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Myrtle hydroalcoholic extracts obtained from different selections of *Myrtus communis* L.

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Abstract

Hydroalcoholic extracts obtained from myrtle berries of five different selections were studied to obtain unique clones. During the typical 40 day maceration period, dry matter, pH, colour, and anthocyanins, were analyzed. Variability among myrtle selections was observed both in fresh berries (yield berries/plant, weight of the berries, number and weight of seeds per berry) and in the hydroalcoholic extract physical–chemical characteristics (dry matter, colour intensity, tint and anthocyanins). Reversed-phase high performance liquid chromatography (RP-HPLC) analysis with photodiode array detection (PDA) was used for anthocyanins characterization in myrtle hydroalcoholic extracts. Anthocyanin qualitative composition was the same in all selections, but varied strongly in its total amount. D was the selection with the characteristics most suitable for industrial use.

Keywords: *Myrtus communis* L. selection; RP-HPLC-PAD analysis; Anthocyanins

1. Introduction

Myrtle (*Myrtus communis* L.) liqueur is a Sardinian typical spirit, obtained from myrtle berries, and it is characterized by an initial red-purple colour, along with the peculiar plant flavour. In Sardinia the use of myrtle berries to make a liqueur dates back to past centuries. Recently, it has met a remarkable success, crossing the boundaries of Sardinia with a production of more than three million bottles per year. Organic acids, free anthocyanins, hydrolyzed flavonoids, volatile compounds, anions and cations have been extensively investigated in myrtle liqueur (AA.VV., 1998; Alamanni & Cossu, 2004; Mulas, Spano, Biscaro, & Parpinello, 2000). Until now, the majority of studies on myrtle have focussed on the composition of volatile compounds in leaves (Asllani, 2000; Boelens & Jimenez, 1991; Boelens & Jimenez, 1992; Bradesi, Tomi, Casanova, Costa, & Bernardini, 1997; Chalchat, Garry, & Michet, 1998; Jerkovic, Radonic, & Borcic, 2002; Koukos, Papadopoulou, Papagiannopoulos, & Patiaka, 2001; Mazza, 1983; Ozek, Demirci, & Baser, 2000; Pirisino, Mulè, Moretti, & Satta, 1996; Tateo & Picci, 1982; Tuberoso, Barra, Angioni, Sarritzu, & Pirisi, 2006; Vanhaelen & Vanhaelen-Fastrè, 1980) and of the phenolic compounds in leaves and berries (Diaz & Abeger, 1987a, 1987b, 1987c; Martin, Rubio, Villaescusa, Fernandez, & Diaz, 1999; Martin, Villaescusa, De Sotto, Lucia, & Diaz, 1990; Martin, Villaescusa, Diaz, Ollivier, & Delmas, 1997; Montoro et al., in press; Romani et al., 2004; Romani, Pinelli, Mulinacci, Vincieri, & Tattini, 1999; Rosa et al., 2003). Only one paper dealing with the evolution of volatile compounds during maceration was found (Tuberoso et al., 2006). No data can be found on the influence of the type of myrtle berries and of the maceration period on the liqueur composition.

Some years ago, a selection of wild myrtle plants was micro-propagated to find the clones most productive and most suitable for industrial processing. Using myrtle berries obtained from the most interesting selection, a trial

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to evaluate which one could give a final product with the best characteristics, was carried out on laboratory scale. The attention has been concentrated mainly on the compounds that can influence colour and antioxidant properties. The trial was carried out on the hydroalcoholic extracts, as the final liqueur is obtained simply by dilution with syrup (water and sugar).

2. Material and methods

2.1. Myrtle samples

Myrtle berry samples were collected from an experimental field located at Uta (Cagliari, Italy), owned by CRAS (Centro Regionale Agrario Sperimentale della Sardegna, Sardinian Regional Agricultural Experimental Centre). Harvest was carried out in December 2003, at the industrial ripeness, when berries are fully dark-violet pigmented. Three years plants of five different selections, with a planting distance of 2×3 m, were obtained by micropropagation of wild plants collected in south-west Sardinian sites. Two kilogrammes of berries from each selection, were collected using a random-block design with four replications. The berries were frozen, stored at −18 °C, and used after two months. Table 1 shows the main characteristics of the berries.

