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Some aspects of the quantitative/qualitative assessment of commercial anthocyanin-rich extracts

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Abstract

Colour assessment of anthocyanin-rich extracts according to the legal CE normative involves some important limitations: Lack of objectivity in the assessment of samples with different moisture contents and/or absorption maximums, and inability to identify possible fraudulence due to copigmentation phenomena. To overcome these limitations the following corrections were introduced: Colour intensity $CI = CI_{\lambda max}$, referred to 1 g dry matter; when $Abs_{780} > 0.001$, $CI = CI_{\lambda max} - CI_{780}$, followed by determination of total anthocyanins (TA) by any method using a known standard reference. Pigment qualities were best described by the use of spectral colorimetric assessment. Best objectivity was achieved by the use of the homogeneous CIELAB colour space. Separation of anthocyanins as monomer, and red and yellow–brown polymer fractions by open column chromatography gave important information about the structure/quality relationships of the anthocyanin pigments. HPLC analysis contributed to the identification of the extracts with high acylated anthocyanin contents, related to high physicochemical stability. Determination of total dry matter and total soluble and suspended solids gave a good indication of the purity of the anthocyanin pigments and the possible haze contamination of the coloured product, whereas determination of total catechins and condensed tannins were indicative of possible taste alterations. The main drawback, limiting the use of most of the examined qualitative parameters, was the lack of normalized reference data for anthocyanin-rich extracts.
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1. Introduction

Anthocyanins (E-163) are among the most widespread naturally occurring food colorants. They are obtained by extraction with sulphurous and/or acidulated water, carbon dioxide, methanol or ethanol from different edible fruits and vegetables and can contain other compounds, proper for the original material, such as organic acids, tannins, carbohydrates and minerals in proportion not necessarily the same as the original source (EC Directive 95/45/EC). Anthocyanin-rich extracts are available in liquid concentrate or dried powder forms from a wide range of natural materials: Grape skins, currants, elderberries, cranberries, bilberries, roselle, maize, cabbage, and carrots. The most abundant and cheapest are anthocyanins from grape pomace (enocyanin or enocianina). Their main application is in low pH beverages (syrups, soft and alcohol drinks), foods (jams, jellies, confectionery, sweet dressings, dairy products, powder mixes, water ice, and bakery topics) pharmaceuticals (Henry, 1996; Jackman & Smith, 1996) and red wines for colour-reinforcing in the countries where this manipulation is not restricted.

Chemically, anthocyanin extracts are an intricate mixture of glycosylated (Gl) polyhydroxy and polymethoxy flavilium salts, acylated (Ac) by *p*-coumaric, ferulic, sinapic, caffeic or other organic acids, where pelargonidin (Pl), peonidin (Pn), petunidin (Pt), delphinidin (Dp), cyanidin (Cy) and malvidin (Mv) are the most frequently encountered aglycones (Brouillard, 1982). Their red colour is a net result of all monomer, oligomer and polymer anthocyanins, together with their copigmented forms and intensity and hue depend on factors such as the nature and the concentration of the

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individual anthocyanins and their degree of degradation, temperature, pH, nature of the solvent, presence of SO_2, oxigen, enzymes, copigments, sugars, etc (Boulton, 2001; Brouillard, 1982; Haslam, 1998; Henry, 1996; Jackman & Smith, 1996; Osawa, 1982; Mazza & Miniati, 1993).

Colour assessment of anthocyanin extracts, according to the legal requirements in Spain (Real Decree No. 2107/1996) comprises only the measurement of the colour intensity (CI) of 1% extract solution (pH 3.0) in a 1 cm glass cell, at wave length (λ) of 520 nm (CI_{520}), whereas the European Community (EC) Directive 95/45/EC specifies values for λ between 515 and 535 nm and defines the absorbtivity value of the pure pigment solution ($E^{1\%}_{1\,cm}$) as 300.

Nowadays, it is known that extracts from different plant materials are different in their composition and present different colouring properties and stabilities. Thus, extracts rich in Pl and/or Cy glycosides exhibit more orange–red colours, whereas extracts rich in Pn glycosides are deep red and those containing Dp, Pt and/or Mv glycosides are bluish-red (Henry, 1996; Jackman & Smith, 1996). On the other hand, acylated anthocyanins have higher chemical stability than their nonacylated analogues (Bridle & Timberlake, 1997; Yoshida, Kondo, & Goto, 1991); also, polymer anthocyanin are more stable than their monomers (Francis, 1993; Gao, Girard, Mazza, & Reynolds, 1997). Furthermore, addition of even small amounts of some flavonoids, organic acids, alkaloids, aminoacids, polysaccharides or metal ions, known as copigments, to anthocyanin-containing solutions can cause an important colour enhancement (hyperchromic effect) (Jackman & Smith, 1996; Mazza & Miniati, 1993; Osawa, 1982). The presence of some copigments, such as flavonols in anthocyanin extracts, also, may improve their chemical stability (Malien-Aubert, Dangles, & Josèphe Amiot, 2002). Contrary to this, high concentrations of catechins and/or proanthocyanidins can impart unwanted bitterness and/or astringency to the final product, whereas high concentrations of proteins and/or polysaccharides can provoke haze formation. High concentrations of catechins are also, related to low colour stability of anthocyanin drink models (Malien-Aubert, Dangles, & Josèphe Amiot, 2001).

Taking in account these considerations, it is clear that defining quality aspects between different anthocyanin extracts and especially, their protection from adulteration with other, usually cheaper pigments and/or copigments, meets the need for seeking new analytical alternatives which can give additional information about the authenticity, the virtues and the possible limitations (defects) of each extract.

Bibliographic data showed that, total anthocyanins (TA) are determined most frequently by the method based on the bleaching effect of SO_2 (Ribéreau-Gayon, 1982) and the "pH differentiation" method (Ribéreau-Gayon & Stonestreet, 1965). Both methods cause a certain deviation from the real results: The first overestimates the anthocyanin content due to the lower sensitivity of the polymerised anthocyanin forms to the bleaching effect of SO_2 and the second underestimates it because of the incomplete conversion of the copigmented anthocyanin forms to free monomer anthocyanins at pH 1 (Boulton, 2001).

