Evaluation of processing effects on anthocyanin content and colour modifications of blueberry (*Vaccinium* spp.) extracts: Comparison between HPLC-DAD and CIELAB analyses

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ABSTRACT

Colour is the first organoleptic property that consumers appreciate of a foodstuff. In blueberry (Vaccinium spp.) fruits, the anthocyanins are the principal pigments determining the colour as well as many of the beneficial effects attributed to this functional food. Commercial blueberry-derived products represent important sources of these healthy molecules all year round. In this study, blueberries were produced into purees comparing two homogenization methods and further heated following different thermal treatments. All the supernatants of the homogenates were monitored for pH. Then, the hydroalcoholic extracts of the same samples were characterized by CIELAB and HPLC-DAD analyses. These analytical techniques provide complementary information on fruit pigments content as a whole and on quali-quantitative profile of the single bioactive colorants. These data could be very interesting to know the best manufacturing procedure to prepare blueberry-derived products, well accepted by the consumers, while maintaining their healthy properties unaltered.

Keywords: Blueberry, Vaccinium spp., Anthocyanins, CIELAB, HPLC-DAD

1. Introduction

The worldwide dietary guidelines recommend a daily consumption of plant foods rich in phytochemicals able to prevent the onset of chronic and degenerative diseases and maintain a state of wellbeing (Zhang et al., 2015). Among all the recommended plant foods, blueberries (Vaccinium spp.) raised considerable interest in view of the high content in bioactive molecules, in general, and anthocyanins, in particular (Cerletti et al., 2016; Fang, 2015; Zafra-Stone et al., 2007). These phenolic compounds, mainly located in the peel of the fruit, provide blueberries with their typical purple-blue colour and account for most of their beneficial qualities for health (Nile & Park, 2014; Zafra-Stone et al., 2007). The radical scavenger and the metal ions-chelating abilities of anthocyanins and, at the same time, their involvement in cell signalling pathways, gene expression, DNA repair, and cell adhesion processes, may explain the key role of these molecules as health-promoting agents. In fact, several observational, clinical and experimental studies have attributed to blueberry anthocyanins many different biological activities, including genoprotective, anticancer, cardioprotective, anti-inflammatory, antithrombotic, antihypertensive, lipidlowering, hypoglycemic, antiobesity, ocularprotective, neuroprotective and antimicrobial effects (Chu, Cheung, Lau, & Benzie, 2011). Like many other seasonal fruits, all year round availability of fresh Vaccinium berries is greatly limited, and for this reason people make frequent use of derivative products, such as jams, juices, fruit canned, and jellies. As consumption of these industrial products is driven not only by their palatability, but also by their health properties, preserving high levels of bioactive molecules is of fundamental importance (Howard, Prior, Liyanage, & Lay, 2012; Michalska & Łysiak, 2015). Focusing the attention on the anthocyanins, different stages of the manufacturing and distribution process of blueberry-derived foodstuffs, beginning from harvesting, continuing through all the postharvest operations, until the shelf-life of the end products, may be involved in the loss of these healthy molecules (Howard et al., 2012; Michalska & Łysiak, 2015). Therefore, in order to ensure high quality to blueberry-based prod ucts, adequate controls of the manufacturing process are required in order to obtain increased stability of anthocyanins. Although processing of most industrial and domestic forms of blueberry preserves generally involves crushing steps for the preparation of fruit purees, a limited number of studies have investigated the effect of different grinding methods on possible modifications of the organoleptic and nutritional properties of blueberries while processed into finished products. For this reason, in this work the effect of two different homogenization techniques on the anthocyanin content was studied by comparing a coarse blueberry puree prepared in a food mixer with a more finely crushed puree obtained with a high-speed Ultraturrax_ homogenizer. Moreover, with the purpose to provide insight into the effects that key stages of the manufacturing process of blueberry derived products may have on their final

anthocyanin content, beside homogenization, also different thermal procedures were investigated. In particular, a steam pre-crushing heating (85 °C for 3 min) was compared to dry post-crushing heatings carried out at different temperatures and for different times (70 °C for 15 s; 70 °C for 2 h; 40 °C for 1 h). More specifically, the precrushing blanching at 85 °C for 3 min as well as the postcrushing rapid pasteurization at 70 °C for 15 s were carried out in order to assess their capacity to preserve anthocyanins from enzymatic degradation, potentially operated by different enzymes, such as polyphenol oxidase, during fruit processing (Brambilla, Lo Scalzo, Bertolo, & Torreggiani, 2008; Del Bo' et al., 2012; Sablani et al., 2010; Skrede, Wrolstad, & Durst, 2000). Instead, the treatment at 70 °C for 2 h accounts for the processing scheme adopted in the industrial manufacturing of jams and jellies, where, during the phase of concentration in vacuum evaporators, the operating temperatures are first in the range 60–65 °C, and then raise above 80 °C for foam removal and hot filling of collection containers. This sort of prolonged pasteurization has the purpose of giving an insight into the thermal degradation effect on the anthocyanin content of blueberry purees (Patras, Brunton, O' Donnell, & Tiwari, 2010). Finally, the moderate heating at 40 °C for 1 h, simulates the enzymatic maceration step aimed to degrade the fibrous materials, such as pectin, hemicellulose and cellulose, often carried out during the industrial preparation of fruit juices. This procedure greatly increases the yield of juice from the pressed puree, but it may otherwise promote the activity of endogenous enzymes, such as polyphenol oxidase, which are likely to cause oxidative degradation of anthocyanins contained in the fruit (Skrede et al., 2000). Based on these considerations, extracts obtained from purees of fresh and ripe blueberries were used to monitor the impact of different processing techniques (mixing and heat treatment) on the anthocyanin content, colour changes and their interrelationship. For this purpose, HPLC-DAD and CIELAB analyses were carried out and compared.

2. Materials and methods

2.1. Materials

Ethanol (>96%), formic acid (>85%), and double-distilled water were purchased from Carlo Erba (Milan, Italy). Glacial acetic acid and ethyl acetate were obtained from Fluka (Milan, Italy). Acetonitrile RS for HPLC was purchased from Sigma-Aldrich (Milan, Italy). Blueberry fruits, labelled as coming from the Trentino region of Italy, were purchased in a local market. Commercial standards of malvidin-3-O-glucoside chloride (>95%), cyanidin-3-O-galactoside chloride (>97%), and delphinidin-3-O-glucoside chloride (>95%) were purchased from Extrasynthese (Lyon, France). Malvidin-3-Ogalactoside chloride (>95%) and delphinidin-3-O-galactoside chloride (>95%) were supplied by PhytoLab (Vestenbergsgreuth, Germany). Chlorogenic acid (>95%) was purchased from Sigma-Aldrich.

