Innovative Treatment of Bilberry By-Products for a Selective Recovery of Anthocyanin Compounds

Renata Adami[a]*, Giuseppe Salvo[a], Miguel Meneses[a,b], Eila Järvenpää[c], Rainer Huopalahti[d], Libero Sesti Osséo[a], Ernesto Reverchon[a]

[a]Department of Industrial Engineering. University of Salerno
Via Ponte Don Melillo, 84084 Fisciano (SA), ITALY
[b]Departamento de CienciasAgropecuarias y de Alimentos, UniversidadTécnicaParticular de Loja (UTPL), Apartado 1101608. Loja, ECUADOR
[c]MTTAgrifoodResearch Finland, Biotechnological and Food Research, Jokioinen, FINLAND
[d]Department of Food Chemistry, University of Turku, FINLAND
*radami@unisa.it

Industrial interest on phenolic and anthocyanin compounds, recovered from fruits or vegetables, is growing. Comparison of bilberry and lingonberry by-product with different matrices, extracted by different ethanol solutions was done. Bilberry press-cake matrix, extracted by ethanol solution 70% in volume wasthe most interesting by-product in terms of potential industrial uses.From this solution, processed by Supercritical Antisolvent Extraction (SAE)technique, was possible to concentrate the anthocyanins, in all conditions of temperature and pressure tested. Therefore,process conditions suitable to improve the value of by-products were investigated.SAE conditions that give a product with highest concentration of anthocyanins are 40°C and 100 bar. The temperature used does not cause degradation of the active compounds, and the corresponding supercritical fluid density (622 Kg/m^3) does not allowanthocyanins dissolution in SC-CO_2.The powder obtained by SAE preserves anthocyanin antioxidant activity of feed ethanol extract.

Keywords: bilberry,lingonberry, anthocyanins, antioxidant, supercritical antisolvent, extraction

1. INTRODUCTION

The functional ingredients market is a growing sector by the increasing interest of customers to improve health and to prevent degenerative illness. The epidemiological evidence supports the health benefits of a diet rich in polyphenols and, as a consequence, the high development of food products with functional properties.Bilberry (*Vacciniummyrtillus* L.) and lingonberry (*Vacciniumvitis-idaea* L.) areamong the most abundant wild berries native in boreal forest and arctic tundra throughout the northern hemisphere from Eurasia to North America[1].In these countries they are widely consumed as part of daily diet by their recognized health benefits, for example to improve the night vision [2], antioxidant properties [3]; from literature it is known that anthocyanins are the main compounds in berry [1] with related pharmaceutical effect [4]. Recently agroindustrial by-products have been identified as good source of functional ingredients [5]. Inberryjuice production a solid residue is generated from the pressing of fruits, it can be interesting use this by-product as source of phenolic compounds[6].Laaksonen, et al. [7] have reported, the presence of important quantities of anthocyanins in residues of bilberry juice production(2308.2 mg/100 g) compared with the one of juice (444.5 mg/100 g)and five main types of anthocyanins: delphinidin, cyanidin, petunidin, peonidin and malvidin, all of them linked to 3-O galactoside, 3-O glucoside or 3-O arabinoside. A process set up to recover phenolic compounds from berry by-products seems to be very interesting.

Traditional extraction techniques have been used for recovering of anthocyanin compounds with use of water, ethanol and methanol. The most common techniques for extraction of polyphenol correspond to dynamic maceration andsoxhlet[8]. In the traditional processes the extract is obtained in liquid form and post processing is necessary for final use. Frequently, the solvent toxicity and/or operating conditions that denature phenolic compoundsrepresent limitations for efficient post processing.

Recently,Supercritical Assisted Extraction(SAE) has been applied for recovery of antioxidant compounds starting from ethanolic solution of grape residues [9].This innovative technique shows important advantages referred to complete elimination of the organic solvent, moderate and not denaturing operating conditions and the production of extracts as free powder.This work is focused on the processing of bilberryand lingonberryby-products, currently regarded as waste, in order to identify a green process for the recovery of phenolic and anthocyanin compounds.

