Reliability of Analytical Methods for Determining Anthocyanins in Blood Orange Juices

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Quantitative analysis of anthocyanins was performed on a series of blood orange juices according to various spectrophotometric and HPLC methods, and the causes for different concentration resulting from the application of such procedures were investigated. Spectrophotometric methods utilizing aqueous ethanol as a solvent provided an anthocyanin content higher than that determined by HPLC. Discrepancies were ascribed to the use of impure standards and/or unsuitable calibration lines. The most consistent results with the HPLC findings were obtained by a method utilizing water as a solvent and cyanidin-3-glucoside as a standard. Actual concentration of anthocyanins in blood orange juice was remarkably lower than that currently determined by procedures used in the juice producing factories.

Keywords: *Anthocyanins; blood orange juice; cyanidin-3-glucoside; quantitative analysis*

INTRODUCTION

Anthocyanins are responsible for the blood color of some sweet oranges (*Citrus sinensis*, L. Osbeck, var. Tarocco, Moro, and Sanguinello) grown in Italy (Maccarone et al., 1983, 1985). These water soluble pigments are scavengers of radical species (Miller et al., 1995; Wang et al., 1997) and contributors to antioxidant activity (Bonina et al., 1998; Rapisarda et al., 1999; Arena et al., 1999), thus constituting useful markers to enable recognition and evaluation of quality of fresh and processed products. Blood orange juices contain several 3-glucosides and 3,5-diglucosides of the most common anthocyanidins, but there is a predominance of cyanidin-3-glucoside and cyanidin-3-(6″-malonyl)-glucoside (Maccarone et al., 1998). Their content depends on genetic and physiological factors, such as genotype and ripening (Rapisarda and Giuffrida, 1992). Quantitative analysis can be performed by spectrophotometry of absorption in the visible region, but it needs a suitable standard to calibrate concentration (Francis, 1982). Fortunately, such anthocyanins have similar spectroscopic properties and comparable molecular mass, consequently concentration can be correctly expressed in terms of cyanidin-3-glucoside, available as a pure standard.

Anthocyanin content is the most important factor in determining color of orange juices, but self-association and copigmentation with different polyphenolic substances can affect shade and intensity of color (Brouillard, 1988). Therefore, absorbance may, or may not, be correlated with actual concentration. It is necessary to use a solvent to dilute juice and minimize interference due to effects of the above cited phenomena. Moreover, the equilibrium between colored and colorless forms of anthocyanins

$$F^+ + 2H_2O \rightleftarrows FOH + H_3O^+$$

depends on hydrogen ion concentration, shifting to the red flavylium cation (F^+) in highly acidic medium. p*K* of such hydration equilibrium for cyanidin-3-glucoside is 3.05 at 20 °C (Maccarone et al., 1992); therefore, absorbance must be measured in a pH ∼1 solution where anthocyanin is present only as flavylium salt to detect latent red color stored in the colorless chromenol (FOH).

Five different spectrophotometric methods are utilized for determining anthocyanins in blood orange juices. Three of them are described in the literature (Di Giacomo et al., 1989; Rapisarda et al., 1994; Trifirò et al., 1996), the remaining two are currently used in the Italian juice producing factories (Ruby and Parmalat, private communications). Methods are based on the measurement of absorbance at λ_{max} of juice after dilution with an acidic solvent, but procedures to obtain an adjusted value of absorbance and to calibrate concentration of anthocyanins are typical of each method. The present work is aimed at investigating causes of the different anthocyanin content resulting from the application of different methods. Anthocyanins were determined in a series of blood orange juices according to the various procedures, including an independent HPLC analysis (Rapisarda et al., 1994), more expensive and time-consuming than spectrophotometric methods, but reliable as a reference point.

MATERIALS AND METHODS

Sixteen different blood orange juices (12 from Moro and four from Tarocco fruits) were prepared from fruits harvested in 1998 at the *Palazzelli* experimental field of the Istituto Sperimentale per l'Agrumicoltura (Acireale, Catania, Italy) in the territory of Lentini (Siracusa, Italy). Juices were obtained using a domestic squeezer, poured into 500 mL bottles, and

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stored at −18 °C. Before analysis, juices were defrosted and centrifuged at 6000 rpm for 30 min. Surnatant was used to prepare samples for spectrophotometric and HPLC analyses. A UV−vis Lambda 2 spectrophotometer (Perkin-Elmer, Milan, Italy) and 1 cm quartz cells were used throughout. Cyanidin-3-glucoside chloride standard (purity >95%) was purchased from Extrasynthèse (Genay, France). 95% Ethanol for spectrophotometry and purity grade HPLC solvents were used.