2.2. Sample preparation

Fresh and fully ripe blueberries were washed and carefully dried on paper towel at room temperature. According to the flow chart shown in Fig. 1, one half of them was subjected to a blanching process (steamed for 3 min at 85 °C), rapidly ice-cooled and then homogenized at room temperature for 2 min either by a domestic mixer at 16,000 rpm (blanched sample MB) or by a T18 Ultraturrax_ homogenizer (IKA_, Staufen, Germany) at 10,000 rpm (blanched sample UB). The other half of blueberries was first homogenized (domestic mixer or Ultraturrax homogenizer) for 2 min, and subsequently the resulting fruit puree was divided into four aliquots submitted to different heat treatments: (a) not treated (samples M and U); (b) treated at 70 °C for 15 s (rapidly pasteurized samples MRP and URP); (c) treated at 40 °C for 1 h (moderately heated sample MMH) and (d) treated at 70 °C for 2 h (prolonged pasteurized sample MPP). After each type of thermal treatment, the purees were rapidly ice-cooled, freeze-dried and stored at ‑18 °C until the extraction procedure.

2.3. Extraction of polyphenols

According to Li, Feng, Huang, and An (2013) with minor changes, an aliquot of freeze-dried sample (1.2 g) was extracted for 2 h, at room temperature and in the dark, under stirring with 50 mL of a hydroalcoholic acidified mixture composed of ethanol: 0.5% acetic acid (70:30, v/v). Then, the suspension was centrifuged at 12,000g for 10 min at 4 °C and the supernatant collected. The sample was concentrated under reduced pressure at 40 °C with a rotary evaporator and added with ethanol:0.5% acetic acid (70:30, v/v) to a final volume of 20 mL. The obtained solution was analyzed for anthocyanin content by HPLC-DAD and for colour parameters.

2.4. HPLC-DAD analysis

HPLC analysis of the hydroalcoholic filtered extracts was carried out by a Perkin-Elmer apparatus equipped with a series LC 200 pump, a series 200 diode array detector and a series 200 autosampler. Data acquisition and processing were carried out with a Perkin-Elmer Totalchrom software. The chromatographic separation was performed as previously described (Masci et al., 2016), with minor changes, using a LiChrosorb RP18 column (250 x 4.6 mm, i.d. 5 lm). The mobile phase consisted of acetonitrile (solvent A) and a 5% (v/v) formic acid water solution (solvent B) which in 40 min is changed from 5% A and 95% B to 20% A and 80% B, with a decreasing flow rate from 1 to 0.5 mL/min. The detection wavelength was selected at 520 nm for anthocyanins and 320 nm for chlorogenic acid. The injection volume of the blueberry extract was 20 μL. Peaks were identified on the basis of their ultraviolet‑visible spectra, co-chromatography respect to commercial standards, when available, and by comparison with elution order as reported in other published studies (Borges, Degeneve, Mullen, & Crozier, 2010; Brambilla et al., 2008). All the detectable anthocyanins identified in each sample, namely delphinidin-3-Ogalactoside (Del-3-gal), cyanidin-3-O-galactoside

(Cya-3-gal), delphinidin-3-O-arabinoside (Del-3-ara), petunidin-3-Ogalactoside (Pet-3-gal), cyanidin-3-O-arabinoside (Cya-3-ara), petunidin-3-O-arabinoside (Pet-3-ara), malvidin-3-O-galactoside (Mal-3-gal), malvidin-3-O-glucoside (Mal-3-glu) and malvidin-3- O-arabinoside (Mal-3-ara), were quantified by an external-matrix matched calibration method on the basis of the area ratios respect to the pure chemical standard Del-3-gal and reported as its equivalents (mg/100 g dry fruit). The total anthocyanin content was calculated as the sum of all the chromatographic peaks identified, with the exception of the glucoside derivatives of delphinidin and petunidin because their peaks cannot be integrated. The concentration of chlorogenic acid was determined by measuring detector response to the corresponding standard and expressed as mg/100 g dry fruit. Stock solutions of pure Del-3-gal and chlorogenic acid were prepared in the same solvent of samples, namely ethanol:0.5% acetic acid (70:30, v/v).

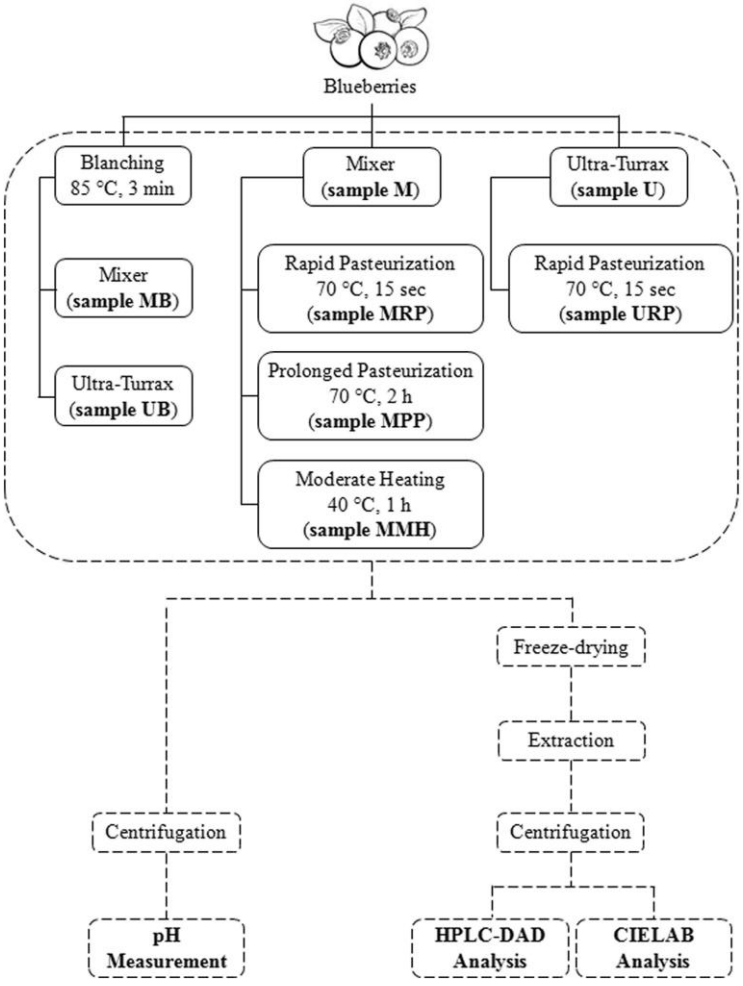

Fig. 1. Flow chart of both the processing steps applied to blueberry fruits and of the analyses performed on the different samples obtained

2.5. pH measurement

The blueberry puree was centrifuged at 12,000g for 10 min at 4 °C. The pH of the supernatant, once

returned to room temperature, was determined using a Metrohm E632 pH-meter (Metrohm Italiana S.r.l., Rome, Italy).

2.6. Colour analysis

CIELAB parameters (L*, a*, b*, C*ab and hab) were determined on the hydroalcoholic extracts using a colorimeter X-Rite SP-62 (XRite Europe GmbH, Regensdorf, Switzerland), equipped with a D65 illuminant and an observer angle of 10°. Colour description is based on three parameters: L_ that defines the lightness and varies between 0 (absolute black) and 100 (absolute white), a* that measures the greenness (_a*) or the redness (+a*) and b* that measures the blueness (_b*) and the yellowness (+b*). C* ab (chroma, saturation) expresses a measure of colour intensity and hab (hue, colour angle) is the attribute of appearance by which a colour is identified according to its resemblance to red, yellow, green, or blue, or a combination of two of these attributes in sequence. Cylindrical coordinates C*ab and hab are calculated from the parameters a* and b* using the equations $C^*_{ab} = (a^{*2} + b^{*2})^{1/2}$ and $h_{ab} = \tan^{-1}(b^*/a^*)$ (Clydesdale, 1978).