2.MATERIALS AND METHODS

2.1 Materials

Fruit samples of bilberry and lingonberrycultivated in Piikkio at MTT Agrifood Research Finland and harvested in August 2008, and frozen bilberry fruits bought in a food shop (SurgelatiIacovazzoS.r.l., Italy) were used in this work. From the juice extraction a solid matrix was obtained; part was used as it is and part was used dried, while dry fruit was obtained by drying the whole fruit at 30 °C in an electrical oven with forced flow of air.

All reagents and chemical were of analytical grade.Delphinidin-3-O-glucoside chloride, cyanidin-3-O-galactoside chloride and malvidin-3-O-glucoside chloride (Extrasynthese, France) were used as standards.

2.2 Solvent Extraction

Ethanol was used as solvent for extracting phenolic compounds.Different aqueous solutions (90, 70 and 50 % v/v)were used to compare the extraction yield; each extraction was repeated three times, using each time fresh ethanol-water solvent.For press-cake, dry press-cake and dry fruits,10 g of sample were extracted with 100 mL of ethanolic solution under continuous stirring by 30 minutes, and then this solution was filtered, while the solid matrix was extracted again two times.

2.3 Sugar elimination

Adsorption/desorption technique was adopted using a Waters Sep-Pak® Vac C18 cartridge (20 cm^3/5g 55–105 µm) to eliminate co-extracted sugars.Prior tothe adsorption, the ethanolic solution was acidified to pH 5 using tartaric acid; then the ethanol was eliminated by evaporation and the aqueous solution was diluted with distillated water in 1:4 ratio.The tartaric solution (60 mL) was flushed through the cartridge. The compounds adsorbed in the cartridge were washed with 10 mL of water to separate sugar compounds and finally weredesorptedwith 30 mL of ethanol (99%). This procedure was optimized to avoid artifacts and improve the yield of C18 cartridge; the procedure was repeated to obtain enough solution to perform SAE experiments.

2.4 Supercritical AntisolventExtraction (SAE)

The SAE process consisted of three steps. In the first one, SC-CO_2 was pumped into the precipitation vessel with a membrane pump until the operating conditions of pressure were achieved. In the second one, theethanolic solution was fed with a piston pump through an injector of 180 µm to obtain a spray dispersion characterized by a high contact surface between SC-CO_2 and liquid solution. The two phases arranges a new equilibrium between ethanol and SC-CO_2; this condition is required for the precipitation of the insoluble compounds in SC-CO_2. The ethanol and compounds dissolved in SC-CO_2 pass through a filter

with a porosity of 1 µm and are recovered in a separator by depressurization of CO_2 at low pressure (30 bar). The final step consisted of a flow of SC-CO_2 to remove the excess of solvent in the precipitates.**Figure 1** shows a schematic representation of the process.

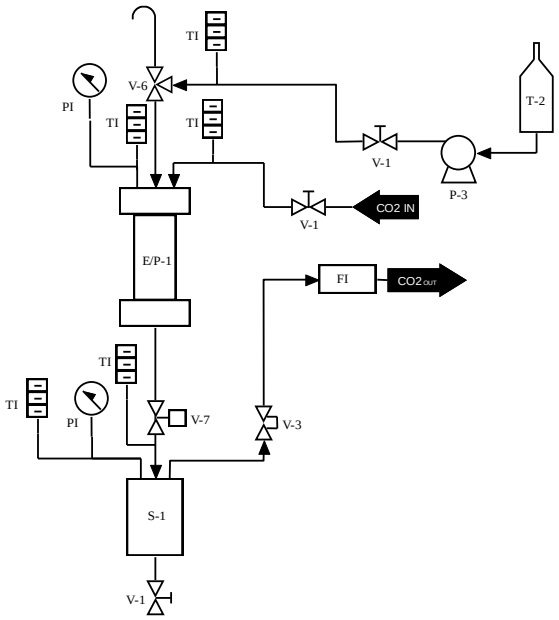

Figure 1. Schematic representation of SAE process.