Spectrophotometric Methods. *Method 1 (Di Giacomo et al., 1989).* Ten milliliters of clear juice was diluted to 100 mL using a 20.3/79.7 (v/v) mixture of 95% ethanol and 37% HCl. Absorbance was measured at 535 nm. Concentration of anthocyanins was calculated by the equation

$$C_{mg/100mL} = (Abs/0.1) \times 10$$

where 0.1 is the slope of a calibration line obtained from standard solutions of a pigment isolated from blood orange juice, 10 is the dilution factor (DF). Concentration in milligrams per liter for a generic *DF* is given by the equation $C_{mg/L} = Abs \times 100 \times DF$.

Method 2 (Rapisarda et al., 1994). An aliquot of juice (2 mL) was diluted up to 25 mL with a pH 1 solution (125 mL of 0.2 M KCl and 375 mL of 0.2 M HCl). A second aliquot (2 mL) was diluted up to 25 mL with a pH 4.5 buffered solution (400 mL of 1 M CH_3CO_2Na, 240 mL of 1 M HCl, and 360 mL of H_2O). Absorbance of the solutions was measured at 510 nm. Concentration of anthocyanins was calculated by the equation

$$C_{mg/L} = (Abs_{pH1} - Abs_{pH4.5}) \times 484.82 \times 1000/24825 \times DF$$

where the term in parentheses is the difference of absorbance at 510 nm between pH 1 and pH 4.5 solutions, 484.82 is the molecular mass of cyanidin-3-glucoside chloride, 24 825 is its molar absorptivity (ϵ) at 510 nm in the pH 1 solution, and DF is the dilution factor.

Method 3 (Trifirò et al., 1996). Twenty-five milliliters of juice was diluted to 100 mL using a 80/20 (v/v) mixture of 95% ethanol and 37% HCl. Absorbance was measured at 420, 530, and 620 nm. Concentration of anthocyanins was calculated by the equation

$$C_{mg/L} = \text{Net } Abs_{530}/\text{slope} \times DF$$

where Net Abs_{530} is given by $Abs_{530} - (Abs_{420} + Abs_{620})/2$, slope is the angular coefficient of a calibration line of Net Abs_{530} of standard solutions of cyanidin-3-glucoside in 80/20 solvent mixture, and DF is the dilution factor.

Method 4 (Ruby Co., Catania, Italy, private communication). Sample preparation and solvent composition were the same as method 3. Absorbance was measured at 535 nm. Concentration of anthocyanins was calculated by the equation

$$C_{mg/L} = Abs/98.2 \times 10\,000 \times DF$$

where 98.2 is the absorbance of a solution containing 1% (w/v) of cranberry anthocyanins, 10 000 is the conversion factor from g/100 mL to mg/L, and DF is the dilution factor.

Method 5 (Parmalat, Parma, Italy, private communication). Sample preparation and solvent composition were the same as methods 3 and 4. Absorbance was measured at 532 nm. Concentration of anthocyanins was calculated by the equation

$$C_{mg/L} = Abs/402.3 \times 10\,000 \times DF$$

where 402.3 is the absorbance of a solution containing 1% (w/v) of blood orange juice anthocyanins; 10 000 is the conversion factor from g/100 mL to mg/L, and DF is the dilution factor.