2.7. Statistical analysis

Each sample was prepared in duplicate and the experiments were repeated at least in triplicate. All the results are expressed as the mean value ± standard deviation (SD). Statistical comparison between groups was made using unpaired Student’ s t-test. P values ≤0.05 were regarded as significant. The correlation values between experimental data were evaluated by the Pearson coefficient.

3. Results and discussion

3.1. Experimental design

Fresh and ripe blueberry fruits, selected and cleaned, were homogenized following two different procedures in combination with several heat treatments, as reported in Fig. 1. The obtained homogenates were other centrifuged and the pH values of the supernatants measured or freeze-dried, extracted with a hydroalcoholic mixture, filtered and analyzed both for the chromatographic profile of their phenolic fraction by HPLC-DAD analysis and for colour by CIELAB analysis.

3.2. The pH values of blueberry purees

The pH values measured on supernatants obtained from the different blueberry purees are reported in Table 1. It can be observed that the pH ranged from 3.35 to 3.69, without significant differences between the two procedures of homogenization, when equal heat treatment is considered. The blanching treatment, carried out on the whole fruits before homogenization, has no significant effect on the pH value for MB (3.59) compared to M sample (3.49), although an upward trend was observed (3%). A slight increase (6%, $P \leq 0.05$) from 3.42 to 3.63 was also found for U vs UB samples. These results are in partial accordance with previous literature data (Del Bo’ et al., 2012), which reported

a mild increase by 4% measured in the pH value of blueberry purees produced with a food domestic mixer from blanched fruits, respect to non-heat-treated samples. Instead, the rapid pasteurization procedure carried out after the coarse (M series) or fine (U series) homogenization processes did not produce significant changes of the pH value, while the prolonged one tended to increase pH by about 6% (from 3.49 to 3.69 for M vs MPP, $P \leq 0.05$). Finally, any significant change of the pH value was observed, when thermal conditions promoting polyphenol oxidase activity were applied to blended fruits (from 3.49 to 3.41 for M vs MMH). In general, irrespective from the thermal or homogenization procedure applied, the pH of the fruit purees was always lower than 3.7, therefore it can be expected that the blue quinoidal forms of anthocyanins are predominant in all the samples produced, although a slight hypochromic effect in the absorbance intensity could be observed when the pH value changes from 3.35 to 3.69 (Castañeda-Ovando et al., 2009; West & Mauer, 2013).

3.3. Qualitative and quantitative HPLC-DAD analysis of polyphenols in blueberry extracts

The HPLC-DAD analysis carried out on all the filtered hydroalcoholic extracts, obtained from the differently homogenized and thermally treated blueberry samples, revealed a rich and specific content in anthocyanins. A typical chromatogram recorded at 520 nm is reported in Fig. 2. It can be observed that the more representative anthocyanins resulted the galactoside and the arabinoside derivatives of delphinidin, petunidin and malvidin aglycones (92% of total detected anthocyanins) followed by a smaller content of Cya-3-gal, Cya-3-ara and Mal-3-glu. The profile of the anthocyanic components of the blueberry extracts produced in the present study well overlaps, from a qualitative point of view, polyphenolic patterns already reported for similar Vaccinium corymbosum's extracts (Borges et al., 2010; Brambilla et al., 2008). Moreover, the chromatographic results obtained in this study were in agreement with previous NMR metabolic profiling studies for total anthocyanin composition of blueberry extracts (Capitani et al., 2014). After identification, the detectable anthocyanins were quantified on the basis of the area ratios of the chromatographic peaks respect to pure Del-3-gal, used as a standard, and the corresponding results are reported in Table 2. The total anthocyanin content found in the URP sample was about 5% higher in respect to the corresponding not pasteurized U sample (2740 vs 2602 mg/100 g dry fruit, P _ 0.05). Similar results were obtained with the samples crushed in the domestic blender, as total anthocyanins in MRP sample are about 6% higher compared to M (1527 vs 1435 mg/100 g dry fruit, P _ 0.05). These results are in agreement with the effect of pasteurization reported by Skrede et al. (2000) who observed a 4% higher content of anthocyanins in pasteurized blueberry juices compared to unpasteurized samples. Our data confirm their hypothesis that a rapid pasteurization treatment of a blueberry juice is able to inactivate enzymes, such as polyphenol oxidase, involved in the anthocyanin degradation, thus preserving these healthy molecules, while assuring prolonged shelf-life to the commercial product. On the

contrary, when the blueberry purees were thermal stressed at 70 C for 2 h, >25% loss of anthocyanins was observed (1039 vs 1435 mg/100 g dry fruit for MPP and Mrespectively, P 0.001). This result shows that, under prolonged pasteurization conditions, beside the inactivation of oxidative enzymes, thermal degradation of anthocyanins prevails over their preservation. Therefore, attention should be paid when prolonged thermal processing procedures, aiming to extend the shelf-life of a foodstuff, are applied to blueberries or other anthocyanin containing foods, because these treatments could compromise the nutritional properties of the manufactured products. Even the moderate heating of blueberry purees at 40 C for 1 h produced significant loss (about 10%, $P < 0.01$) of anthocyanins (from 1435 to 1293 mg/100 g dry fruit, for M vs MMH samples, respectively). It is likely that, in this case, the decrease in the total anthocyanin content is determined by the enzymatic degradation operated by polyphenol oxidase, placed in its optimal catalytic conditions. These thermal conditions are frequently applied in the food industry, during the enzymatic hydrolysis of the fibrous part of the fruit, aimed to promote the squeezing out of the juice. Therefore, it has to be considered that although this procedure may result in extraction of a higher volume of juice from the vegetable matrix considered, it should be counterproductive in terms of final concentration of bioactive molecules. In view of that, it should be opportune to find mechanical crushing procedures that facilitate the recovery of a high yield of juice, while preserving important micronutrients from enzymatic degradation. Interestingly, our findings suggest the utility of a high shear disperser respect to a blade mixer, for a fine breaking of plant tissues.

Table 1
pH and colour data of blueberry samples differently processed.