The hydroalcoholic extracts were prepared according to the industrial process: 140 g of berries plus 330 ml of ethanol 95% (v/v) and 50 ml of water, were stored for 40 days in dark bottles fitted with special caps that allowed 5 ml to be taken for sampling of the hydroalcoholic extract without opening the bottle. Each experiment was triplicated. During the 40 days of maceration, five samplings at 7, 14, 21, 28 and 40 days were performed. The analytical parameters studied were: for berries, yield berries/plant, fresh and dry weight, number and weight of seeds; for the hydroalcoholic extract, dry matter, pH, chromatic indices and anthocyanins.

2.2. Analytical procedures

All samples were filtered through 0.45 μm cellulose acetate septa VariSep (Supercrom, Milan, Italy) before the analyses. Dry matter content was assessed by drying 1 ml of the hydroalcoholic extract for at least 2 h in a thermostatic oven at 105 ± 1 °C and weighing after it reached a constant temperature. Dry weight was determined in the same way, but using 10 g of berry sample. pH measurements were carried out with an Orion 420 pH meter (Orion Research Inc. Beverly, MA, USA). The measure of the optical density at 420, 520 and 620 nm were performed with a Cary 50 spectrophotometer (Varian, Milan, Italy), using quartz cells of 0.5 cm path length. Intensity and tint were determined, respectively, as sum of the optical densities at 420, 520 and 620 nm and as relationship between the optical density at 420 and 520 nm (Ribéreau-Gayon, Glories, & Maujean, 2000). Free anthocyanins were determined by HPLC, a Waters LC fitted with a multisolvent delivery system 600 and column heater set at 35 °C, an autosampler 717 plus a 50 μl loop and a PDA detector 996, were employed (Waters S.p.A., Vimodrone, Milan, Italy). A Symmetry C18 (250×4.6 mm, 5 μm, Waters) column was used. The solvents used were H_3PO_4 0.2 M (A) and 80% CH_3CN + 20% H_3PO_4 0.2 M (B), using a linear

Table 2
Evolution of dry matter (g/l), pH, colour intensity ($E^{1\,cm}_{420} + E^{1\,cm}_{520} + E^{1\,cm}_{620}$), and tint ($E^{1\,cm}_{420}/E^{1\,cm}_{520}$) during the maceration

Selection	Maceration (days)				
	7	14	21	28	40
Dry matter (g/l)					
A	19.3[a]	20.7[a]	21.1[a]	20.1[a]	20.3[a]
B	25.2[b]	26.3[b]	25.6[b]	24.4[b]	24.5[b]
C	13.8[c]	15.3[c]	14.9[c]	14.3[c]	14.6[c]
D	30.6[d]	32.1[d]	32.5[d]	31.4[d]	31.2[d]
E	15.8[e]	17.1[e]	17.6[e]	17.0[e]	17.4[e]
pH					
A	5.38[a]	5.38[a]	5.45[a]	5.56[ab]	5.46[a]
B	5.48[b]	5.39[a]	5.39[ab]	5.55[a]	5.57[b]
C	5.37[a]	5.35[a]	5.32[b]	5.46[c]	5.51[ab]
D	5.73[c]	5.58[b]	5.57[c]	5.65[d]	5.66[c]
E	5.61[d]	5.55[b]	5.46[a]	5.62[bd]	5.66[c]
Colour intensity ($E^{1\,cm}_{420} + E^{1\,cm}_{520} + E^{1\,cm}_{620}$)					
A	3.364[ab]	4.754[a]	4.902[a]	5.352[a]	5.326[a]
B	3.416[b]	5.348[b]	5.510[b]	5.824[b]	5.802[b]
C	3.328[a]	4.782[a]	5.056[c]	5.426[a]	5.376[a]
D	6.210[c]	8.402[c]	8.300[d]	8.330[c]	8.194[c]
E	3.900[d]	5.256[d]	5.630[e]	6.070[b]	6.042[d]
Tint ($E^{1\,cm}_{420}/E^{1\,cm}_{520}$)					
A	2.269[a]	2.314[a]	2.557[a]	2.980[a]	3.432[a]
B	1.974[b]	1.980[b]	2.285[b]	2.610[b]	2.775[b]
C	2.349[c]	2.449[c]	2.678[c]	2.798[c]	3.132[c]
D	1.435[d]	1.458[d]	1.604[d]	1.834[d]	2.161[d]
E	3.138[e]	3.689[e]	3.889[e]	4.129[e]	4.656[e]

Values within a column for each selection having different letters are significantly different from each other at $p < 0.05$.