Molar Absorptivity of Cyanidin-3-glucoside. This was determined in various aqueous ethanol solvents according to the following procedure. A weighed quantity of cyanidin-3-glucoside chloride was dissolved in a known volume of a 99/1 (v/v) mixture of 95% ethanol and 37% HCl. In such mother solution, HCl was 0.1 M and the final composition of solvent was ~95/5 (v/v) ethanol/water. Five solutions were prepared by diluting different volumes of the mother solution with the same solvent (99/1) up to a known final volume. Aliquots of these solutions were then diluted up to known final volumes using aqueous 0.1 M HCl. Thus, five solutions at different concentration of cyanidin-3-glucoside chloride were obtained (from 5.16×10^{-6} to 6.23×10^{-5} M) for each of five solvent mixtures having variable composition of ethanol and water (from 95/5 to 47/53, v/v) and constant concentration of HCl (0.1 M). A second series of solutions was prepared using a different mother solution. Absorbance was measured at 530 nm in all solutions, and molar absorptivity of cyanidin-3-glucoside (ϵ) was calculated from the slope of the plot between absorbance and molar concentration for each solvent system.

Table 1. Quantitative Determination of Anthocyanins (mg/L) in Sixteen Blood Orange Juices by Different Analytical Methods

	methods						
	spectrophotometric						
sample[a]	1	2	3	3A	4	5	HPLC
M1	720	104.3	175.3	104.3	734	178.8	102.6
M2	754	106.5	186.0	110.2	768	188.7	103.5
M3	542	76.5	127.6	77.6	552	134.1	72.4
M4	686	96.3	165.0	98.5	698	167.1	92.8
M5	534	74.8	124.7	76.0	543	132.6	73.9
M6	371	50.2	78.5	50.2	378	92.2	49.1
M7	643	90.9	155.7	93.3	655	160.3	84.2
M8	443	60.9	98.9	61.6	452	111.0	62.6
M9	729	104.3	182.5	108.3	743	182.5	89.6
M10	429	59.0	97.7	60.9	437	107.0	56.3
M11	311	42.2	81.2	51.7	316	77.0	36.8
M12	573	81.9	153.4	92.0	584	142.7	80.5
T1	327	40.5	67.1	43.8	334	81.6	42.0
T2	245	32.9	45.6	31.8	249	60.9	37.4
T3	201	26.8	31.9	24.2	204	49.9	28.6
T4	304	38.3	59.9	39.8	309	75.6	37.4
mean	488	67.9	114.4	70.3	497	121.4	65.6
sd[b]	184	27.6	51.1	28.6	188	45.8	25.2

[a] M. Moro and T. Tarocco. [b] sd, standard deviation.

HPLC Method. Samples for HPLC analysis were prepared according to Rapisarda et al. (1994). Ten milliliters of centrifuged juice was passed through a 5 mL syringe fitted with a Sep Pack C_{18} cartridge (Waters). Adsorbed anthocyanins were washed with 5 mL of distilled water and then desorbed using 5 mL of 0.1% HCl-CH_3OH. Methanol solution was evaporated at 35 °C under vacuum and residue was dissolved in a known final volume of the mobile phase solvent B (as described later). Analysis was performed by a liquid chromatograph (Waters, model 600E) equipped with a W 410 UV−vis detector and a W 746 peak area integrator. Column was a 250 × 4 mm Hypersil ODS 5 μm (Phenomenex, Torrance, CA) and solvent system was the following. Solvent A, H_2O:CH_3CO_2H (85/15, v/v), and solvent B, H_2O:CH_3CO_2H:CH_3OH (65/15/20, v/v/v). Percentage of B increased linearly from 1 to 100% at a flow rate of 1.0 mL/min. Samples of 20 μL were injected and monitored at 520 nm. Concentration of anthocyanins was calculated by the equation

$$C_{mg/L} = (\text{area/slope}) \times DF$$

where area is total HPLC area of anthocyanin peaks, slope is the angular coefficient of a calibration line of standard solutions of cyanidin-3-glucoside, and DF is the dilution factor.

RESULTS AND DISCUSSION

Table 1 reports concentration of anthocyanins in 16 blood orange juices resulting from the application of different analytical procedures as described in the Materials and Methods. Methods 1 and 4 yield similar results (mean content is 488 and 497 mg/L, respectively)

Table 2. Molar Absorptivity (ϵ) of Cyanidin-3-glucoside Chloride in 0.1 M HCl Water−Ethanol Mixtures

		statistical data[a]	
water % (v/v)	molar absorptivity ($cm^2 \times mol^{-1}$)	standard deviation	*t*-test
5	32 518	605	53.7
20.8	32 678	562	58.2
32.8	29 322	161	181.5
42	28 924	195	147.8
53	27 876	369	75.6
100[b]	25 740		
100[c]	24 825		