Sample	Heating	pH		Colour coordinates									
				L^*		a^*		b^*		C^*_{ab}		h_{ab}	
Mixer													
M	–	3.49 ± 0.07	a^{Δ}	26.18 ± 0.13		5.69 ± 0.64	$a^{\Delta}b^{\Delta}c^{\Delta}d^{\#}$	1.51 ± 0.17	$a^{\Delta}b^{\Delta}c^{\Delta}d^{\#}$	5.89 ± 0.66	$a^{\Delta}b^{\Delta}c^{\Delta}d^{\#}$	14.86 ± 0.05	$a^{\#}b^{\Delta}c^{\#}$
MRP	70 °C, 15 s	3.37 ± 0.05	$b^{\#}c^{\#}$	26.08 ± 0.56		5.30 ± 0.70	$e^{\Delta}f^{\Delta}$	1.40 ± 0.13	$e^{\Delta}f^{\Delta}g^{\#}$	5.48 ± 0.71	$e^{\Delta}f^{\Delta}$	14.85 ± 0.60	
MB	85 °C, 3 min	3.59 ± 0.06	$b^{\#}d^{\Delta}$	26.59 ± 0.28	a^{Δ}	4.31 ± 0.45	$a^{\Delta}g^{\Delta}$	1.09 ± 0.12	$a^{\Delta}e^{\Delta}h^{\Delta}$	4.45 ± 0.47	$a^{\Delta}g^{\Delta}$	14.19 ± 0.10	$a^{\#}d^{\#}e^{\#}$
MMH	40 °C, 1 h	3.41 ± 0.06	$d^{\Delta}e^{\#}$	26.12 ± 0.46		3.96 ± 0.58	b^{Δ}	1.11 ± 0.14	b^{Δ}	4.11 ± 0.60	b^{Δ}	15.69 ± 0.28	$b^{\Delta}d^{\#}$
MPP	70 °C, 2 h	3.69 ± 0.07	$a^{\Delta}c^{\#}e^{\#}$	26.07 ± 0.25		3.69 ± 0.42	$c^{\Delta}e^{\Delta}$	1.00 ± 0.10	$c^{\Delta}f^{\Delta}$	3.82 ± 0.43	$c^{\Delta}e^{\Delta}$	15.18 ± 0.18	$e^{\#}$
Ultraturrax													
U	–	3.42 ± 0.06	f^{Δ}	26.27 ± 0.63		2.82 ± 0.28	$d^{\#}$	0.62 ± 0.08	$d^{\#}$	2.89 ± 0.29	$d^{\#}$	12.38 ± 0.30	$c^{\#}f^{\#}g^{\#}$
URP	70 °C, 15 s	3.35 ± 0.06	$g^{\#}$	26.51 ± 0.16		3.06 ± 0.38	f^{Δ}	0.76 ± 0.07	$g^{\#}$	3.15 ± 0.38	f^{Δ}	13.98 ± 0.44	$f^{\#}$
UB	85 °C, 3 min	3.63 ± 0.07	$f^{\Delta}g^{\#}$	25.85 ± 0.35	a^{Δ}	2.71 ± 0.40	g^{Δ}	0.68 ± 0.09	h^{Δ}	2.79 ± 0.41	g^{Δ}	14.11 ± 0.24	$g^{\#}$

Values are expressed as mean ± SD ($n = 6$).
Values with the same letters are significantly different ($^{\Delta}$, $P < 0.05$; $^{\#}$, $P < 0.01$).

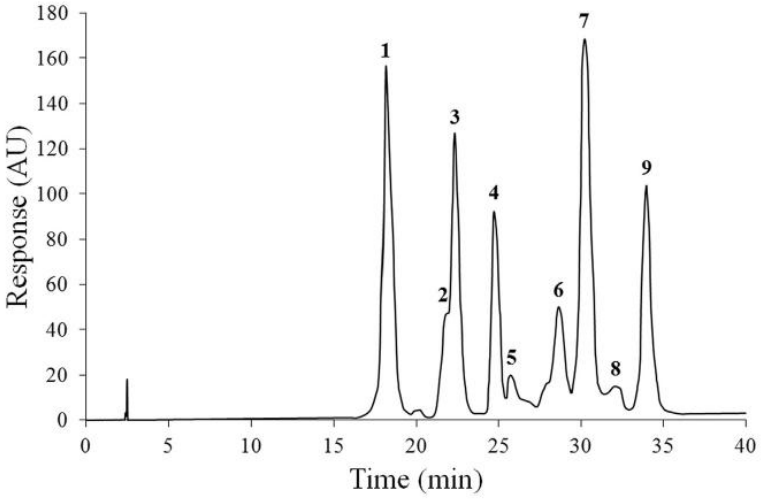

Fig. 2. Chromatographic pattern of the anthocyanins monitored at 520 nm in a hydroalcoholic extract of the analyzed blueberry samples. Peaks assignment: 1, delphinidin-3-*O*-galactoside; 2, cyanidin-3-*O*-galactoside; 3, delphinidin-3-*O*-arabinoside; 4, petunidin-3-*O*-galactoside; 5, cyanidin-3-*O*-arabinoside; 6, petunidin-3-*O*-arabinoside; 7, malvidin-3-*O*-galactoside; 8, malvidin-3-*O*-glucoside and 9, malvidin-3-*O*-arabinoside.

Table 2
Anthocyanins and chlorogenic acid content of blueberry samples differently processed.

Sample	Heating	Anthocyanins content										Chlorogenic acid (mg/100 g[C])	
		Del-3-gly (%)[A]		Cya-3-gly (%)[A]	Pet-3-gly (%)[A]		Mal-3-gly (%)[A]		Total (mg DGE[B]/100 g[C])				
Mixer													
M	–	28.79 ± 0.78	$a^{\#}b^{\#}$	5.81 ± 0.41	18.36 ± 0.34	a^{Δ}	47.05 ± 1.54	$a^{\#}b^{\#}$	1435 ± 8	$a^{\Delta}b^{\Delta}c^{\#}d^{\S}e^{\S}$	1.82 ± 0.06	$a^{\S}b^{\#}$
MRP	70 °C, 15 s	28.76 ± 1.62	$c^{\#}d^{\Delta}$	6.20 ± 0.22	17.90 ± 0.45	b^{Δ}	47.14 ± 1.12	$c^{\#}d^{\Delta}$	1527 ± 31	$a^{\Delta}f^{\#}g^{\S}h^{\S}i^{\S}$	1.83 ± 0.13	$c^{\#}d^{\Delta}$
MB	85 °C, 3 min	28.83 ± 0.47	$e^{\#}f^{\#}$	5.55 ± 0.76	19.83 ± 0.68	$a^{\Delta}b^{\Delta}c^{\Delta}$	45.80 ± 0.57	$e^{\#}f^{\Delta}$	1363 ± 30	$b^{\Delta}f^{\#}j^{\S}k^{\S}$	1.69 ± 0.07	$e^{\S}$
MMH	40 °C, 1 h	21.46 ± 1.57	$a^{\#}c^{\#}e^{\#}g^{\#}$	5.98 ± 0.32	17.67 ± 0.05	c^{Δ}	54.90 ± 1.20	$a^{\#}c^{\#}e^{\#}g^{\#}$	1293 ± 27	$c^{\#}g^{\S}l^{\S}$	1.62 ± 0.11	$f^{\#}$
MPP	70 °C, 2 h	28.01 ± 0.78	$g^{\#}$	5.98 ± 0.54	18.35 ± 0.65		47.67 ± 1.76	$g^{\#}$	1039 ± 23	$d^{\S}h^{\S}j^{\S}l^{\S}$	ND[D]	$a^{\S}c^{\#}e^{\S}f^{\#}$
Ultraturrax													
U	–	37.06 ± 1.27	$b^{\#}h^{\Delta}$	5.48 ± 0.97	18.31 ± 0.67		39.15 ± 0.37	$b^{\#}h^{\S}$	2602 ± 49	$e^{\S}m^{\Delta}n^{\#}$	2.23 ± 0.11	$b^{\#}g^{\#}$
URP	70 °C, 15 s	34.43 ± 2.22	d^{Δ}	5.81 ± 0.84	18.19 ± 0.50		41.57 ± 2.44	d^{Δ}	2740 ± 53	$i^{\S}m^{\Delta}o^{\#}$	2.23 ± 0.15	$d^{\Delta}h^{\Delta}$
UB	85 °C, 3 min	31.31 ± 0.17	$f^{\#}h^{\Delta}$	5.58 ± 0.64	18.92 ± 0.44		44.19 ± 0.17	$f^{\Delta}h^{\S}$	2039 ± 6	$k^{\S}n^{\#}o^{\#}$	1.74 ± 0.04	$g^{\#}h^{\Delta}$