Table 1
Characteristics of myrtle sample berries

Selection	Yield (kg berries/plant)	Average weight of berry (g)	Berry dry weight (%, w/w)	Seeds weight (%, w/w)	Number of seeds/berry
A	2.76[a]	0.41[a]	30.5[a]	19.5[a]	8[a]
B	3.26[b]	0.41[a]	31.5[b]	19.5[a]	7[a]
C	2.93[c]	0.30[b]	33.1[c]	23.4[b]	16[b]
D	3.91[d]	0.47[c]	31.3[b]	13.2[c]	5[c]
E	2.49[e]	0.19[d]	34.9[d]	23.6[b]	4[c]

Values within a column for each selection having different letters are significantly different from each other at $p < 0.05$.

gradient as follows: $t = 0$ A:B (90:10; v/v), reaching 80:20 (v/v) in 15 min, then 60:40 (v/v) in 10 min, and finally 30:70 (v/v) in 20 min. Before each injection, the LC system was stabilized for 15 min with the initial A/B ratio (90:10; v/v). The flow rate was 1 ml/min. The anthocyanin analysis was performed at $\lambda = 520$ nm, according to their maxima of absorbance. Chromatograms and spectra were elaborated with a Millenium[32] Waters data system.

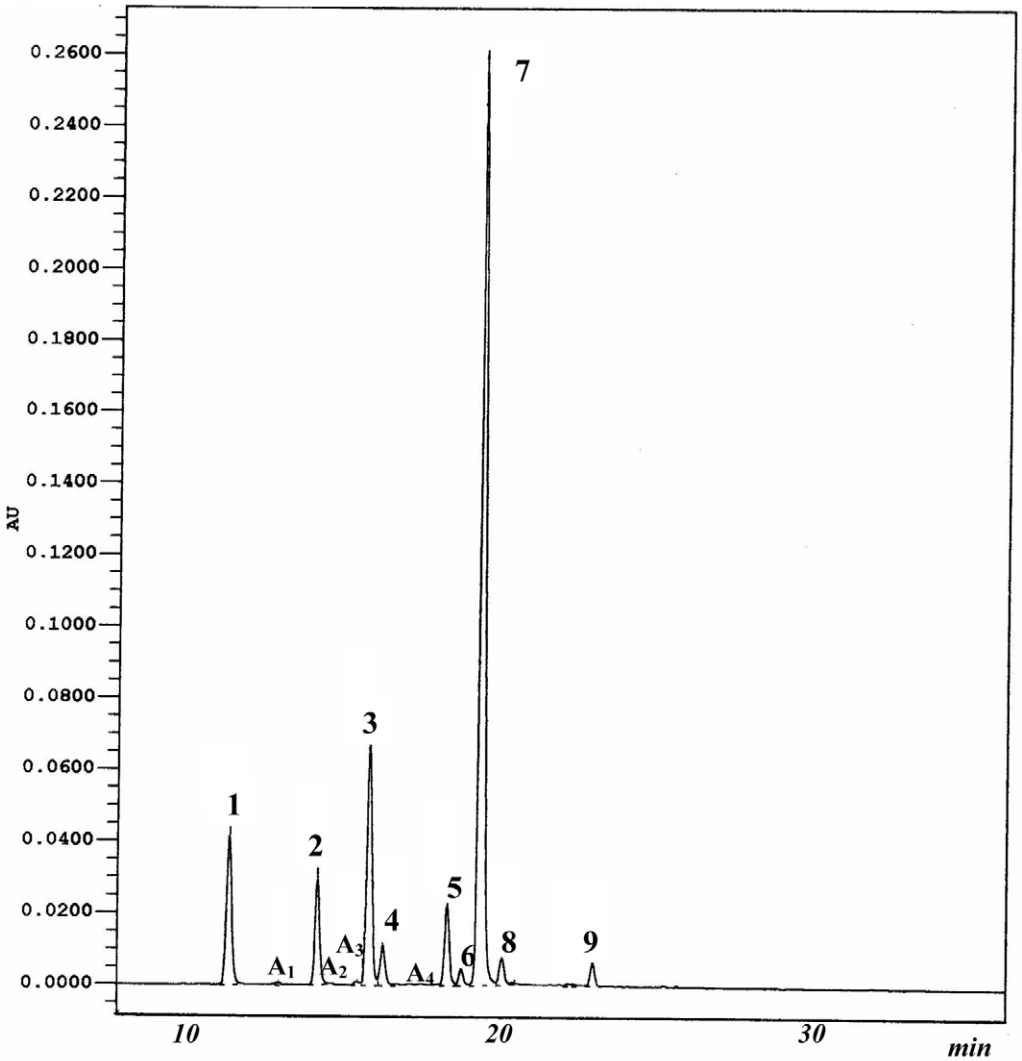
2.3. Chemicals

Standards of malvidin, cyanidin, delphinidin, peonidin, petunidin, malvidin-3-glucoside, cyanidin-3-glucoside, peonidin-3-glucoside, malvidin-3,5-diglucoside and cyanidin-3,5-diglucoside, were purchased from Extrasynthese (Genay, France). Stock standard solutions of the anthocyanins (~500 mg/l each) were prepared in methanol. Working standard solutions were prepared daily by diluting aliquots of stock solution with the initial mobile phase (A/B, 90:10).

Phosphoric acid (85%), methanol and acetonitrile were of HPLC grade (Merck, Milan, Italy). HPLC water was distilled and filtered trough a Milli-Q apparatus (Millipore, Milan, Italy) before use. Ethanol (95%) (v/v) was for food use.

2.4. Statistical analysis

Analysis of variance (ANOVA) was carried out and the average values were compared with Duncan's test at



1) delphinidin-3-O-glucoside 2) cyanidin-3-O-glucoside 3) petunidin-3-O-glucoside 4) delphinidin-3-O-arabinoside 5) peonidin-3-O-glucoside 6) cyanidin-3-O-arabinoside 7) malvidin-3-O-glucoside 8) petunidin-3-O-arabinoside 9) malvidin-3-O-arabinoside
Peaks from A_1 to A_4 are unknown supposed to be anthocyanins.

Fig. 1. HPLC separation of myrtle anthocyanins under the operating conditions described in the text.