[a] Number of points: 10 for each solvent system. Concentration of cyanidin-3-glucoside: 2.5−30 mg/L. Correlation coefficients > 0.999. $P > 0.000$. [b] McClure, 1967. [c] Rapisarda et al., 1994.

overestimating anthocyanins by a factor ∼7.5 with respect to HPLC data. Methods 3 and 5 also yield comparable results (mean content is 114 and 121 mg/L, respectively), overestimating anthocyanins by a factor ∼1.8. Results of method 2 are similar to the HPLC ones (mean content is 67.9 and 65.6 mg/L, respectively). These results indicate that most of spectrophotometric methods are affected by systematic errors. Possible sources of these deviations are now separately examined.

Overestimation of anthocyanins by method 1 is due to the use of an impure pigment as the calibration standard. In fact, the molar absorptivity of this pigment was calculated by us from the reported data (Di Giacomo et al., 1989), and a too low value for a pure anthocyanin was found ($\epsilon = 4800$). Molar absorptivity of a pure standard of cyanidin-3-glucoside was then determined in the same solvent system of method 1, and a much higher value ($\epsilon = 32\,700$) was found. Use of a 7-fold lower calibration line accounts for a 7-fold increase of anthocyanin concentration in orange juice.

Despite using a pure standard of cyanidin-3-glucoside to calibrate both methods 2 and 3, the respective results differ by a factor ∼1.8. Regarding method 3, we observed that absorbance of standard solutions was measured in ∼80/20 ethanol−water system, whereas absorbance of juice solutions was measured in a medium where actual percentage of water was significantly higher (∼35%). A different composition of medium can influence molar absorptivity of standard giving rise to an incorrect application of Beer's law. Molar absorptivity of cyanidin-3-glucoside was then determined in various ethanol/water mixtures, where percentage of water was changed from 5 to ∼50% and HCl concentration was 0.1 M in each mixture (Table 2). The ϵ value remains unchanged up to 20% water ($\epsilon = 32\,600$), but it decreases with increasing water percentage, until a value of ∼24 800 in pure water (Rapisarda et al., 1994). Reliability of observed ϵ values is supported by literature data reporting 34 300 in 1% HCl methanol (Siegelman and Hendricks, 1958) and 25 740 in aqueous 0.1 M HCl (McClure, 1967). Anthocyanin concentration was recalculated for orange juices according to method 3, but using a calibration line derived from standard solutions of cyanidin-3-glucoside in a ∼67/33 ethanol−water solvent. Mean content for anthocyanins now becomes 70.3 mg/L (Table 1, column 3A), analogous with that found by HPLC and method 2. This result suggests that calibration line must be obtained in a solution where solvent composition is similar to that resulting after dilution of the juice, to obey Beer's law which imposes constant ϵ value in absorbance-concentration correlation. Method 2 does not suffer such a limiting factor because water is utilized as a solvent for diluting both standard and juice.

The calibration factor 98.2 of method 4 is improperly used, because original papers of Fuleki and Francis (1968a,b) report a value of 982 (not 98.2) as absorbance at 535 nm of a solution containing 1% (w/v) of cranberry anthocyanins. Moreover, cranberry anthocyanins are an unsuitable standard because these are different from those of blood orange juice (Zapsalis and Francis, 1965). Finally, the data of method 5 result from the application of the factor 402.3, i.e., the absorbance at 532 nm of a solution containing 1% (w/v) of blood orange juice anthocyanins, but such a value is not supported by any experimental evidence.

CONCLUSION

The more reliable spectrophotometric procedure appears to be method 2, where aqueous acidic solutions as a solvent and cyanidin-3-glucoside as a standard are used. Accuracy of results is supported by independent HPLC data. Nevertheless, method 3 also provides reliable results if solvent used for calibration has the same composition as the juice solution. Actual content of anthocyanins in the blood orange juices is remarkably lower than that currently obtained by procedures used in the juice producing factories. Standardization of color is often the limiting factor in juice production, therefore analyses for anthocyanins are routine. Obviously, it is desirable to have a rapid and inexpensive method, but simplicity and low cost of analysis must be coupled with reliability of the results, to avoid disputes in trade exchanges and misleading data in research work.