Values are expressed as mean ± SD ($n = 6$).
Values with the same letters are significantly different ($^{\Delta}$, $P < 0.05$; $^{\#}$, $P < 0.01$; §, $P < 0.001$).
[A] Values represent the proportion of the anthocyanidin glycosides respect to the total anthocyanins.
[B] DGE = delphinidin-3-*O*-galactoside equivalents.
[C] Data refer to 100 g of dry fruit.
[D] ND = not detectable.

Irrespective from the pasteurization treatment, the anthocyanin content decreased by 45% when mixer instead of Ultraturrax_ was used for blending. In specific, an anthocyanin content of 2602 mg/100 g dry fruit was found in the U sample, whereas 1435 mg/100 g dry fruit was observed in the M sample ($P \leq 0.001$). In the same way, the differently homogenized samples subjected to rapid pasteurization process, resulted in anthocyanin content of 2740 and 1527 mg/100 g dry fruit for URP and MRP, respectively ($P \leq 0.001$). A 33% decrease in the anthocyanin content was also observed when mixer instead of Ultraturrax_ was applied to the blanched samples (1363 and 2039 mg/100 g dry fruit for MB and UB samples respectively). This important difference may be probably related to the different grinding capacity of the two homogenization systems considered (blade mixer vs high shear disperser). In particular, the Ultraturrax_ may enable a finer crushing of the cell-wall

material in the epidermal tissue of the fruits where anthocyanins are more concentrated (Skrede et al., 2000), thus assuring higher extraction yield of these pigments from the corresponding puree. It is also likely that the high shear disperser involves lower incorporation of free oxygen during the homogenization process with respect to the blade mixer and a consequent lower loss caused by the oxidative process. The blanching treatment applied to the whole fruit before preparation of the puree, was not effective in reducing the enzymatic degradation of anthocyanins, if compared to the rapid pasteurization carried out immediately after the homogenization. In fact, considering the set of the M samples, lower yield of anthocyanins was obtained from the blanched fruits than the corresponding non-heat-treated sample (5% decrease from 1435 to 1363 mg/100 g dry fruit for M and MB respectively, $P \leq 0.05$) or the rapidly pasteurized one (11% decrease from 1527 to 1363 mg/100 g dry fruit for MRP and MB respectively, $P \leq 0.01$). Also Del Bo' et al. (2012) observed that the blanching pretreatment of blueberries is not able to effectively preserve anthocyanins from degradation in phenolic extracts obtained from purees produced with a domestic mixer (corresponding to our MB and M samples). When the blanching treatment was associated with the Ultraturrax_ homogenization, the decrement of the anthocyanin content in the corresponding extracts becomes even more evident, if compared to the M, MRP and MB samples. In particular, the total anthocyanin content showed a 22% decrease ($P \leq 0.01$) for UB vs U (from 2602 to 2039 mg/100 g dry fruit), and a 26% decrease ($P \leq 0.01$) for UB vs URP (from 2740 to 2039 mg/100 g dry fruit). Overall, the blanching process seems to have a destabilizing effect on the anthocyanin pigments, especially in those extracts obtained from purees produced using the Ultraturrax_ homogenization system. To our knowledge, no previous reports have studied the effect of the blanching process in association with a high shear homogenization system and it is worth of further investigation. Our HPLC-DAD data about total anthocyanin content (expressed as Del-3-gal equivalents) in blueberry extracts obtained from not heat-treated M (1435 mg/100 g dry fruit) and U (2602 mg/100 g dry fruit) samples fall in the range of values reported by Müller, Schantz, and Richling (2012) for different cultivars of V. corymbosum (1570–2762 mg/100 g dry weight) extracted for 3 min with a solvent mixture of 87% acetonitrile/3% water/10% formic acid (v/ v/v). Lower values (681–972 mg/100 g dry weight) were observed by Giovanelli and Buratti (2009) from four varieties of cultivated blueberries (V. corymbosum) extracted for 1 h with 96% acetonitrile/ 4% acetic acid (v/v). It is likely that the acidified hydroalcoholic mixture (0.5% acetic acid aqueous solution/ethanol, 30/70, v/v) adopted in our study has a higher extraction capacity than an almost exclusively organic medium. When we considered the percentages of anthocyanins found in our blueberry extracts, we observed that the glycosides of the four predominant anthocyanidins showed a different profile according to the type of processing procedure applied to the sample (Table 2). Cyanidin and

petunidin derivatives were the most stable, whereas delphinidin and malvidin glycosides exhibited the greatest modifications. In particular, as already reported in literature (Brambilla et al., 2008; Del Bo' et al., 2012), the profile of delphinidin and malvidin derivatives was changed in the opposite manner as a function of the applied treatment. In fact, comparing M and MMH samples, a decrease from 29% to 21% ($P \leq 0.01$) against an increase from 47% to 55% ($P \leq 0.01$) was observed for delphinidin and malvidin glycosides, respectively. Furthermore, a downward (37% vs 34% vs 31%) against a rising (39% vs 42% vs 44%) trend resulted for delphinidin and malvidin derivatives in U, URP and UB samples, respectively. In addition, comparing M vs U, MRP vs URP and MB vs UB samples, delphinidin glycosides showed an increase (29% vs 37%, P _ 0.01; 29% vs 34%, P _ 0.05; 29% vs 31%, P _ 0.01) while malvidin glycosides were decreased (47% vs 39%, P _ 0.01; 47% vs 42%, P _ 0.05; 46% vs 44%, P _ 0.05) when the Ultraturrax_ was employed, instead of the domestic food mixer. These fluctuations of the anthocyanin profile have to be considered when the influence of the processing procedures on the nutritional properties of blueberry-derived foodstuffs are evaluated. In fact, literature data point out that malvidin derivatives show about 40% reduced antioxidant activity compared to delphinidin ones (Borges et al., 2010). Our study shows that the Ultraturrax_ treatment, if compared to the domestic food blender homogenization, not only promotes a more effective extraction of total anthocyanins, but also causes an increase in the ratio of Del-3-gly/Mal-3-gly, thus proving to be an advantageous crushing method of a vegetable matrix for the preservation of high antioxidant capacity. Considering the data relative to the extracts obtained from the blueberry purees prepared with the domestic food blender, no effects were observed in the anthocyanin profile following high temperature treatments (70 °C), irrespective from their duration (15 s or 2 h). On the contrary, the moderate heating of the blueberry purees evidenced an important change in the percentage ratio of delphinidin and malvidin derivatives respect to the total anthocyanins, as previously discussed. This result could indicate that the relative levels of delphinidin and malvidin glycosides may be regulated by enzymatic activities, which should remain longer active only in the MMH sample treated at 40 _C for 1 h. Taking into account the overall percentage profile either of galactoside or arabinoside derivatives of the different anthocyanidins, negligible changes can be appreciated among the different samples. For a comprehensive evaluation of the effect of various processing procedures on the recovery of bioactive compounds in blueberry-derived products, the chlorogenic acid, the most representative cinnamic acid derivative in blueberries, was also quantified and the results reported in Table 2. In fact, it has been reported that the antioxidant capacity of blueberry juices is better correlated (linear regression analysis coefficient, rxy = 0.97) with the sum of chlorogenic acid and the total glycosylated anthocyanins and flavonols (quercetins), than the content of total glycosylated anthocyanins alone (rxy = 0.75) (Brambilla et al.,