In red wines, colour strength is measured by the Sudraud's ($Abs_{420} + Abs_{520}$) (Sudraud, 1958) or the OIVs ($Abs_{420} + Abs_{520} + Abs_{620}$) (OIV, 1978) colour density (CD) indices. However, both indices have limited application in quantitative assessment of anthocyanin extracts because they over-emphasise the yellow–brownish red components, which are not the usual tonalities for anthocyanin applications. For grape skin extracts, Knuthsen (1987) measured the colour strength at pH 1.0, assuming that, at this pH, the anthocyanins are basically in their monomeric forms and that their maximal $E^{1\%}_{1\,cm}$ is 500. Using the same assumption, Henry (1996) calculated that liquid concentrate and solid powder grape skin extracts, with 600 and 1200 absorbance units/g ($E^{1\%}_{1\,cm}$ 6 and 12), had respectively, 2% and 4% anthocyanins.

High performance liquid chromatography (HPLC) has been used, as well, for quantification of anthocyanins in grapes, wines, fruits, juices, and flowers. Nevertheless, this technique separates and quantifies only the individual monomer and oligomer anthocyanin forms which makes it more feasible in the analysis of fresh fruits and juices, where anthocyanin polymerisation is not yet initiated (Bakker, Preston, & Timberlake, 1986).

Quality characterization of anthocyanin extracts is based mainly on measurements of their visible spectra. An important parameter derived from them is the colour tonality which defines the proportion of the red-brownish to the red colours ($CT = Abs_{420}/Abs_{520}$) (Glories, 1984). Other spectral derivatives, such as the Brown (Abs_{430}/Abs_{520}) and the Violet (Abs_{620}/Abs_{520}) indices are used with less extension. The precision and the objectivity of the colour characterization of some colorants (Casiraghi, Lucisano, & Pompei, 1984; Maga & Kim, 1990; Malien-Aubert et al., 2001), model anthocyanin and wine solutions (Bakker, Picinelli, & Bridle, 1993; Heredia, Francia-Aricha, Rivas-Gonzalo, Vicario, & Santos-Buelga, 1998), fruit juices (Cheynier, Rigaud, Souquet, Duprat, & Moutounet, 1990; Hong & Wrolstad, 1990a; MacDougall, 2000; Skrede, 1985) and wines (Garijo, Salinas, Pardo, & Alonso, 1997; Gómez-Cordovés & González-San, 1995; Heredia & Guzmán, 1995; Huerta, Salinas, Masoud, & Alonso, 1998; Negueruela, Echávarri, Ayala, & Lomas, 1995) was improved thanks to the introduction of several colour meter systems: CIEXYZ, CIELUV and CIELAB.

Table 1
Technical specifications of solid (SS1–SS4) and liquid (LS1–LS4) commercial anthocyanin-rich extracts

Sample	Density (g/ml)	CI_{520} (−)	CD (−)	Moisture (%)	pH (−)	Total SO_2 (ppm)	Source	Comments
SS1	–	12.0 ± 1.0	–	<7	–	–	Elderberry (*Sambus nigra* L.)	Free flowing powder, drying carrier – maltodextrin
SS2	0.35	12.0 ± 0.5	–	–	–	–	Grape skins (*V. vinifera* L.)	Free flowing powder, obtained by drying liquid extract
SS3	–	8.0 ± 0.4	–	<5	–	–	Grape skins (*V. vinifera* L., *V. labrusca* L. and *V. riparia* L.)	Free flowing powder, drying carrier – maltodextrin
SS4	–	12.0 ± 0.5	24.0 ± 2.0	<8	<3.5	<2500	Grape skins	Free flowing powder, more than three years old
LS1	1.17 ± 0.05	3.0 ± 0.1	6.0 ± 0.3	–	<3.5	<1000	Grape skins	Concentrate liquid
LS2	1.17 ± 0.05	3.0 ± 0.1	6.0 ± 0.3	–	<3.5	<1000	Grape skins	Concentrate liquid
LS3	Sample obtained by concentration of young Spanish red wine						*V. vinifera* L.	Concentrate liquid
LS4	No technical description available						Grape pomace	Concentrate liquid

CI_{520} is the absorbance of 1% extract solution (pH 3.0) in a 1 cm glass cell, at 520 nm.
CD, colour density: $Abs_{430} + Abs_{520} + Abs_{580}$ of 1% solution, 1 cm glass cell, pH 3.0.

To estimate the proportion of degraded to non-degraded anthocyanins in fruit juices, Fuleki and Francis (1968) introduced the degradation index, which is calculated from the Abs_{520} of one anthocyanin solution at pH 1.0 and 4.5. Nevertheless, the use of this index is not free of errors due to the incomplete release of the co-pigmented forms at pH 1 (Boulton, 2001). Contrary to this, fractionation of anthocyanins into monomers and red and brown polymers on silica gels has been found to contribute to the quality assessment of wines (Díaz-Plaza, Reyero, Pardo, & Salinas, 2002) and anthocyanin extracts (Knuthsen, 1987).

Identification of individual anthocyanins by paper, thin layer and liquid chromatography has been successfully used in the authenticity determination of grapes, fruit juices and colorants (Hale, Francis, & Fagerson, 1986; Hong & Wrolstad, 1990a, 1990b; Knuthsen, 1987; Singleton, Draper, & Rossi, 1966).

The aim of this work was to estimate the adequacy and efficacy of some analytical parameters, such as the spectrophotometric indices, CI_{520} and CT, CIELAB colour space, total and individual anthocyanins, their fractionation on total monomers and red and yellow–brown polymers, total catechins, total condensed tannins and some physical characteristics, such as total soluble solids, total suspended solids and moisture, in the quantitative, qualitative and authenticity assessment of commercial anthocyanin-rich extracts.

2. Materials and methods

2.1. Anthocyanin samples

Commercial anthocyanin-rich extracts (E-163), one from elderberries (solid free flowing powder), and six from grape skins (three solids and three liquid concentrates), were purchased from several European companies: Chr. Hansen (Spain), Expafruit (Spain), Exquim (Spain), Materias Primas Alimenticias (Spain) and Avialsa (Spain). The samples were stored at 4 °C in darkness. Individual technical specifications for all studied samples are described in Table 1. Data about their heavy metal contents (ppm) were available only for samples SS1 from elderberry and SS3 from grape skins and their cell count for samples SS1 and SS4 (yeasts $<10^2$ and total cell count $<10^3$ cells/g). The ash content of SS3 sample was also specified (<9%).