LITERATURE CITED

Arena, E.; Fallico, B.; Maccarone, E. L'attività antiossidante dei succhi di arance pigmentate. *Proc. 4th Ital. Congress Food Sci. Technol.*; Cernobbio, Italy, Sept. 16−17, 1999.

Bonina, F.; Saija, A.; Tomaino, A.; Lo Cascio, R.; Rapisarda, P.; Dederen, J. C. *In vitro* antioxidant activity and *in vivo* photoprotective effect of a red orange extract. *Int. J. Cosmet. Sci.* **1998**, *20*, 331−342.

Brouillard, R. Anthocyanins and flower colour. In *The Flavonoids. Advances in Research since 1980*; Harborne, J. B., Ed.; Chapman and Hall: London and New York, 1988; pp 525−538.

Di Giacomo, A.; Calvarano, M.; Calvarano, I.; Di Giacomo, G.; Belmusto, G. Il succo delle arance pigmentate italiane. *Essenze, Deriv. Agrum.* **1989**, *59*, 273−289.

Francis, F. J. Analysis of anthocyanins. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; Academic Press: New York and London, 1982; pp 181−207.

Fuleki, T.; Francis, F. J. Quantitative methods for anthocyanins. 1. Extraction and determination of total anthocyanin in cranberry. *J. Food Sci.* **1968a**, *33*, 72−77.

Fuleki, T.; Francis, F. J. Quantitative methods for anthocyanins. 2. Determination of total anthocyanins and degradation index for cranberry juice. *J. Food Sci.* **1968b**, *33*, 78−83.

Maccarone, E.; Maccarrone, A.; Perrini, G.; Rapisarda, P. Anthocyanins of the Moro orange juice. *Ann. Chim.* **1983**, *73*, 533−539.

Maccarone, E.; Maccarrone, A.; Rapisarda, P. Acylated anthocyanins from oranges. *Ann. Chim.* **1985**, *75*, 79−86.

Maccarone, E.; Dugo, P.; Passerini, A. Effects of phenolic copigments in the flavylium-chromenol equilibrium of anthocyanins. *Ital. J. Food Sci.* **1992**, *4*, 247−258.

Maccarone, E. Rapisarda, P.; Fanella, F.; Arena, E.; Mondello, L. Cyanidin-3-(6″-malonyl)-glucoside. An important anthocyanin of blood orange juice. *Ital. J. Food Sci*. **1998**, *10*, 367−372.

McClure, J. W. Photocontrol of Spirodela intermedia flavonoids. *Plant Physiol*. **1967**, *43*, 193−200.

Miller, J.; Diplock, A. T.; Rice-Evans, C. A. Evaluation of the total antioxidant activity as a marker of the deterioration of apple juice on storage. *J. Agric. Food Chem*. **1995**, *43*, 1794−1801.

Rapisarda, P.; Giuffrida, A. Anthocyanin level in Italian blood oranges. *Proc. Int. Soc. Citriculture* **1992**, *3*, 1130−1133.

Rapisarda, P.; Fallico, B.; Izzo, R.; Maccarone, E. A simple and reliable method for determining anthocyanins in blood orange juices. *Agrochimica* **1994**, *38*, 157−164.

Rapisarda, P.; Tomaino, A.; Lo Cascio, R.; Bonina, F.; De Pasquale, A.; Saija, A. Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. *J. Agric. Food Chem*. **1999**, *47*, 4718−4723.

Siegelman, H. W.; Hendricks, S. B. Photocontrol of alcohol, aldehyde and anthocyanin production in apple skin. *Plant Physiol*. **1958**, *33*, 409−413.

Trifirò. A.; Postorino, E.; Grandi, R.; Gionfriddo, F.; Ragonese, C.; Ivaldi, L.; Gherardi, S.; Zoni, C.; Bovalo, F. Caratterizzazione del succo di arancia rossa refrigerato ottenuto non da concentrato. *Essenze, Deriv. Agrum*. **1996**, *66*, 267−278.

Wang, H.; Cao, G.; Prior, L. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem*. **1997**, *45*, 304−309.

Zapsalis, C.; Francis, F. J. Cranberry anthocyanins. *J. Food Sci.* **1965**, *30*, 396−399.

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