2008). Furthermore, the quinone form of chlorogenic acid takes part in the enzymatic oxidation of anthocyanins, drastically increasing the rate of pigments degradation (Kader, Rovel, Girardin, & Metche, 1997). Great variability exists in the content of chlorogenic acid in blueberries from different cultivars. Literature data report values of chlorogenic acid content ranging from 21 to 85 mg/100 mL in V. corymbosum pasteurized juices prepared pressing blended and depectinized purees obtained from steam-blanched fruits (Brambilla et al., 2008). Moreover, Rodriguez-Mateos, Cifuentes-Gomez, Tabatabaee, Lecras, and Spencer (2012) report a chlorogenic acid concentration variable from about 40 to 80 mg/100 g fresh weight in extracts prepared from freeze-dried powder of other six highbush blueberry varieties. In our blueberry extracts obtained from purees of fresh fruits, the concentration of chlorogenic acid ranged from undetectable values to 2.23 mg/100 g dry fruit. These values are obviously much lower than those reported by studies which analyzed extracts of frozen fruit powder such as 27.4 mg/100 g fresh weight determined by Skrede et al. (2000), or 30.09 and 40.51 mg/100 g fresh weight quantified by

Del Bo' et al. (2012) for unblanched and blanched samples, respectively. On the other hand, our findings are fully comparable to those reported by Borges et al. (2010) who analyzed an extract obtained using a methanol:formic acid (99:1, v/v) mixture on a puree produced homogenizing fresh blueberries in Ultraturrax for 1 min. Under the adopted conditions, these authors found a chlorogenic acid content of 8 nmol/g fresh weight that corresponds to 1.78 mg/100 g dry fruit, if a correction factor of 6.3 is applied, considering a juice yield of 84% (Müller et al., 2012; Skrede et al., 2000). This result can be explained considering the rapid degradation kinetic of chlorogenic acid evidenced by Kader et al. (1997) when fresh blueberries are homogenized. Comparing our URP vs U (2.23 vs 2.23 mg/100 g dry fruit) and the MRP vs M (1.83 vs 1.82 mg/100 g dry fruit) samples, no changes in the chlorogenic acid content were observed as a consequence of the rapid pasteurization procedure. This result differs from a previous observation (Skrede et al., 2000) showing a higher chlorogenic acid content in a blueberry pasteurized juice respect to the corresponding unpasteurized sample. The presence of chlorogenic acid was not detected in the extract obtained from MPP sample. The prolonged heating at high temperature probably caused a complete thermal degradation of the cinnamic acid derivative. The temperature and pH conditions of our MMH puree, optimal for polyphenol oxidase activity, associated to the sufficiently long time of incubation, may explain the partial decrease of chlorogenic acid content (11%, from 1.82 to 1.62 mg/100 g dry fruit, in M and MMH samples, respectively), although it is not statistically significant. Similarly to what observed for anthocyanins, the use of an Ultraturrax disperser instead of a domestic blender resulted in extracts containing higher amount of chlorogenic acid (2.23 vs 1.82 mg/100 g dry fruit for U vs M, P 0.01, and 2.23 vs 1.83 mg/100 g dry fruit for URP vs MRP, P 0.05). However, while all the U samples

showed around 44% higher anthocyanin content compared to the M ones, only a 22% increase was observed for chlorogenic acid. Considering the high solubility of chlorogenic acid and its localization in the berry pulp vacuoles, it is likely that it can be easily extracted even when a domestic blender is used. On the contrary, as the anthocyanins are localized in the epidermal tissue and associated with cell-wall materials, they can benefit from a high shear homogenization system which may facilitate their extraction, as a consequence of the finer puree produced. Comparably with the results observed for the total anthocyanin concentration, the quantification of chlorogenic acid revealed a lower content in extracts obtained from blanched samples (a decrease by about 7% from 1.82 to 1.69 mg/100 g dry fruit for M and MB respectively, P = 0.07; a decrease by about 22% from 2.23 to 1.74 mg/100 g dry fruit for U and UB respectively, P 0.01).

3.4. Colorimetric CIELAB analyses

It is well known that colour plays a crucial role in the acceptance of foods by consumers. Anthocyanins, besides their healthy properties, are the pigments responsible for the attractive colour of juices, or other derivatives obtained from berry fruits, such as blueberry. The tristimulus colorimetry was employed in this study to evaluate the colour properties of blueberry derivatives with the aim to better understand if heating and homogenizing treatments may have an impact on colour appearance and if a correlation exists among colorimetric parameters, anthocyanin content and qualitative pattern. In general, it is not very easy to give an interpretation to the CIELAB colorimetric analyses, both because only few data are available in literature about colorimetric studies on blueberries (Casati et al., 2012) or bilberries (Nour, Trandafir, & Cosmulescu, 2015; Pop, Lupea, Popa, & Gruescu, 2010) and also because the obtained colorimetric parameters are deeply influenced by several factors, including type of cultivar, pH, temperature, oxygen content, light exposition, metals and processing, such as clarification and/or pasteurization (Delgado-Vargas, Jiménez, & Paredes-López, 2000; Turfan, Türkyilmaz, Yemis, & Özkan, 2011). In addition, it is fundamental to consider that the illuminant and the observer angle have to be setted equal in order to make colorimetric data comparable. In this work a D65 illuminant, sunlight simulating, with an observer angle of 10was used. These conditions make our results only partially comparable with Casati et al. (2012) who carried out a shelf-life study on blueberry juices, under accelerated storage conditions, using the same illuminant, but an observer angle of 2. Moreover, even considering other anthocyanin-containing matrices, such as grape pomace, purple corn, black rice and maqui berries, only few data have been published investigating colour using a D65 illuminant, with an observer angle of 10 (Gironés-Vilaplana, Calín-Sánchez, Moreno, Carbonell-Barrachina, & García-Viguera, 2015; West & Mauer, 2013). Therefore, under the adopted operating conditions, this work represents a new and useful contribute for the analysis of colorimetric parameters of blueberry samples. In

particular, for the first time, a correlation among colorimetric parameters, anthocyanin content and composition of blueberries, has been attempted, with the final aim of demonstrating whether colour analysis may provide reliable information on the composition of blueberry-derived products. Colorimetric parameters of our blueberry puree extracts, obtained according to the different thermal and homogenizing treatments studied, are reported in Table 1. Quite low values of the L parameter (25.85–26.59) were measured for all the extracts, thus denoting very dark samples. Moreover, only slight variations were found among the samples and the only significant difference ($P < 0.05$) was observed between the MB (26.59) and UB (25.85) samples, which showed the highest and the lowest L values, respectively.