Sample SS4 was more than three years old, kept in a well sealed pot and refrigerated all of time. This sample was selected as a reference for old (deteriorated) sample. For sample LS4 we had only the producer's qualification that it was "crude extract" and "not very good quality". SS1 sample was from elderberries and was collected as a reference for anthocyanin extract different from the grape skin extracts. This sample was specified as a powerful anthocyanin pigment and its price was several times higher than those of enocyanins. Finally, we introduced a sample obtained by concentration of a young common, unfiltered Spanish red wine (LS3) in a laboratory rotary evaporator (20 °C) until it reached a density similar to those of the commercial samples, $D = 1.17$ g/ml.

2.2. Analytical methods

Moisture (M) was determined by drying the samples in a drying oven at 105 °C to constant mass ($M = 100 - \mathrm{DM}$ (%), where DM is the dry matter) (the liquid samples were first kept at 70 °C until dryness and after that the temperature was brought to 105 °C for their drying to constant mass. Total soluble solids

(TSoS) were measured by weighing the dry matter, obtained after evaporation of an aliquot of 1% aqueous extract solution, previously filtered on a 0.22 μm cellulose membrane filter. Total suspended solids (TSpS) were calculated as a difference: TSpS(%) = DM(%)−TSoS (%).

Spectroscopic analyses were carried out on a Lambda 20 UV/VIS spectrophotometer (Perkin–Elmer, Norwalk, USA) and data were recorded by Perkin–Elmer's Spectroscopy Software (version 3.20). Samples were prepared according to Real Decree No. 2107/1996 and the technical specifications provided by the producers: One gram (1 ml) of each solid (liquid) sample was dissolved in sodium citrate/citric acid buffer solution, pH 3.0, and made up to 100 ml in a volumetric flask with the same buffer. Ten ml of these solutions were centrifuged on a laboratory centrifuge at 4000 rpm for 10 min in order to remove possible haze. Colour intensity (CI_{520}) index was taken from the visible spectra as Abs_{520}. Colour density (CD) and colour tonality (CT) were calculated, respectively, as $Abs_{420} + Abs_{20} + Abs_{620}$ and Abs_{420}/Abs_{520} (Glories, 1984). Tristimulus parameters (a^*, b^*, L^*, C^*, H^* and S^*) were calculated using "Colour of Wines-2001" Perkin–Elmer's software (Perkin–Elmer Hispania, Madrid, Spain) based on CIELAB (CIE, 1976) colour space with reference standard observer of 10° visual field and reference to a standard illuminator D65 at $\Delta\lambda$ of 5 nm.

Total anthocyanins (TA) were determined according to the Ribéreau-Gayon (1982) method by decolorization with sodium hydrogen sulphite and fractionation into monomers and red and yellow–brown polymers were carried out by open column chromatography (OOC) according to Ribéreau-Gayon, Pontallier, & Glories (1982). Total catechins (TC) were determined by the method of Swain & Hillis (1959) and total condensed tannins (TCT) by the method of Ribéreau-Gayon & Stonestreet (1966). TA and separations of anthocyanins into monomer (MAF), red (RPAF) and yellow–brown (YBPAF) fractions, TC and TCT were carried out on a 1 g/100 ml sample solution (sodium citrate/citric acid buffer (pH 3.0)) for the solid samples and 5 ml/100 ml sample solution of the same buffer for the liquid samples.

Individual anthocyanin analysis was carried out as follows: One gram (ml) of each anthocyanin extract was dissolved in distilled water and adjusted to 100 ml final volume. Purification of the solutions was performed according to Rosillo, Alonso, Garijo, & Salinas (1998) by Sep-Pak C-18 cartridges. The purified samples were filtered on cellulose acetate membrane filters (0.45 μm pore size) and analysed by HPLC. Chromatographic conditions were as described by Johnston & Morris (1997). Peaks were identified by retention time and by comparison of their spectral characteristics with standards and bibliographic data (Hebrero, Santos-Buelga, & Rivas-Gonzalo, 1988; Santos-Buelga, 1997). Quantification was carried out using malvidin-3-glucoside (Mv-3-Gl) chloride (Extrasynthese, Genay, France) as external standard.

In order to avoid misleading results from the different moisture contents of the samples, the results were recalculated on a dry weight base using as factors of conversion, $F_m = 1/(M_s - M_w)$ for the solid samples and $F_m = 1/(V_s D - M_w)$, for the liquid samples (M_s is the sample mass (g), M_w is the sample moisture (g), V_s is the liquid sample volume (ml) and D is the sample density (g/ml)).

2.3. Statistical analysis

Data were subjected to normalized analysis of variance, ANOVA (SPSS statistic packet for Windows, version 10.0) using Duncan's test for estimation of significant differences between samples with a probability of 99.5% (SPSS Inc. Chicago, Illinois, USA).

3. Results and discussion

3.1. Quantitative analysis

Results for the colour strength of 1% solid and liquid anthocyanin extract solutions, obtained according to the legal requirements in Spain (sodium citrate/citric acid buffer, pH 3.0, 1 mm quartz cell) (Real Decree No. 2107/1996), are shown in Table 2. Numbers in brackets show the sample rating in decreasing order of richness. Samples SS2 and SS1 had the highest colour intensity (CI_{520}), and LS3, LS1 and LS4 – the lowest, varying from 12.0 to 0.9. It can be clearly, seen that all solid samples had higher absorbancies than the liquid, due to the differences in their moisture contents. Thus, for the correct comparison of the samples, their net anthocyanin content was placed on an equivalent dry matter basis. CI_{520} corrected values for 1 g dry matter by the moisture correction factor F_m are shown in the same Table 2. In this case, samples SS2, LS2 and SS1 had the highest CI_{520} and LS3 and LS4 – the lowest. Fig. 1(A) and (B) shows the direct and the corrected (by F_m) full visible spectra of all studied samples, which confirm the already established quantitative ranking.