The effect of the main parameters, pressure and temperature, with respect to recovered powder and to anthocyaninsconcentration was analyzed. The selected operating conditions ranged from 80 to 150 bar for pressure and from 36 to 45 °C for temperature.

2.5 Analytical methods

The FolinCiocalteu method was used to quantify the total phenol content of extracts[7]. A calibration curve, using gallic acid as standard, with concentrations from 0.1 to 1.2 mg/mL was used for quantification of total phenols. The absorbance was measured in a spectrophotometer (Perkin-Elmer Lambda 3B) at 750 nm. The results were expressed as mg of gallic acid equivalent/g solid matrix.The quantification of anthocyanins was made by high performance liquid chromatography (HPLC) using an Agilent HPLC equipped with diode array detector (DAD). Phenolic compounds were separated using a Phenomenex Prodigy RP-18 ODS-3 (250 mm, 4.60 mm i.d., 5 µm) column.For the elution two organic phases were used; solution A consisted of 5% formic acid in water (v/v), and acetonitrile as solution B. with the following gradient: 0-5 min, 5-10% B; 5-10 min, 10%B; 10-25 min, 10-40% B; 25-35 min, 40-90% B; 35-40 min, 90-5% B; 40-45 min, 5% B. The flow rate of the mobile phase was 1.0 mL/min.The compounds were identified, by comparing the retention times and UV-vis spectral characteristicswith the corresponding standards, and quantified at 520 nm using calibration curves.Morphological characteristics of the powder samples were analyzed by Scanning Electron Microscope (SEM, mod. 420, LEO) The DPPH assay was used to evaluate the radical scavenging activity of extracts and to compare the effect of SAE process on it. The method used was according to Thaipong, et al. [10].To quantify the radical scavenging power every sample was analyzed in solution at concentrations between 0.01 to 1 mg/mL.

RESULTS AND DISCUSSIONS

The humidity of bilberry and lingonberry press-cake was 55.75 ± 6.76 % and 68.62 ± 0.36 respectively. For bilberry and lingonberry extraction,the solvent with the highest

extractionyield corresponded to ethanol 70% for all three kinds of matrixes (fresh press-cake, dried press-cake, and dried berries). Bilberry showed the highest amount of polyphenols.

Table 1. Influence of matrix and ethanol percentage on total phenol content in bilberry and lingonberry extracts

Ethanol percentage	Extraction	Bilberry			Lingonberry		
		wetpress-cake	dry press-cake	dry fruits	wetpress-cake	dry press-cake	dry fruits
		mg/g FM	mg/g FM	mg/g FM	mg/g FM	mg/g FM	mg/g FM
90	I	15.7	8.9	6.4	13.1	7.2	5.4
	II	3.7	2.3	1.3	4.5	1.8	2.1
	III	1.5	1.6	0.6	1.0	1.0	1.2
70	I	18.9	17.3	9.0	15.7	16.0	10.5
	II	4.3	2.9	1.4	4.2	2.6	2.4
	III	1.4	1.2	0.5	1.5	1.2	0.8
50	I	18.7	19.8	9.4	15.5	19.3	11.6
	II	4.6	2.9	1.3	3.5	2.5	1.9
	III	1.7	1.2	0.4	2.0	1.2	0.5

mg/g FM: mg equivalent of gallic acid/ gram of fresh matter

As showed in**Table 1**, the first extraction, when fresh feed was used,allows to obtain the highest concentration for all matrices. If the first extraction is analyzed for wet press-cake matrix there was not effect of solvent concentration on the extraction of phenol compounds, while for dry press-cake and dry fruits the 70 and 50% of ethanol concentration shows better results for total content of phenols than 90%.The general trend shows a better extraction of phenolic compounds from wet press-cake, maybe because the content of water improves the polarity of ethanolic solution.Comparing the extracts from bilberry and lingonberry by-products, the ethanolic extract (70%) of bilberry press-cake showed the highest content of anthocyanins and total phenol content with high antioxidant/free radical scavenging activity. Therefore, the wet press-cake of bilberry was selected for the SAE experiments for the recovery of antioxidant compounds.