The positive a values (2.71–5.69, in the red region of the colorimetric space) and the positive, near to zero, b* values (0.62–1.51, in the yellow region of the colorimetric space) indicate the red-dark appearance of our blueberry extracts. The M series samples showed a and b, and consequently C ab values, higher than the ones of the corresponding U series samples. In specific, the following values were measured for the C ab parameter: 5.89 vs 2.89 for M and U (P 0.01); 5.48 vs 3.15 for MRP and URP (P 0.05); 4.45 vs 2.79 for MB and UB (P 0.05). Therefore, the use of an Ultraturrax homogenizer respect to a blade mixer, provides blueberry purees with lesser visual palatability because of the faded red colour of the corresponding samples, particularly pronounced in the thermally untreated ones. These findings suggest the importance of a careful selection of the crushing method adopted in the industrial manufacturing of blueberry-derived products, as it may deeply affect their appearance and the consequent acceptance by consumers. The hue angle, hab; values of all our samples fall in the range 12.38–15.69, thus indicating red–purple extracts with high content of anthocyanin pigments. In fact, it was reported in literature that partially ripe, less red cherries had higher hue angle values than the ripe ones, and this colour appearance was correlated to a lower anthocyanin content (Gonçalves et al., 2007). The blanching treatment applied to blueberries before shredding in the blade mixer results in reduction of all the colorimetric parameters, a, b, C ab and hab respect to the non-heat-treated sample. The fading of the red–purple colour may depend on the already discussed loss of total monomeric anthocyanins (Table 2), as well as on a browning phenomenon consequent to the heat treatment (Zorenc, Veberic, Stampar, Koron, & Mikulic-Petkovsek, 2017). The same effect was not observed when the blanching treatment was combined with the Ultraturrax homogenization system, as the hab value of the UB sample (14.11) showed a slight increase (14%, $P \leq 0.01$) with respect to the U sample (12.38). The variation of the extract composition in anthocyanins may account for the shift of the red–purple hue towards red. In fact, results reported in Table 2 showed decreased percentage of the red-blue delphinidin glycosides and increased percentage of the red–purple malvidin glycosides. Extracts obtained from blueberry

purees subjected to moderate or intense and prolonged heat treatment showed a decrease of a; b and, consequently, of C ab values, when compared to the unheated M sample. A proportional loss of total anthocyanin content, more important in MPP (thermal degradation) than in MMH (mainly enzymatic degradation) samples (Table 2), corresponds to the modification of the colour parameters. However, as already stated, it is likely, that also the browning process may play an important role in the colour changes of thermally stressed samples. Again, the decrease of the Del-3-gly/Mal-3-gly ratio may account for the increase in the hab value (6%, $P < 0.05$) observed for the MMH (15.69) compared to the M (14.86) samples.

The correlation data between changes of the colorimetric parameters and the content of anthocyanins, totally and individually considered, of all our samples, are reported in Table 3. The L values showed no correlation neither with the total anthocyanin content, nor with the percentage of the individual anthocyanidin glycosides. The high and substantially comparable darkness of all the blueberry extracts investigated, may be the consequence of such an enrichment in anthocyanins that makes L value almost insensitive to variations in concentration of these pigments (Liang et al., 2011).

Table 3
Factors of the Pearson correlation matrix.

	L^*	a^*	b^*	C^*_{ab}	h_{ab}	Del-3-gly	Cya-3-gly	Pet-3-gly	Mal-3-gly	Del-3-gly/Mal-3-gly	Total anthocyanins
L^*	1.00										
a^*	0.03	1.00									
b^*	−0.04	0.99***	1.00								
C^*_{ab}	0.03	0.99***	0.99***	1.00							
h_{ab}	−0.30	0.53	0.65	0.54	1.00						
Del-3-gly	0.26	−0.48	−0.59	−0.48	−0.91**	1.00					
Cya-3-gly	−0.30	0.54	0.62	0.54	0.75*	−0.57	1.00				
Pet-3-gly	0.38	−0.11	−0.17	−0.11	−0.30	0.21	−0.67	1.00			
Mal-3-gly	−0.30	0.46	0.58	0.47	0.91**	−0.99***	0.61	−0.31	1.00		
Del-3-gly/Mal-3-gly	0.28	−0.52	−0.64	−0.53	−0.94***	0.99***	−0.60	0.19	−0.98***	1.00	
Total anthocyanins	0.26	−0.61	−0.69	−0.62	−0.79*	0.83*	−0.48	−0.06	−0.79*	0.86**	1.00

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
***Correlation is significant at the 0.001 level (2-tailed).