From Fig. 1(A) and (B), it can be seen as well, that the SS2 sample showed the highest absorbance, not only at 520 nm, but all over the visible spectra. The huge experience accumulated in anthocyanin colour validation proves that the absorbance at $\lambda \geqslant 700$ nm should be zero or very close to it (Wrolstad, 1976). After discarding possible errors from the detector output signal, it was concluded that the observed deviation could be related to any untypical chemical changes taking place in the SS2 solution, most probably due to a provoked colour enhancement (addition of pigments or copig-

Table 2
Colour strength (CI_{520}) of solid (SS1–SS4) and liquid (LS1–LS4) commercial anthocyanin-rich extracts carried out according to the Real Decree No. 2107/1996 (1% solution, 1 mm glass cell, sodium citrate/citric acid buffer, pH 3.0), referred to 1 g dry weight sample by the use of F_m and adjusted to a 0 spectrophotometric baseline by Δ, together with their colour strength at the maximal wave length ($CI_{\lambda max}$), CD and total anthocyanin (TA) content

Sample	λ_{max} (nm)	CI_{520} (−)	$CI_{520}(F_m)$ (−)	$CI_{520}(F_m, \Delta)$ (−)	$CI_{\lambda max}$ (−)	$CI_{\lambda max}(F_m, \Delta)$ (−)	CD (−)	$CD(F_m, \Delta)$ (−)	TA[a] (g/100 g)
SS1	515	11.2 (2)[b]	11.6 (3)[b]	11.5 (1)[b]	11.2 (2)[b]	11.5 (1)[b]	18.4 (2)[b]	18.8 (3)[b]	3.47 ± 0.13 (1)[b]
SS2	535	12.0 (1)	14.1 (1)	11.2 (2)	12.3 (1)	11.5 (1)	28.1 (1)	24.3 (1)	1.53 ± 0.07 (4)
SS3	525	9.1 (3)	10.4 (4)	10.1 (4)	9.2 (3)	10.2 (4)	15.5 (3)	16.7 (4)	2.65 ± 0.04 (2)
SS4	530	6.2 (4)	7.3 (6)	7.2 (6)	6.3 (6)	7.3 (6)	10.6 (4)	12.1 (6)	1.32 ± 0.06[a] (5)
LS1	530	3.2 (6)	9.6 (5)	8.7 (5)	3.2 (5)	8.7 (5)	5.9 (6)	15.1 (5)	1.32 ± 0.10[a] (5)
LS2	530	3.5 (5)	12.0 (2)	10.9 (3)	3.6 (4)	11.3 (3)	6.7 (5)	19.8 (2)	2.11 ± 0.16 (3)
LS3	530	0.9 (8)	2.3 (8)	2.3 (8)	0.9 (8)	2.3 (8)	1.4 (8)	3.7 (8)	1.00 ± 0.02[b] (7)
LS4	530	3.2 (6)	6.2 (7)	6.1 (7)	3.3 (7)	6.3 (7)	5.6 (7)	10.5 (7)	1.08 ± 0.02[b] (7)

TA are expressed as g of Cy-3-Gl/100 g of dry matter.
CI_{520} is the absorbance of 1% extract solution (pH 3.0) in a 1 cm glass cell, at 520 nm.
$CI_{\lambda max}$ is the absorbance of 1% extract solution (pH 3.0) in a 1 cm glass cell, at λ_{max}.
CD is colour density: $(Abs_{420} + Abs_{520} + Abs_{620})$ of 1% extract solution (pH 3.0) in a 1 cm glass cell.
F_m is moisture correction factor.
Δ is base line correction factor $= Abs_{\lambda j} - Abs_{780}$ ($j = 380$–780 nm).
The same superscript in the same column means no significant differences ($p \leqslant 0.05$).
[a] Mean value of at least three determinations ± SD.
[b] Numbers in brackets indicate ranking order of quantity.

ments). The existence of a certain batocromic shift of this spectrum in comparison to those of the rest of the grape skin samples (Table 2) makes it likely that it is related to the addition of copigments (Boulton, 2001; Haslam, 1998; Mazza & Miniati, 1993; Osawa, 1982). This result seriously questions the pure natural origin of the SS2 extract. In this case, a correction of the spectra with the Abs_{780} value (correction factor Δ) normalizes the spectral line to zero at the end of the visible spectra (780 nm). All corrected absorbancies at 520 nm (by Δ) of the studied extracts are shown in Table 2 and the corresponding full visible spectra in Fig. 1(C). Thus, Δ improved the ranking of the "normal" samples which did not show significant deviations from the spectral base line, as SS1 is in front of the others with spectral distortions as SS2 and LS2. It is noteworthy that the introduction of both correction factors, F_m and Δ, allows quantitative comparison of anthocyanin extracts, independently of their moisture content, and gives higher confidence of the spectrophotometric measurement, discounting possible apparatus background noise and sample colour anomalies.

Table 2 (Fig. 1) also, shows that λ_{max} of all studied samples differed from 520 nm. Thus, comparison of $CI_{520}(F_m, \Delta)$ and $CI_{\lambda max}((F_m, \Delta)$ shows that the $CI_{\lambda max}$ was higher in five of the eight studied samples. Even through these differences were very small, their significance can be of first order of importance, as they determine the extract absolute colour strength which is further shown in the extract final price. For example, a difference of 0.4 absorbance units, found for sample LS2, supposes an underestimation of its real value by 3.7%. Thus, the final order of rating showed that samples SS1, SS2 and LS2 had the highest colour strength and the sample LS3 (concentrated wine) the lowest. All these considerations suggest that the correct estimation of anthocyanin colour strength must be carried out by measuring the extract CI at λ_{max}, which is in agreement with the EC legislation and that the corresponding Spanish normative should be adapted to it.

With respect to the technical specifications shown in Table 1, it can be seen that samples SS2, SS3, LS1 and LS2 (Table 2) fulfilled the specified values for their CI_{520} and samples SS1 and SS4 had, respectively, 93% and 61% of their specified colour intensities. The same was valid for samples which had specification for their CD. In this case, sample SS4 showed only 44% of the specified CD, which can be explained by the long storage time that this sample had.

Sample ranking, according to their $CD(F_m, \Delta)$, was in a good agreement with those for $CI_{\lambda max}(F_m, \Delta)$ (Table 2), giving certain priority to the pigments with higher proportions of yellow-brownish red components, such as SS2 and LS2. This suggests that the $CD(F_m, \Delta)$ parameter may have major use in cases where specific brownish red tonalities have to be reinforced and it should be considered more qualitative than a quantitative parameter.

