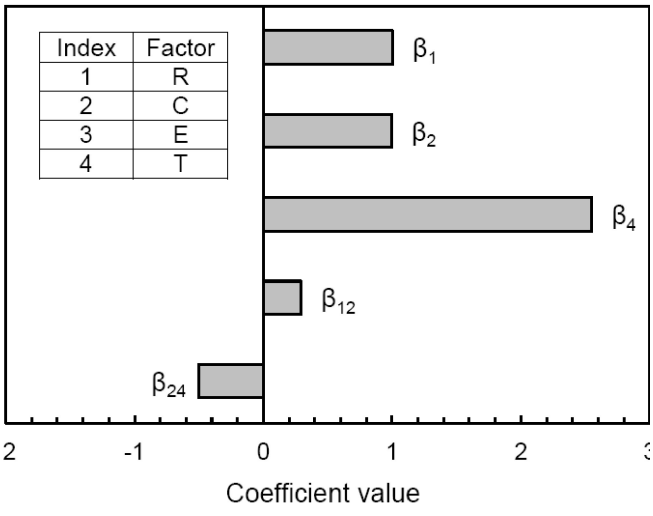

Figure 2 : Pareto chart showing the effects of the significant model coefficients on the phenolic extraction yield.

The positive effects of temperature and liquid-to-solid ratio can be explained by considering that an increase in temperature facilitates the release of phenolic compounds from the plant tissue and that higher liquid-to-solid ratios improve the mass-transfer of the dissolved substances from the solid to the solvent[27].

Finally, the observed enhancement in yields at higher ethanol concentration is in agreement with the results of studies on the extraction of phenolics from other types of materials, such as peanut skins[28], olive leaves[29] and byproducts of kiwifruit juicing[30]. Such effect is probably due to an averagely higher affinity of phenolic compounds for ethanol than for water[31]. However, other solvent-related mechanisms, such as the swelling of the plant matrix, could also be involved. Swelling results from the adsorption of solvent components, particularly those with small molar volume, high hydrogen bonding capability and large basicity, such as ethanol, on the hydroxyl and carboxyl groups of cellulose fibers[32]. The adsorbed solvent molecules produce a partial separation of the fibers, which increases solvent penetration into the matrix and favors the recovery of extractable compounds[33].

By removing the non-significant terms from the full model, the following simplified expression was derived:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_4 x_4 + \beta_{12} x_1 x_2 + \beta_{24} x_2 x_4 \quad (3)$$

The six coefficients in Eq. (3) were estimated from the experimental data by least-squares regression analysis. A very good agreement was found between experimental and calculated yields (Figure 3), with an average percentage error of 1.8% and an R^2-value of 0.977.

To further validate the model, the results of experiments performed outside the region of experimentation delimited by the factorial points, under the conditions reported in Table 4, were compared with the values predicted by Eq. (3).

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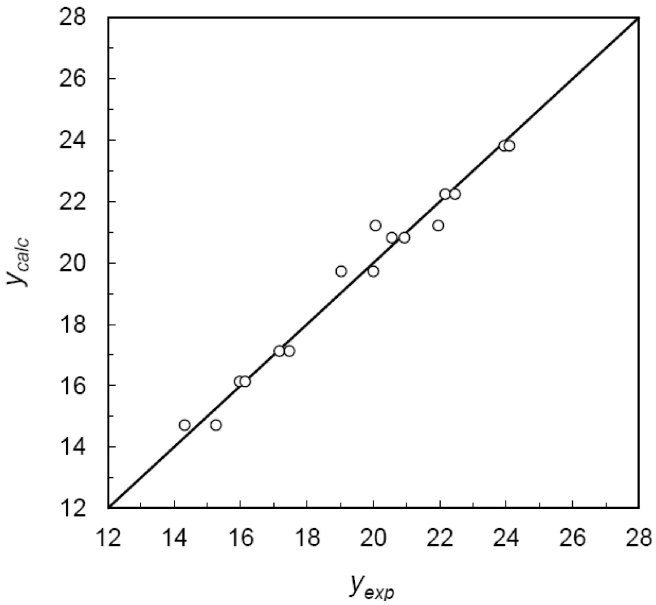

Figure 3 : Experimental and calculated (by Eq. 3) phenolic extraction yields.