The sugar elimination from the bilberry extract was performed for all the ethanolic extractsbefore to be processed by SAE.The efficacy of C18 cartridge was evaluated by HPLC analyses of inlet solution (C1), the liquid recovered from the initial adsorption flush down (C2) and the ethanolic solution from desorption (C3). From HPLC chromatogram of C2 there was no evidence of anthocyaninsleakage with washing solution, which means the correct binding of anthocyanins in C18 column. The quantity of the main anthocynins identified is shown in **Figure 2** for C1 and C3 solutions.

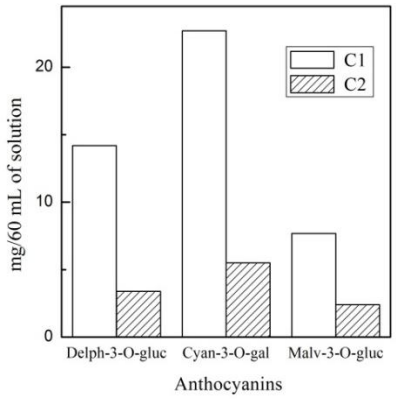

Figure 2. Anthocyanin content in bilberry solutions

From the **Figure 2**, it can be observed the lower recovery or desorption of anthocyanins from C18 cartridge, in fact the recovery was near to 50% of initial quantity. This procedure was repeated to obtain an enough quantity of bilberry anthocyanin solution for SAE experiments.
For each SAE experiment 30 mL of purified extract were used. As result from the SAE experiments two products were obtained, a powder inside the precipitator and the ethanolic solution in the separator vessel. The fractionation temperature was varied at 36, 40 and 45°C at a fixed pressure (100 bar) to study the effect over the selectivity. Then at fixed temperature (40°C) the pressure was changed at 80, 100 and 150 bar. In that way the CO_2 density was varied from 281 to 780 kg/m^3.Temperatures higher than 45°C are not recommended since the degradation of polyphenols takes place.
The powder obtained at 36°C 100 bar remained stuck in the wall of precipitator. The experiment carried out at 40°C 150 bar produced dried powder composed by spherical particles with size of around 200 nm (**Figure 3**). This result is typical of Supercritical Antisolvent technique (SAS), when full developed supercritical conditions are used to obtain nanometric particles[11].

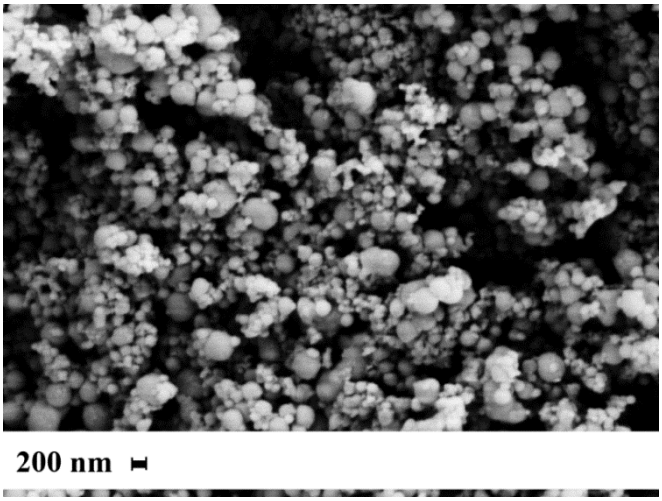

Figure 3. SEM photomicrograph of powder fractionated at 150 bar, 40°C

In **Table 2** the HPLC analyses of SAE experiments are reported and shows an increase in the concentration of anthocyanins in the powder product compared to the starting ethanolic extract for all operating conditions. Therefore the variation in CO_2 density allows maximizing the recovery of anthocyanins.