The results for total anthocyanins (TA) showed that the elderberry extract (SS1) had the highest content (3.47 g/100 g), followed by SS3 (2.65 g/100 g) and LS2 (2.11 g/100 g) ($P \leqslant 0.05$) (Table 2). LS3 (concentrated red wine) and LS4 (low quality) samples had the smallest pigment contents of 1.00 and 1.08 g/100 g ($P \leqslant 0.05$), respectively. With the exception of samples SS2 and SS3, these results are in a reasonable agreement with those obtained for $CI_{\lambda max}(F_m, \Delta)$, confirming the already obtained rating order. It is interesting to note that this method found the anthocyanin content of sample SS2 to be considerably lower than those of samples SS1 and SS2, which is contrary to that found by

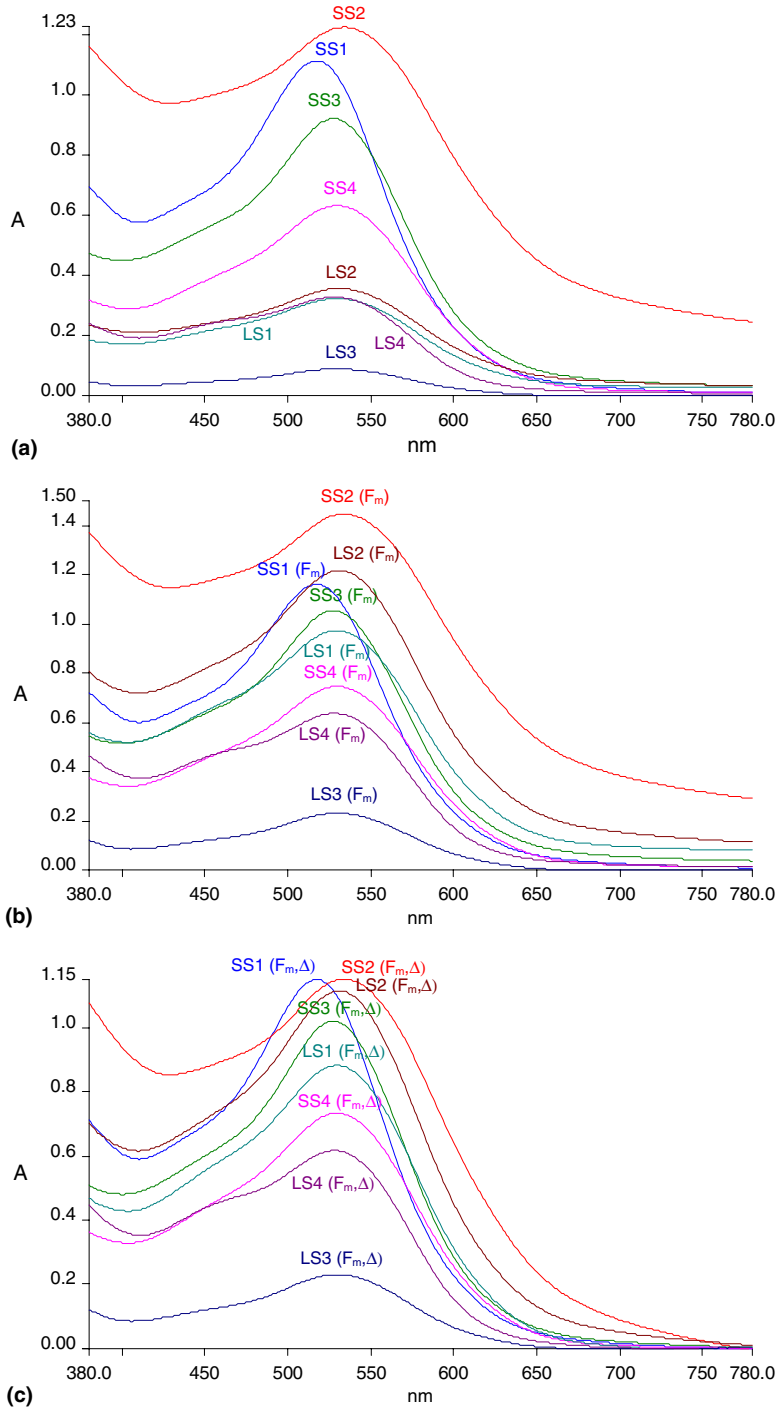

Fig. 1. UV/VIS spectra of 1% solution of solid (SS1–SS4) and liquid (LS1–LS4) commercial anthocyanin-rich extracts carried out according to the Real Decree No. 2107/1996 (1 mm glass cell, sodium citrate/citric acid buffer, pH 3.0) (A), referred to 1 g dry weight matter (B) and adjusted to 0 spectrophotometric baseline by Δ correction factor (C).

the $CI_{\lambda max}(F_m, \Delta)$. This finding confirms our suspicion that sample SS2 had been adulterated. Therefore, we can say that, $CI_{\lambda max}(F_m, \Delta)$ determination alone is unable to discriminate the copigmented part of the anthocyanin red colour and an additional determination of TA by the Ribéreau-Gayon method can be a great help in the authentic identification of anthocyanin extracts, especially in the cases, when Abs_{780} is very different from the spectroscopic zero.

3.2. Qualitative measurements

As anthocyanin colour is strongly dependent on the pH and the composition of the food (beverage) to which

the extract will be added, the most usual tests for its acceptability are carried out directly on the studied products and are completely empirical. Nevertheless, in the previous selection of anthocyanin pigments, some analytical measures can be very useful. Thus, the first step in the quality evaluation of anthocyanin extracts comprises the examination of their colour characteristics: The full visible spectrum or the most significant points of it.

Table 3 shows the results obtained for the colour tonality (CT). The values varied from 0.44 for sample LS3 to 0.82 for sample SS2. The CT index was first introduced in red wine quality assessment, especially in the control of aging processes (Glories, 1984) and gives the relationship of the yellow-brownish to the red colours. Smaller CT values refer to young wines and higher to older and/or oxidized wines. If we apply this criterion to the studied anthocyanin extracts, we can see that best evaluated samples were LS3, SS4 and SS3, whereas SS2, which was one of the best quoted from quantitative point of view, became the worst (quality ranking is noted by Roman numbers in brackets in decreasing order). It is surprising that the three year old sample SS4 was classified as one of the best (Tables 1 and 2). This result suggests that, even though this sample had lost 39% of its colouring power, it still kept its colour characteristics without substantial changes.

Furthermore, Table 3 presents the results for the uniform CIELAB colour space parameters a^*, b^*, L^* (lightness), C^* (chroma), H^* (hue) and S^* (saturation) already adjusted by both correction factors, F_m and Δ. There are large variations of all studied parameters for the different samples. Thus, the most quantitative variables L^*, C^* and S^* varied, respectively, in the ranges 37.9–0.3, 67.8–2.3 and 1.8–7.5 and their ranking orders were in good agreement. Similar trend between L^*, C^* and S^* parameters were found by Rhim, Nunes, Jones, & Swartzel (1989) for red grape juices. The most qualitative parameter H^* which specifies the exact extract colour, comprised values from 10.3 to 34.3. As H^* is a function of a^* and b^* ($H^* = \text{arc tg}(b^*/a^*)$), it can be considered that samples with red-violet colours, typical for fresh (young) extracts can reach values for H^* of around 30°, whereas higher values should be related to older (oxidized) extracts. In this sense, samples LS3 (concentrated wine), SS2 and LS2 had preferable colour characteristics.