$p \leqslant 0.05$ using GenStat v 7.1 software (VSN International Ltd., Herts, UK).

3. Results and discussion

Table 1 shows that selection D was the clone with the highest berries production, almost 4 kg/plant. This sample had the best berry characteristics: highest average fresh weight (0.47 g) and low seeds amount (13.2%, w/w), both as total weight and number of seeds/berry (five). Selection E showed the highest value for the dry weight, but this was attributable to the high seeds weight amount (23.6%, w/w of the fresh berry). Selection C showed the highest amount of seeds (sixteen), almost double or triple those of the other selections, very small and with a weight (23.4% w/w) similar to selection E.

Table 2 shows dry matter variation during maceration and it gives clues about the extraction process yield from the berries. All samples reached the maximum level of extraction of dry matter between the second and the third week of extraction. Strong differences depending on the selection, were noticed: for instance, after 21 days, selection D had the highest value (32.5 g/l), about twice higher than selection C and E (14.9, and 17.6 g/l, respectively).

pH is strictly connected with the organoleptic aspects of the liqueur, such as sour taste and colour. pH affects the molecular structure of anthocyanins, modifying the colour of the solution (Ribéreau-Gayon et al., 2000), and allowing anthocyanins to form chemical bonds with other compounds in the extract, forming tannins-related compounds with tawny colour. Table 2 shows pH values during maceration. Selection D showed the highest value with 5.73, while selection A and C showed the lowest, almost 5.37. As is shown in Table 2, pH values underwent small changes within each selection, during maceration.

Colour intensity (measure of the colour amount) showed, after seven days of maceration similar values for selections A, B, C, while selections D and E were statistically different (Table 2). Selection D showed the highest value, 6.210. During the maceration process, the values increased for all samples with the same ratio and the differences among the selections were maintained. Tint gives an indication of the type of colour, low values denote a