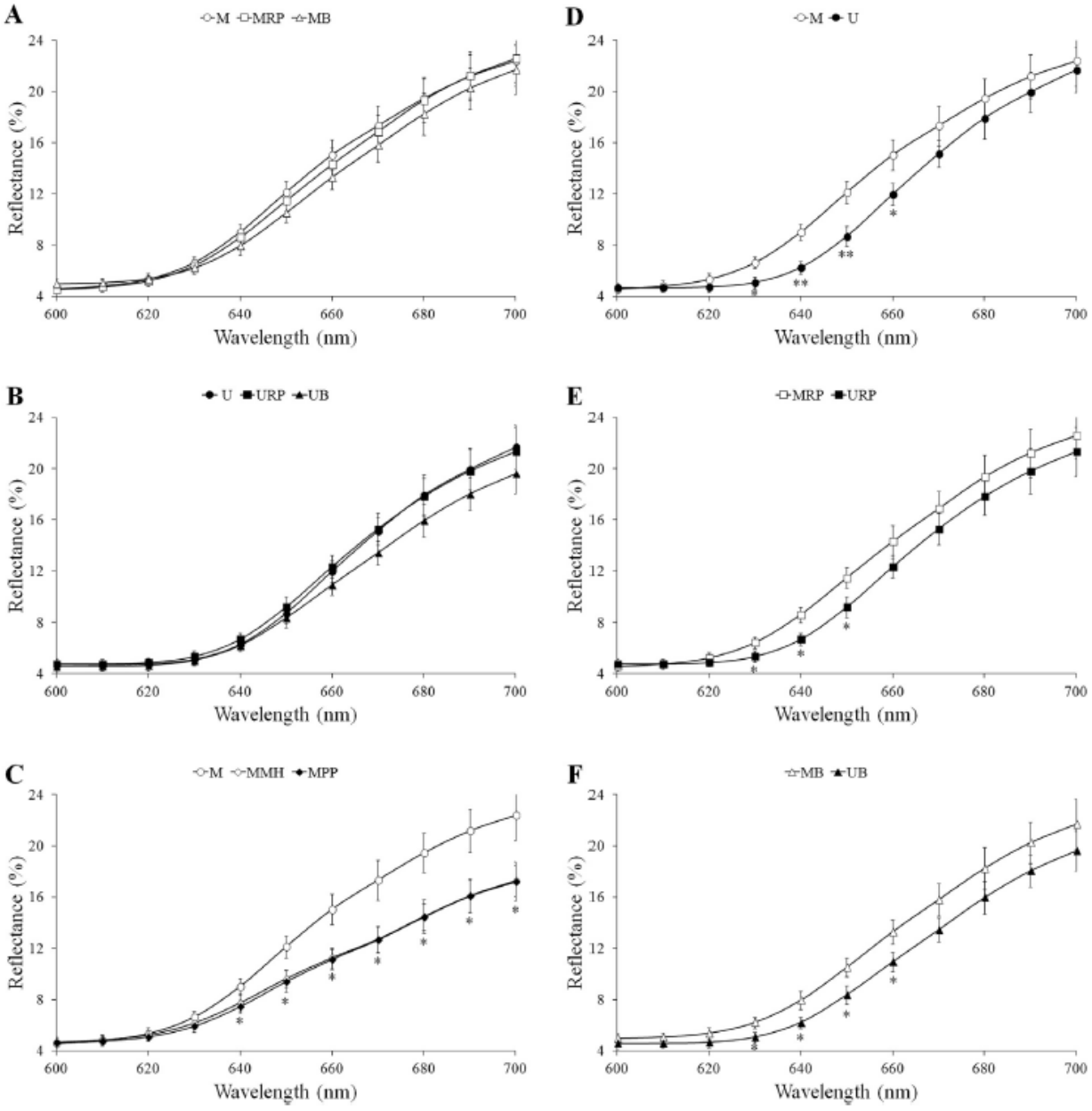

Fig. 3. Spectral reflectance curves obtained by CIELAB colour analysis applied to extracts of different processed blueberry purees. Compared samples: (A) MB *vs* M and MRP; (B) UB *vs* U and URP; (C) MMH and MPP *vs* M; (D) U *vs* M; (E) URP *vs* MRP; (F) UB *vs* MB. Date are presented as mean ± SD ($n = 6$). The significant differences between the compared samples are indicated ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$). Samples identified in Fig. 1.

The a and b values correlated positively with each other and, as expected, based on the mathematical relationship that binds them, with Cab. The hab values correlated negatively with the total anthocyanin content of the blueberry extracts. This finding disagrees with the data of Liang et al. (2011) concerning grape skin, but are in agreement with Gonçalves et al. (2007) relative to whole cherries. This result can be explained as a natural consequence of the ability of red–purple coloured anthocyanins, such as those predominant in blueberry (malvidin, cyanidin and petunidin glycosides), to confer a colour nuance, red–purple precisely, to the extracts of the corresponding fruit purees proportional to their concentrations. Therefore, to an increased content in anthocyanins corresponds

a decrease in the hab parameter. Very important correlations were found between the hab parameter and the changes of the individual anthocyanidin glycoside levels. More specifically, a positive correlation was observed with Mal-3-gly (q = 0.91) and Cya-3-gly (q = 0.75), whereas hab negatively correlated with Del-3-gly (q = –0.91). These results can be explained on the basis of the content of the red–purple coloured Mal-3-gly and Cya-3-gly, and the red-blue coloured Del-3-gly. In fact, while Mal-3-gly and Cya-3-gly determine an increase of the hab value, Del-3-gly tends to decrease it. In addition, since a high negative correlation (q = –0.99) exists between the delphinidin and malvidin glycosides percentages, the negative correlation existing between the Del-3-gly/Mal-3-gly ratio and the hab values is even greater (q = –0.94) than the variations of Del-3-gly and Mal-3-gly separately considered. To an increment in total anthocyanin content correspond an increase of the percentage of the delphinidin glycosides (q = 0.83) and a decrease of the malvidin glycosides (q = –0.79), in our blueberry extracts. To account for the browning effects that may affect the heat treated samples analyzed in this study, the comparison of the reflectance curves in the range of wavelength 600–700 nm is reported in Fig. 3. These data were analyzed considering that the slope of the reflectance curve decreases with increasing content of the polymeric forms of the anthocyanins. According to this consideration, the overlap observed among the M series (M, MRP and MB) as well as the U series (U, URP and UB) samples (Fig. 3A and B), denotes a weak influence of the rapid pasteurization or the blanching treatment on the browning effect. Instead, prolonged thermal stresses, moderate in the MMH sample or high in the MPP sample, caused a great decrement of the slope of the corresponding reflectance curves respect to the non-heat-treated M sample (Fig. 3C). These results may be ascribed to the enzymatic and nonenzymatic (thermal) degradation of the anthocyanins for the MMH and MPP samples, respectively, and their consequent polymerization in brown pigments (Kader et al., 1997; Patras et al., 2010). Finally, comparing U vs M, URP vs MRP and UB vs MB samples (Fig. 3D–F), a decrease in the slope of the reflectance curves can be observed in the U series samples, which indicates the important difference in the total monomeric anthocyanins content between each pair of samples considered (higher in U than in M series samples).

4. Conclusions

In this study the effect of different thermal treatments and homogenization systems were evaluated. To our knowledge no previous studies are reported about the effects of blanching process in association with a high sheer homogenization system. Our results show that Ultraturrax promotes not only a more effective extraction of total anthocyanin content respect to classic blending, but also increases the Del-3-gly/Mal-3-gly ratio demonstrating to be a better crushing method for the preservation of a higher antioxidant capacity. Here a new and useful contribute for the evaluation of

colour of blueberry samples is also reported. For the first time a correlation has been shown among colour parameters, total anthocyanin content and qualitative pattern. The hab values negatively correlates with the total anthocyanin and delphinidin glycosides content. On the contrary a highly positive correlation exists between hab parameter and malvidin glycosides content, therefore an even greater correlation exists between hab and Del-3-gly/Mal-3-gly ratio (q = –0.94). Tristimulus colorimetry represents a rapid, simple and economic analytical method, as it can be directly applied on purees or unclarified/unfiltered fruit juice samples. HPLC-DAD data and CIELAB colour analyses are able to give interesting and sometimes complementary information. The overall findings suggest that attention should be paid when blanching procedure or prolonged thermal treatments, aiming to extend the foodstuff shelf-life, are applied in the manufacturing of blueberry jellies or juices, because these treatments could seriously affect the nutritional properties of the final product. The maximum preservation of anthocyanins is reached when a rapid pasteurization treatment is associated with a high speed homogenization. It is likely that these observations may be extended to other anthocyanin rich fruits.

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