Bibliographic reports of simple or tristimulous colorimetric parameters in anthocyanin extracts were unsystematic and referred mostly to technological treatments and stability of fruit juices or model solutions. In addition, they were obtained by different colorimetric systems in which tristimulous parameters do not have equivalent colorimetric meanings (Bakker et al., 1993; Cheynier et al., 1990; Heredia et al., 1998; Hong & Wrolstad, 1990a; Malien-Aubert et al., 2001; Skrede, 1985). Contrary to this, the accumulation of important empirical data for diverse categories and qualities of wines, confirmed that it was possible to make a distinction between wines from different varieties, growing regions, ages, and technological treatments (Garijo et al., 1997; Gómez-Cordovés & González-San, 1995; Heredia & Guzmán, 1995; Huerta et al., 1998; Negueruela et al., 1995), or simply to determine how far one wine may be different from a set of standards, both in colorimetric and visual acceptability terms. Unfortunately, similar data for anthocyanin extracts are not available, or at least, are not published (to our knowledge, some anthocyanin extract manufacturers use tristimulous colorimetric parameters for quality control of their products). Thus, with the data shown here for CT, and especially for CIELAB colour space, it is difficult to make correct quality estimations, because of the lack of systemised knowledge (quality scale) and because of the limited number of the studied samples.

Fractionation of anthocyanins into monomer (MAF), red (RPAF) and yellow–brown polymer (YBPAF) fractions by open column chromatography showed a large variation in the obtained results (Table 4). Elderberry anthocyanins were composed mainly of MAF and RPAF (98.4%). Among the grape extracts, the MAF varied from 55.2–54.0% (SS4 and SS3) to

Table 3
Colour characterization of 1% solution of solid (SS1–SS4) and liquid (LS1–LS4) commercial anthocyanin-rich extracts using CT and CIELab parameters, referred to 1 g dry weight sample and adjusted to 0 spectrophotometric baseline (1 mm glass cell, sodium citrate/citric acid buffer, pH 3.0)

Sample	CT	a^*	b^*	L^*	C^*	H^*	S^*	Colour
SS1	0.53 (IV)[A]	44.0 (III)[A]	21.9 (VII)[A]	12.7 (III)[A]	49.1 (III)[A]	26.5 (VII)[A]	3.9 (III)[A]	Red violet
SS2	0.82 (VIII)	2.2 (VIII)	0.5 (I)	0.3 (VIII)	2.3 (VIII)	13.4 (II)	7.5 (VIII)	Red violet
SS3	0.52 (III)	38.6 (VI)	14.7 (III)	8.5 (VI)	41.3 (VI)	20.9 (IV)	4.8 (VI)	Red violet
SS4	0.50 (II)	43.8 (IV)	20.4 (VI)	11.9 (IV)	48.3 (IV)	25.0 (VI)	4.1 (IV)	Red violet
LS1	0.56 (VII)	40.7 (V)	16.2 (V)	9.4 (V)	43.8 (V)	21.7 (V)	4.7 (V)	Red violet
LS2	0.60 (VI)	12.9 (VII)	3.2 (II)	1.8 (VII)	13.3 (VII)	13.8 (III)	7.2 (VII)	Red violet
LS3	0.44 (I)	65.4 (I)	11.8 (III)	37.9 (I)	66.5 (II)	10.3 (I)	1.8 (I)	Red violet
LS4	0.63 (VII)	56.1 (II)	38.2 (VIII)	22.2 (II)	67.8 (I)	34.3 (VIII)	3.1 (II)	Purple

CT, colour tonality.
[A] Roman numbers in brackets indicate ranking order of quality.

Table 4
Total monomer (MAF), red (RPAF) and yellow–brown polymer (YBPAF) anthocyanin fractions of commercial anthocyanin-rich extracts

Sample	MAF[A] (%)	RPAF[A] (%)	MAF + RPAF (%)	YBPAF[A] (%)
SS1	76.0 ± 2.5 (I)[B]	22.4 ± 2.4^{a}	98.4 (I)[B]	1.6 ± 0.2
SS2	$42.1 \pm 2.6_{1}^{a,b}$ (IV)	$42.5 \pm 3.5_{1}$	84.6 (V)	15.4 ± 1.0^{a}
SS3	54.0 ± 0.4^{c} (II)	31.3 ± 1.2^{b}	85.3 (IV)	14.7 ± 1.6^{a}
SS4	55.2 ± 1.5^{c} (II)	32.2 ± 1.6^{b}	87.4 (III)	12.6 ± 0.4
LS1	31.2 ± 0.5 (VIII)	39.5 ± 0.3	70.7 (VII)	29.3 ± 0.8
LS2	$41.1 \pm 0.8^{a,d}$ (IV)	36.7 ± 0.6	77.8 (VI)	22.2 ± 0.8
LS3	46.3 ± 2.0^{b} (V)	51.0 ± 1.7	97.3 (II)	2.7 ± 0.3
LS4	39.4 ± 0.4^{d} (VI)	22.8 ± 0.5^{a}	62.0 (VIII)	37.8 ± 0.5

The same superscript in the same column means no significant differences ($p \leqslant 0.05$).
The same subscript in the same row means no significant differences ($p \leqslant 0.05$).
[A] Mean value of at least three determinations ± SD.
[B] Roman numbers in brackets indicate ranking order of quality.

31.2% (LS1), the RPAF was between 51% (LS4) and 22.4–22.8% (SS1 and LS4) and the YBPAF was from 37.8% (LS4) to 2.7% (LS3). The LS4 sample showed the lowest MAF + RPAF and the highest YBPAF proportion. As monomer anthocyanins contribute violet-bluish red shades, considered as preferable for most of the applications, it is seen that all grape skin samples had quality characteristics much lower than the elderberry one. However, in complex vegetable extracts, such as wines and juices, anthocyanins can condensed themselves or together with other phenolics, forming more stable anthocyanin polymers and causing hypsochromic shift of their λ_{max} toward the red colours (Francis, 1993; Gao et al., 1997; Mazza & Miniati, 1993). According to this, preferable and stable pigments are associated with high proportions of both, monomer and red polymer anthocyanins and degraded pigments are associated with high proportions of yellow–brown red colours. Taking this into account, it is noteworthy that the difference between the elderberry and most of the grape skin extracts (LS3, SS4, SS3 and SS2) was minimal. In another study on grape skin extracts, Knuthsen (1987) found still larger variation of the MAF, extending from 0 to 75%. This author found that three of the ten studied samples contained more than 70% MAF, which indicates that, grape skin anthocyanins can have similar levels of monomers to the elderberry in our study. It is note worthwhile that Knuthsen (1987) considers only the monomer anthocyanin fraction, as important for the pigment quality which we think disfavours samples with higher proportion of stable red polymers.