Table 3
Anthocyanins (mg/l) during hydroalcoholic maceration

Time of maceration (days)	D-3glu	A1	C-3glu	A3	Pt-3glu	D-3ara	A5	Po-3glu	C-3ara	M-3glu	Pt-3ara	M-3ara	Total
Selection A													
7	46.0^{a}	1.1^{a}	16.3^{a}	0.3^{a}	46.5^{a}	10.3^{a}	0.2^{a}	11.7^{ab}	2.1^{a}	140^{ab}	4.4^{a}	3.0^{a}	282^{a}
14	43.3^{a}	0.8^{b}	16.8^{a}	0.3^{a}	46.6^{a}	9.7^{a}	0.2^{a}	12.1^{a}	2.1^{ab}	144^{b}	4.4^{a}	3.0^{a}	283^{a}
21	37.2^{b}	0.6^{c}	16.0^{a}	0.3^{a}	43.4^{a}	8.2^{b}	0.2^{a}	11.7^{ab}	1.9^{abc}	140^{ab}	4.2^{b}	3.1^{a}	267^{a}
28	27.6^{c}	0.3^{d}	13.7^{b}	0.2^{ab}	35.7^{b}	6.3^{c}	0.1^{b}	10.2^{b}	1.7^{bc}	123^{a}	3.7^{c}	2.5^{b}	226^{b}
40	23.9^{c}	0.2^{d}	13.7^{b}	n.d.	34.9^{b}	5.5^{c}	n.d.	10.4^{b}	1.7^{c}	130^{a}	3.1^{d}	2.5^{b}	225^{b}
Selection B													
7	59.5^{a}	1.5^{a}	28.4^{a}	0.8^{a}	63.6^{a}	13.2^{a}	0.3^{a}	16.4^{a}	4.2^{a}	155^{a}	5.7^{a}	3.3^{a}	352^{a}
14	53.1^{a}	1.1^{b}	28.1^{a}	0.8^{a}	61.4^{a}	12.1^{a}	0.3^{a}	16.5^{a}	4.1^{a}	155^{a}	5.5^{a}	3.3^{a}	342^{a}
21	41.2^{b}	0.6^{c}	26.2^{b}	0.7^{b}	54.7^{b}	9.4^{b}	0.3^{ab}	15.7^{b}	3.8^{b}	148^{b}	5.0^{b}	3.1^{a}	309^{b}
28	28.5^{c}	0.3^{d}	22.3^{c}	0.5^{b}	44.6^{c}	6.8^{c}	0.2^{b}	13.6^{c}	3.2^{c}	129^{c}	3.9^{c}	2.5^{b}	255^{c}
40	21.9^{c}	0.2^{e}	20.9^{d}	0.4^{c}	40.2^{d}	5.4^{d}	0.1^{c}	13.1^{c}	3.1^{c}	127^{c}	3.7^{c}	2.5^{b}	239^{c}
Selection C													
7	43.0^{a}	1.8^{a}	22.1^{a}	1.3^{a}	43.0^{a}	7.2^{a}	0.3^{a}	10.4^{a}	2.3^{a}	91.2^{a}	2.5^{a}	1.1^{a}	226^{a}
14	40.0^{a}	1.4^{b}	22.3^{a}	1.3^{a}	42.4^{a}	6.6^{b}	0.3^{a}	10.6^{a}	2.4^{a}	92.9^{a}	2.4^{a}	1.1^{a}	224^{a}
21	32.4^{b}	0.9^{b}	20.5^{a}	1.1^{a}	38.6^{b}	5.5^{b}	0.2^{b}	10.0^{a}	2.1^{a}	88.5^{a}	2.2^{a}	1.0^{a}	203^{b}
28	23.9^{c}	0.5^{c}	17.5^{b}	0.9^{b}	31.8^{c}	3.9^{c}	0.2^{b}	8.5^{b}	1.8^{b}	76.5^{b}	1.7^{b}	0.8^{ab}	168^{c}
40	20.5^{c}	0.1^{d}	16.4^{b}	0.7^{c}	29.9^{c}	3.4^{d}	0.1^{bc}	8.0^{c}	1.7^{c}	75.5^{c}	1.6^{c}	0.8^{b}	159^{c}
Selection D													
7	153^{a}	2.8^{a}	41.3^{a}	0.6^{ab}	130^{ab}	27.0^{a}	0.4^{a}	21.6^{a}	4.6^{a}	276^{ab}	8.5^{a}	3.9^{a}	669^{ab}
14	152^{a}	2.2^{b}	43.8^{b}	0.7^{a}	136^{b}	27.1^{a}	0.5^{b}	23.3^{b}	4.8^{a}	296^{c}	8.7^{a}	4.3^{b}	699^{a}
21	135^{b}	1.5^{c}	41.9^{ab}	0.6^{b}	127^{a}	24.1^{b}	0.3^{c}	22.4^{a}	4.6^{a}	286^{ac}	8.2^{a}	4.1^{ab}	656^{b}
28	108^{c}	1.1^{d}	37.6^{c}	0.6^{b}	112^{c}	19.7^{c}	0.3^{c}	20.2^{c}	4.1^{b}	264^{bd}	7.1^{b}	3.6^{c}	578^{c}
40	86.3^{d}	0.6^{e}	34.8^{d}	0.3^{c}	101^{d}	16.3^{d}	0.3^{c}	19.4^{c}	3.9^{c}	258^{d}	6.9^{b}	3.5^{c}	532^{d}
Selection E													
7	32.7^{a}	0.5^{a}	18.9^{a}	0.3^{a}	28.7^{a}	6.0^{a}	0.1^{a}	9.5^{a}	2.3^{a}	66.5^{a}	1.8^{a}	1.2^{a}	169^{a}
14	30.9^{a}	0.4^{b}	18.9^{a}	0.3^{a}	28.4^{a}	5.7^{a}	0.2^{b}	9.6^{a}	2.2^{a}	67.3^{a}	1.8^{a}	1.2^{a}	167^{a}
21	28.3^{a}	0.2^{c}	18.1^{ab}	0.2^{a}	27.2^{a}	5.2^{a}	0.2^{ab}	9.5^{a}	2.2^{ab}	66.0^{a}	1.8^{a}	1.2^{a}	160^{a}
28	18.6^{b}	0.1^{cd}	13.4^{c}	0.1^{b}	19.7^{b}	3.4^{b}	0.2^{b}	7.0^{b}	1.5^{c}	49.9^{b}	1.2^{b}	0.9^{b}	116^{b}
40	19.0^{b}	0.1^{d}	15.1^{bc}	0.1^{b}	22.1^{b}	3.6^{b}	0.3^{c}	8.0^{b}	1.8^{b}	58.4^{ab}	1.5^{c}	1.1^{a}	131^{c}