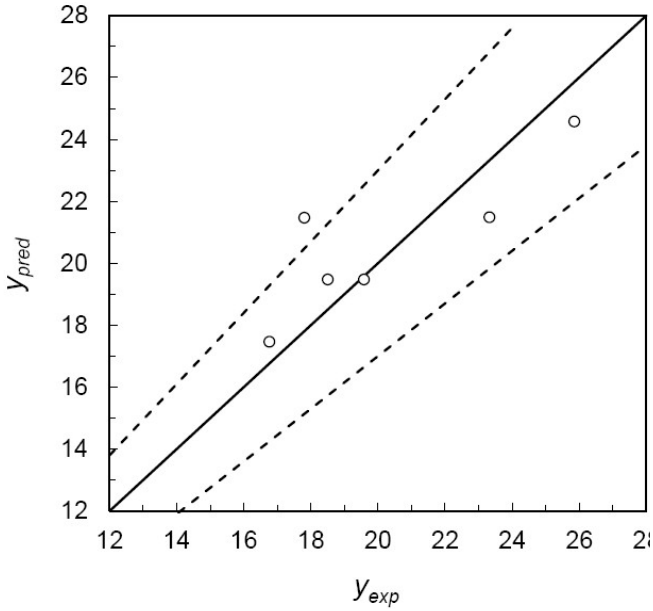

Figure 4 : Experimental and predicted (by Eq.3) phenolic extraction yields. The dashed lines represent ± 15% deviation from the bisecting line.

As apparent from Figure 4, five of the six data points fell within the 15%-deviation band, demonstrating the good predictive capabilities of the model. Finally, the reduced model residuals, defined as the difference between experimental and calculated yields:

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

were calculated and plotted against the corresponding normal-order statistics medians:

$$\mu_i = F^{-1}\left(\frac{i}{n+1}\right) \quad (5)$$

where F is the standard normal cumulative distribution function and n is the total number of experimental points. If the errors were normally distributed, plotting ρ_i against μ_i would give a straight line. In contrast, deviations from linearity would indicate that the model residuals do not follow a normal-probability distribution[34]. From the results in Figure 5 it can be seen that a highly linear pattern (R^2 = 0.956) is obtained. Accordingly, the

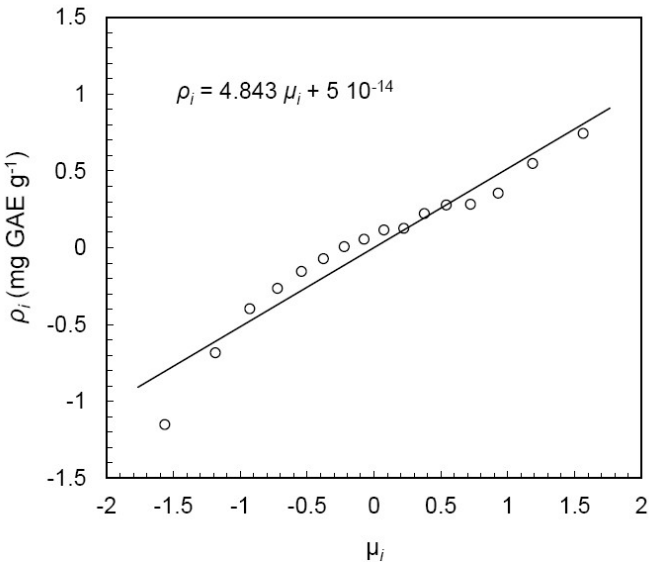

Figure 5 : Normal probability plot showing the dependence of the ordered residuals (ρ_i) on the corresponding normal-order statistics medians (μ_i).

TABLE 4 : Observed (y) and predicted (y_{pred}) extraction yields of phenolic compounds under conditions outside the design space.

Trial	R (mL g^{-1})	C (vol%)	E (min)	T (°C)	y (mgGAE g^{-1})	y_{pred} (mgGAE g^{-1})
A	30	50	150	60	25.85	24.57
B	30	50	30	40	18.50	19.47
C	30	50	270	40	19.59	19.47
D	30	90	150	40	17.80	21.46
E	10	50	150	40	16.77	17.46
F	50	50	150	40	23.33	21.48

simplified model described by Eq. (3) can be considered statistically significant and used to describe the influence of process conditions on the recovery of phenolic compounds from bilberry peels.

CONCLUSIONS

This study demonstrates that the peel fraction of bilberry waste is a rich source of phenolic antioxidants and that these compounds can be easily recovered by an environmentally friendly procedure based on the use of aqueous ethanol as extraction solvent. We have also shown that, by proper choice of process conditions, the recovery of these compounds can reach values as high as 95% of the initial phenolic content. In addition, the influential factor analysis performed and the simplified model developed can provide useful suggestions on how to improve the recovery of phenolic compounds.

At present, considerable amounts of bilberry or other wild berry processing waste are produced in many parts of the world and disposed of as conventional waste. The possibility of using these waste materials as a source of valuable antioxidant compounds could not only provide significant economic benefits to the producers but also contribute to reduce their impact on the environment.