Further examination of Table 4 shows that the SS4 sample, which we had as an example for an old and expired extract, had a relatively low proportion of YBPAF. This finding suggests that anthocyanin degradation of well conserved powdered extracts is going on via colourless degradation products. Contrary to this, the higher proportion of YBPAF found in the liquid extracts, indicates that anthocyanins tend to form large brownish red polymers when they are stored in liquid media. Thus, in light of these results, it can be seen that fractionation of anthocyanin extracts on MAF, RPAF and YBPAF amplifies the information about the structure/quality relationship of the anthocyanin pigments.

Separation of the grape skin pigments by HPLC showed a large variation in their individual (monomer) anthocyanin contents (Table 5) (as the used HPLC method was adapted for separation of *Vitis vinifera* L. anthocyanins, individual elderberry anthocyanins were not monitored). The main anthocyanin was Mv-3-glucoside (Mv-3-Gl), which confirmed the *V. vinifera* origin of most of the samples. The only exception was found for sample LS3, where the main monomer was Dp-3-Gl (it was pointed that this extract was a mixture of *V. vinifera* and other American varieties (Table 1)). Dp-3-Gl and Pt-3-Gl were the most abundant after Mv-3-Gl in all samples. Samples SS4 and LS4 also had a good proportion of Pn-3-Gl. The SS3 sample was found to contain the highest amounts of acylated monomers (AcMA) (139 mg/100 g). Certain amounts of AcMA, basically Dp-3-Gl-Ac, were found in samples SS2 and LS1-LS4, as well. As acylated anthocyanins have been found to have higher chemical stability than the nonacylated (Bridle & Timberlake, 1997; Malien-Aubert et al., 2001; Mazza, 1995; Yoshida et al., 1991), the determination of their content can play an essential role in the selection of anthocyanin extracts. Regarding the sum of the acylated and nonacylated monomers (henceforth total monomer anthocyanins (TMA)), the highest value was found for sample SS3: 2151 mg/100 g d.m (Table 5). The rest of the samples fell into three groups: Intermediate amount anthocyanin-containing group: Samples LS2 and SS2 (657–623 g/100 g), low amount anthocyanin-containing group: LS4 and LS1 (230–220 g/100 g) and very low amount anthocyanin-containing group: LS3 and SS4 (87.6–39.0 g/100 g). It is important to note that the rating for TMA obtained here was in poor agreement with those for MAF (Table 4). This finding can be appreciated more clearly in the sample with the most outstanding rating difference, SS4 (which was also pointed out as three years old). The proportion of 55.2%

Table 5
Individual, total monomer (TMA) and total acylated monomer (TAcMA) anthocyanin contents of commercial anthocyanin-rich extracts

Sample	Dp-3-Gl[A]	Cy-3-Gl[A]	Pt-3-Gl[A]	Pn-3-Gl[A]	Mv-3-Gl[A]	Dp-3-Gl-Ac[A]	Cy-3-Gl-Ac[A]	Pt-3-Gl-Ac[A]	Pn-3-Gl-Ac[A]	Mv-3-Gl-Ac[A]	TMA	TAcMA
SS1	–	–	–	–	–	–	–	–	–	–	–	–
SS2	$82.2 \pm 5.7_1$	15.0 ± 1.7^a	$87.7 \pm 7.2_1$	57.1 ± 2.4^a	350 ± 28.4^a	24.8 ± 0.8	7.0 ± 0.2	n.d	n.d.	n.d.	624 (III)[B]	31.8 (II)[B]
SS3	421 ± 28.9	$57.5 \pm 1.3_1$	303 ± 15.3	136 ± 2.9	1101 ± 58.2	$22.7 \pm 0.4_2$	$19.4 \pm 3.2_{2,3}$	$21.6 \pm 1.5_{2,3}$	$16.8 \pm 3.7_3$	$58.5 \pm 1.8_1$	2151 (I)	139 (I)
SS4	7.1 ± 0.4	n.d.	4.5 ± 0.2	3.3 ± 0.1^b	21.7 ± 1.1	n.d.	n.d.	n.d.	n.d.	2.4 ± 0.1	39.0 (VII)	2.4 (VII)
LS1	58.9 ± 0.4	$8.4 \pm 0.2_1$	63.1 ± 1.2	14.1 ± 0.7	70.3 ± 7.0	4.7 ± 0.4	n.d.	n.d.	n.d.	n.d.	220 (V)	4.7 (V)
LS2	95.5 ± 3.8	14.3 ± 0.2^a	103.9 ± 2.7	55.2 ± 0.5^a	371 ± 1.6^a	17.6 ± 0.7	n.d.	n.d.	n.d.	n.d.	657 (II)	17.6 (III)
LS3	36.6 ± 0.4	4.8 ± 0.3	24.8 ± 5.9^a	2.5 ± 0.1^b	15.4 ± 1.5	3.5 ± 0.1	n.d.	n.d.	n.d.	n.d.	87.6 (VI)	3.5 (VI)
LS4	29.3 ± 0.8	6.8 ± 0.6	33.8 ± 3.4^a	51.5 ± 0.3	100 ± 0.3	9.0 ± 1.2	n.d.	n.d.	n.d.	n.d.	230.4 (IV)	9.0 (IV)

TMA and TAcMA are the sum of all monomer and all acylated monomer anthocyanins, respectively, expressed as mg of Mv-3-Gl/100 g dry matter.
n.d. is not detectible amounts.
Absence of superscript letters in the same column means significant differences ($p \leqslant 0.05$).
Absence of subscript numbers in the same row means significant differences ($p \leqslant 0.05$).
Dp-3-Gl-Ac is Dp-3-Gl-acetate.
[A] Mean value of at least three determinations ± standard deviation, expressed as mg of Mv-3-Gl/100 g dry matter.
[B] Roman numbers in brackets indicate ranking order of quality.