Values within a column for each compound having different letters are significantly different from each other at $p < 0.05$; n.d. not detected.

prevalence of red tone, while higher values show a prevalence of yellow tone. Selection D showed the lowest values (1.435) and a red-violet tonality. Selection A, C, and E showed the highest values and their colour had a tendency to a dark-red tonality. During the maceration period, tint values increased and the colour gradually changed to tawny for all samples. This behaviour was more evident for selections E, A, and C, respectively, while selection D and B with a values of 2.161, and 2.775, respectively, preserved the best initial characteristics.

The HPLC profile of the anthocyanins fraction was the same for all five samples, with the presence of the 3-*O*-glucosidic form of delphinidin, cyanidin, petunidin, peonidin and malvidin, as the most important compounds (Fig. 1). The UV–Vis spectra, indicated that the other eight compounds could be considered as anthocyanins. Recent studies (Montoro et al., in press) proved that four of these compounds were arabinosides of delphinidin, cyanidin, petunidin, and malvidin; for the other four compounds (numbered from A1 to A4), not enough information is available. Anthocyanin arabinosides and unknown compounds represented 7.1%, and 8.2%, respectively, of the total anthocyanins after seven days, and 5.3%, and 6.5%, respectively, after 40 days (Table 3). Differences due to selection type were observed for total anthocyanins and single anthocyanins. Compound A2 was not reported in Table 3 as it quickly disappeared in the hydroalcoholic extracts and only traces can be detected in the first sampling. Total anthocyanin behaviour, during maceration did not correlate with dry matter content; it reached its highest values after 7 days and progressively decreased with a loss of 20–30% in 40 days. Selection D was the richest in total anthocyanins (669 mg/l, after 7 days) while selection E showed the lowest amount (169 mg/l, almost 25% of D); selections A and C had similar values, while selection B was intermediate between D and E selections. Malvidin-3-glucoside was the most important compound in all selections, followed by delphinidin-3-glucoside, petunidin-3-glucoside with similar mean values, and cyanidine-3-glucoside. Relative percentage of single anthocyanins changed during maceration time because each anthocyanin showed a different stability to aging: delphinidin-3-glucoside, and petunidin-3-glucoside and unknown anthocyanins (A1–A4) decreased; cyanidin-3-glucoside, peonidin-3-glucoside and anthocyanin arabinosides increased, while malvidin-3-glucoside was the most stable and, after 40 days, showed values comparable to the first sampling. Selections with higher dry matter amount were also those with the higher free anthocyanins percentages.

The data reported in this paper show that selection D had the choice characteristics as a clone for industry purposes: it produced the best berries (high yield of berries/plant and weight of the fresh berries, and low number and weights of seeds per berry) and the extracts, had higher amounts of dry matter, higher chromatic indices and total anthocyanins. Samples with high amounts of anthocyanins, due to the antioxidant activity of such compounds, can give liqueur more protected from the oxidation processes and so with a longer shelf-life.

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