Table 2. Recovery of anthocyanins from SAE experiments

	SAE feed (ethanolicextract)	80 bar, 40°C	100 bar 45°C	100 bar, 40 °C	100 bar, 36°C	150 bar, 40 °C
CO_2density (kg/m^3)	-	282	496	622	710	780
% ofrecovery	-	65.7	47.2	56.8	68.3	69.5
Polyphenolic compounds(mg/kg)						
Delphinidin-3-O-glucoside	20.7	30.1	39.1	60.2	39.3	36.2
Cyanidin-3-O-galactoside+Delphinidin-3-O-arabinoside	33.5	52.7	66.9	85.9	61.6	71.5
Malvidin-3-O-glucoside	14.4	21.3	21.5	39.0	22.3	21.0

In SAE process the operating parameters cannot be considered separately. Temperature and pressure indicate the distance of the operating point from the mixture critical point, stating the

thermodynamic system at which the particle formation takes place and the presence of one or more phases in the precipitator. The density is responsible of the solubility of the compounds in ethanol + CO_2 solution during the precipitation. The higher concentration of anthocyanins was obtained operating at 40°C and 100 bar, corresponding at a density of 622 kg/m^3. This may be related to the fact that the solubility of compounds decreases with the temperature and the pressure, increasing the concentration of anthocyanins on the precipitated powder.
The antioxidant activity was expressed as the effect of suspension concentration over the inhibition of DPPH radical; was evaluated by IC_{50}. The IC_{50}value was less than 0.14 mg/mL and showedsimilar results for ethanol extract and for powder products (**Figure 4**). Therefore, SAE process does not affect the radical scavenging properties of product.

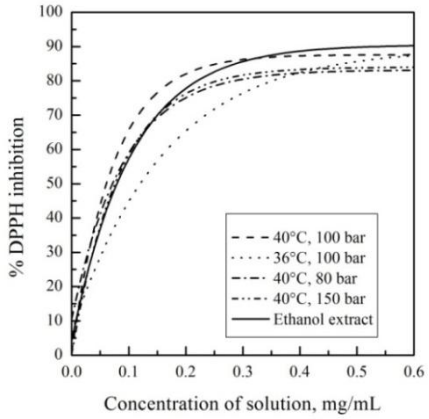

Figure 4. Radical Scavenging power of SAE product.

CONCLUSIONS

SAE process is promising to concentrate anthocyanins from berry by-products of juice factories, therefore it can be seen as a treatment aimed to improve the value of by-products. Sufficient methods for the removal of sugars without compromising anthocyanins prior to SAE requires further studies.

REFERENCES

[1] A. K. Lätti; K. R. Riihinen; P. S. Kainulainen, Journal of Agricultural and Food Chemistry, 56, **2007,**
[2] P. H. Canter; E. Ernst, Survey of Ophthalmology, 49, **2004,**
[3] K. Kaack; T. Austed, Plant Foods Hum Nutr, 52, **1998,**
[4] Y. Yao; A. Vieira, NeuroToxicology, 28, **2007,**
[5] A. Schieber; F. C. Stintzing; R. Carle, Trends in Food Science & Technology, 12, **2001,**
[6] N. Pap; E. Pongrácz; L. Myllykoski; R. Keiski In Waste minimization and utilization in the food industry: Processing of arctic berries, and extraction of valuable compounds from juice-processing by- products., Proceedings of the Waste Minimization and Resources Use Optimization Conference, Oulu, Finland, 2004; E. Pongrácz, Ed. University press: Oulu, Finland, 2004; pp 159-168.
[7] O. Laaksonen; M. Sandell; H. Kallio, Eur Food Res Technol, 231, **2010,**
[8] H. Wijngaard; M. B. Hossain; D. K. Rai; N. Brunton, Food Research International, 46, **2012,**
[9] T. Floris; G. Filippino; S. Scrugli; M. B. Pinna; F. Argiolas; A. Argiolas; M. Murru; E. Reverchon, The Journal of Supercritical Fluids, 54, **2010,**
[10] K. Thaipong; U. Boonprakob; K. Crosby; L. Cisneros-Zevallos; D. H. Byrne, J Food Compos Anal, 19, **2006,**
[11] E. Reverchon, The Journal of Supercritical Fluids, 15, **1999,**