Table 6
Total catechin (TC) and total condensed tannin (TCT) contents of commercial anthocyanin-rich extracts

Sample	TC[A]	TCT[A]
SS1	0.63 ± 0.06	4.12 ± 0.21
SS2	7.36 ± 0.12	12.9 ± 0.13
SS3	8.63 ± 0.12	14.3 ± 0.80
SS4	4.00 ± 0.14	10.1 ± 0.52^a
LS1	2.66 ± 0.22^a	7.62 ± 0.64
LS2	2.72 ± 0.21^a	11.1 ± 0.41^a
LS3	10.6 ± 0.19	6.41 ± 0.25
LS4	8.94 ± 0.12	15.91 ± 0.28

Absence of superscript letters in the same column means significant differences ($p \leqslant 0.05$).
TC is expressed as g of (+)-catechin/100 g dry matter.
TCT is expressed as g of Cy/100 g dry matter.
[A] Mean value of at least three determinations ± standard deviation.

MAF, determined by the method of Ribéreau-Gayon et al. (1982) (Table 4) seemed to be excessively high, which was confirmed by the results obtained for TMA by HPLC. This indicates, that fractionation of anthocyanins by OCC should be taken as a methodology with low precision.

Total catechin (TC) and total condensed tannin (TCT) contents are shown in Table 6. These data confirm again the previous conclusion that elderberry extract was very different from all grape skin extracts, containing the lowest amounts of TC and TCT ($P \leqslant 0.05$). For the grape skin extracts, TC ranged from 2.66–2.72 (LS1 and LS2, respectively), to 10.6 g of (+)-catechin/100 g (LS3) ($P \leqslant 0.05$) and TCT were from 6.41 (LS3) to 15.9 g of cyanidin/100 g (LS4) ($P \leqslant 0.05$). The contribution of these two parameters to the total quality estimation of the studied extracts is somewhat less significant and in some sense controversial. On the one hand, it is found that phenolics such as oligomers and polymers of proanthocyanidins form copigments and/or polymers with Mv-3-Gl, improving the stability of the colour (Malien-Aubert et al., 2002), whereas monomer catechins appeared to have a negative influence on its stability (Malien-Aubert et al., 2001, 2002). In other hand, tannins are known to impart astringency and catechins bitterness, which means that the concentrations of these phenolics should be taken in consideration in the foods where these tastes are unwanted. Unfortunately, the lack of experimental data, on the quantity/taste response of these compounds makes difficult to predict their possible influence in real foods.

Finally, data for total dry matter (DM), total soluble (TSoS) and total suspended solid (TSpS) contents for the studied samples are shown in Table 7. These results showed that the proportion of TSpS in the extract DM was highest in samples LS4, SS4 and LS3 (52%, 49% and 24%, respectively) and lowest in the samples LS1 and SS2 (4% and 7%, respectively). Higher proportions of total suspended solids, such as those for the three years

Table 7
Physical characteristics of solid (SS1–SS4) and liquid (LS1–LS4) commercial anthocyanin-rich extracts

Sample	Moisture[a] (g/100 g)	Dry Matter[a] (g/100 g)	TSoS[a] (g/100 g)	TSpS[a] (g/100 g)
SS1	3.80 ± 0.07	96.2 ± 0.0	82.5 ± 0.2	13.7 ± 0.2
SS2	15.10 ± 2.55	84.9 ± 2.5	79.1 ± 1.2	5.9 ± 1.3
SS3	12.50 ± 0.61	87.5 ± 0.0	71.5 ± 0.1	16.3 ± 0.1
SS4	15.10 ± 0.72	84.9 ± 0.3	43.0 ± 0.1	41.5 ± 0.2
LS1	71.10 ± 3.68	28.9 ± 3.7	27.7 ± 3.5	1.1 ± 0.1
LS2	64.30 ± 0.14	35.7 ± 0.1	29.0 ± 0.7	6.7 ± 0.6
LS3	67.00 ± 0.00	33.0 ± 0.0	25.2 ± 2.7	7.8 ± 2.7
LS4	55.80 ± 0.71	44.2 ± 0.7	21.0 ± 0.1	23.2 ± 0.8

TSoS, total soluble solids.
TSpS, total suspended solids.
[a] Mean value of at list two determinations ± standard deviation.

old powdered sample SS4, should be an indication of the existence of appreciable quantities of complex precipitates, which can increase after prolonged storage or simply that the extracts were not filtered as was the case for samples LS3 (unclarified concentrated red wine) and LS4 (raw extract).

In conclusion, colour assessment of anthocyanin-rich extracts according to the legal Spanish (Real Decree No. 2107/1996) and EC (Directive 95/45/EC) involves some limitations: Lack of objectivity in the assessment of samples with different moisture contents and/or absorption maxima, and inability to identify possible fraudulence due to copigmentation phenomena. To overcome these limitation the following corrections were introduced: $CI = CI_{\lambda max}$, referred to 1 g dry matter, when $Abs_{780} > 0.001$, $CI = CI_{\lambda max} - CI_{780}$, followed by determination of TA by the Ribéreau-Gayon method (1982). Nevertheless, more precise methodology for quantitative assessment of anthocyanins is needed, based on the comparison with known reference standards and able to discriminate colours due to possible copigmentation effects and/or polymer anthocyanin formation.

Pigment qualities were well described by spectral colorimetric assessment. Best objectivity was achieved by the use of tristimulous colorimetry. A CIELAB colour space software, designed for red wine quality assessment, was applied directly to standardized anthocyanin extract solutions as they were wines. Unfortunately, practical interpretation of the results is quite difficult because of the lack of a reference scale about the real meaning of the CIELAB parameters in anthocyanin-rich extracts. That is why we strongly encourage specialists working in the field of anthocyanin pigment quality to strive for the creation of a wide databank, essential for better quality estimation of anthocyanin extracts.

Separation of anthocyanin-rich extracts into monomer, red and yellow–brown polymer fractions by OCC, gave in-precise, but important information about the structure/quality relationships of the anthocyanin pigments. Further, separation of anthocyanins by HPLC amplifies information about their individual constituents, which could be essential in the identification of the grape origin, particularly in processes of colour fortification of monovarietal red wines. Moreover, identification and quantification of acylated anthocyanins contributes to the identification of extracts with higher physicochemical stability.

Determination of total dry matter and total soluble and suspended solids gave a good indication of purity of the anthocyanin pigments and possible haze contamination of the coloured products, whereas determination of catechins and condensed tannins can be indicative of possible taste alterations. Two main drawbacks limit the use of these latter parameters in anthocyanin-rich extracts: The lack of experimental data, relating their quantity/quality response and the relatively high time and labour costs in their analytical assessment.

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