REFERENCES

[1] J.L.Hatfield, D.D.Smith; Carbon Manage., **4**, 203 **(2013)**.
[2] J.Tao, C.-C.Dai, B.J.Jiang; Energy Environ., **20**, 1397 **(2009)**.
[3] M.R.Kosseva; Adv.Food Nutr.Res., **58**, 57 **(2009)**.
[4] A.Zuorro, R.Lavecchia; Biotechnol.Biotechnol. Equip., **24**, 1854 **(2010)**.
[5] Zuorro, M.Fidaleo, R.Lavecchia; Enzyme Microb. Technol., **49**, 567 **(2011)**.
[6] Panusa, A.Zuorro, R.Lavecchia, G.Marrosu, R.Petrucci; J.Agric.Food.Chem., **61**, 4162 **(2013)**.
[7] Zuorro, R.Lavecchia, F.Medici, L.Piga; Chemical Engineering Transactions, **32**, 19 **(2013)**.
[8] X.Wu, R.L.Prior; J.Agric.Food Sci., **53**, 2589 **(2005)**.
[9] N.P.Seeram; J.Agric.Food Sci., **56**, 627 **(2008)**.
[10] M.P.Kähkönen, A.I.Hopia, M.Heinonen; J.Agric. Food Chem., **49**, 4076 **(2001)**.
[11] A.Zuorro, R.Lavecchia; Desalination and Water Treatment (in press), **(2013)**.
[12] C.S.Yang, J.D.Lambert, S.Sang; Arch.Toxicol., **83**, 11 **(2009)**.
[13] S.Quideau, D.Deffieux, C.Douat-Casassus, L.Pouységu; Angew.Chem.Int.Edit., **50**, 586 **(2011)**.
[14] N.P.Seeram; J.Agric.Food Chem., **56**, 630 **(2009)**.
[15] I.F.F.Benzie, S.Wachtel-Galor; Antioxid.Redox Signal., **13**, 1575 **(2010)**.
[16] K.Riihinen, L.Jaakola, S.Kärenlampi, A.Hohtola; Food Chem., **110**, 156 **(2008)**.
[17] J.Lee, R.E.Wrolstad; J.Food Sci., **69**, C564 **(2004)**.
[18] K.Aaby, S.Grimmer, L.Holtung; LWT-Food Sci.Technol., **54**, 257 **(2013)**.
[19] A.Zuorro, R.Lavecchia; J.Clean.Prod., **34**, 49 **(2012)**.
[20] A.Chang, M.Yang, H.Wen, J.Chern; J.Food Drug Anal., **10**, 178 **(2002)**.
[21] V.Louli, N.Ragoussis, K.Magoulas; Bioresour. Technol., **92**, 201 **(2004)**.
[22] P.Chantaro, S.Devahastin, N.Chiewchan; LWT-Food Sci.Technol., **41**, 1987 **(2008)**.
[23] H.H.Wijngaard, C.Rößle, N.Brunton; Food Chem., **116**, 202 **(2009)**.
[24] A.Zuorro, M.Fidaleo, R.Lavecchia; J.Environ. Manage., **127**, 28 **(2013)**.
[25] R.Lavecchia, A.Zuorro; Eur.Food Res.Technol., **228**, 153 **(2008)**.
[26] A.Zuorro, R.Lavecchia; Am.J.Appl.Sci., **10**, 478 **(2013)**.
[27] E.L.Cussler; Diffusion: Mass Transfer in Fuid Systems, 3rd Edition, Cambridge University Press; Cambridge, **(2009)**.
[28] V.Nepote, N.Grosso, C.Guzman; J.Sci.Food Agric., **85**, 33 **(2005)**.
[29] S.Mylonaki, E.Kiassos, D.P.Makris, P.Kefalas; Anal.Bioanal.Chem., **392**, 977 **(2008)**.
[30] D.Sun-Waterhouse, I.Wen, R.Wibisono, L.D.Melton, S.Wadhwa; Int.J.Food Sci.Technol., **44**, 2644 **(2009)**.
[31] F.F.Liu, C.Y.W.Ang, D.Springer; J.Agric.Food Chem., **48**, 3364 **(2000)**.
[32] O.A.El Seoud; Pure Appl.Chem., **81**, 697 **(2009)**.
[33] E.Obataya, J.Gril; J.Wood Sci., **51**, 124 **(2005)**.
[34] R.H.Myers, D.C.Montgomery; Response surface methodology. Process and Product Optimization using Designed Experiments, John Wiley & Sons; New York, **(1995)**.