International Journal of Horticultural Science 2014, 20 (3–4): 93–97.
Agroinform Publishing House, Budapest, Printed in Hungary
ISSN 1585-0404

Optimization of sample preparation for determination of antioxidant parameters from one grape berry

Lukácsy[1], Gy., Stefanovits-Bányai[1], É., Papp,[1] N. & Kocsis,[2]* L.

[1]Corvinus University of Budapest, Faculty of Horticultural Sciences, Budapest, Hungary
*[2]*University of Pannonia, Georgikon Faculty, Keszthely, Hungary, kocsis-l@georgikon.hu*

Summary: Berry quality is an important issue in wineprocessing, however evaluating characteristics in a single berry level is very complex. The Phenolic compound analysis is more challenging because of the limited amount of material and a mixture of skin, pulp and seed. No clear protocol exists for evaluating these compounds from single berries. The aim of our investigation was to develop such a protocol. Single whole grape berries samples were homogenized in 20 replicates by 5 different methods. The most effective method was when berry was placed in a mortar and was crushed with a pestle under liquid nitrogen until seeds were clearly visible. Seeds were then taken from the mortar and crushed between two stainless steel plates with a hammer, and returned to the mortar without residues. Homogenization continued until the sample had a powdery appearance. The homogenized samples were taken for further analysis such as total polyphenols, total anthocyanin contents and total antioxidant capacity. Our results demonstrate that single berries could be processed and that the homogenates were suitable for taking chemically uniform subsamples.

Keywords: homogenization, antioxidant capacity, total phenol, total anthocyanin, grape berry

Introduction

Variability in fruit size and composition can greatly effect wine quality (Fernandez et al., 2006), and the resulting uneven berry ripening generally results in poor fruit quality. Several authors have examined the variability of vineyards on a vine and cluster basis (Kristic et al., 2002, Trought & Tannoc 1996). The variability of berries within clusters due to asynchronous berry development has also been studied (Kasimatis & Vilas 1985; Pagay & Cheng 2010). Non-homogeneous populations of berries or clusters can also elevate the sample size required for accurate vineyard sampling (Kasimatis & Vilas 1985; Wolpert & Vilas 1992). Single berry measurements are required to determine the effect of viticultural practices on berry variability within clusters and the adequate sample size needed for vineyard sampling. Basic quantitative (berry weight, seed number/berry) and qualitative (soluble solids) parameters are routinely examined on cluster level in the practice. Evaluating the phenolic compounds at the single berry level is more complex, due to the presence of seeds and skins. Sub-samples have to be taken to determine several indices from the same berry, which is challenging because of the limited amount of juice and tissue from a single berry.

Several in vitro methods have been developed to evaluate the total antioxidant capacity. However, no standardized method has been adopted because these methods are not able to model the complex processes and reactions in the human body. Methods for measuring total antioxidant capacity have been compared in numerous studies. When applied to food analysis, antioxidant capacity measurements may be different depending on the assay used, thus several methods have been used together. The Ferric Reducing Ability of Plasma (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC) are the most popularly used assays for total antioxidant capacity based on electron transfer. Total Polyphenol Content (TPC) was determined using Folin-Ciocaltue reagent. The main advantages and disadvantages of these methods have been described in various reviews (Apak et al., 2007; Huang et al., 2005; Pérez-Jiménez et al., 2008; van den Berg et al., 1999; Zulueta et al., 2009) and these are compared in Table1.

Measurements of the phenolics from a single berry require the separation of skin, pulp and seeds of berries. Analyses of anthocyanin and phenolic compounds are based on berry parts. However the majority of the analytical techniques used by the grape and wine industry to evaluate these compounds requires the separation and subsequent homogenization of skin, pulp and seeds from at least 50 berries. No clear protocol exists for evaluating these compounds from single berries. The aim of our investigation was to develop such a protocol.

Materials and methods

Sample collection

Berries of cv. Cabernet Sauvignon (*Vitis vinifera* L.) were collected from the experimental vineyard of Corvinus University of Budapest, in Budafok. Two hundred berries

with pedicels were randomly selected, tagged and placed in plastic bags. Samples were put in an insulated cooler and transported immediately to the laboratory for further preparation and analysis. In the laboratory the grapes were kept at –80 °C until preparation.

Chemicals and reagents

The following chemicals and reagents were purchased from Sigma-Aldrich Group (Schnelldorf, Germany): ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), ascorbic acid, Folin -Ciocalteu's phenol reagent, gallic acid, hydrochloric acid, iron(III) chloride hexahydrate, TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine), trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), phosphate buffered saline and potassium persulfate. Acetic acid, ethanol, methanol, sodium-carbonate and potassium-chloride were obtained from Reanal Zrt. (Budapest, Hungary) and sodium acetate was purchased from Merck Labs. (Budapest, Hungary).

Trial of homogenization methods

Single whole grape berries samples were homogenized in 20 replicates by 5 different methods. Berry pedicels were detached by hand and the berry was placed:

- Method "A": in a 15 ml centrifuge tube, and homogenized with a Heidolph Diax 600 homogenizer (Heidolph, Germany) with 10 mm head (24 000 rpm, 3 minutes),
- Method "B": in a 50 ml centrifuge tube, and homogenized with a Heidolph Diax 600 homogenizer (Heidolph, Germany) with 18 mm head (24 000 rpm, 3 minutes),
- Method "C": in a glass ball made by the manufacturer and homogenized with a Warszawa MPW-309 (Precise Mechanics, Warsaw, Poland) with 20 mm glass head (2000 rpm, 3 minutes),
- Method "D": in a mortar and was crushed with a pestle under liquid nitrogen and
- Method "E": in a mortar and was crushed with a pestle under liquid nitrogen until seeds were clearly visible. Seeds were then taken from the mortar and crushed between two stainless steel plates with a hammer, and returned to the mortar without residues. Homogenization continued until the sample had a powdery appearance.

The temperature of the samples after homogenization was determined with a Ebro TFI600 noncontact thermometer (Ebro Electronic Gmbh &Co Ingolstadt, Germany).

Determination the efficiency of homogenization methods

Homogenates from the five methods were visually observed in a Petri dish against a white background. The appearance of observable seed and skin pieces was examined and assigned a score of 1to 2: where 1 meant pieces could not be observed, and 2 meant pieces were observable. Homogenization methods that resulted in a score 1 were further analyzed. Two subsamples were taken from the single berry homogenates in 20 replicates for each analytical method to determine if there was a significant difference between values of the two subsamples. The five analytical methods were used and a total of 100 single berries were processed.

Sample preparation

For the FRAP, TEAC and TPC methods the homogenized samples were transferred to eppendorf tubes, centrifuged (Hettich Mikro 22 R, Tuttlingen, Germany) at 4 °C, for 35 min, at 18 750 g, and the supernatants were use for the measurements. For the anthocyanin assays the homogenized samples were transferred to 15 ml centrifuge tubes and were kept at –32 °C until analyzed.

Determination of total antioxidant capacity by FRAP method

The FRAP method was developed by Benzie & Strain (1996) and is based on the reduction of Fe^{3+} -TPTZ complex to the ferrous form (Fe^{2+} -TPTZ) by the antioxidants. This reaction is followed by colour changes. The increase of the absorbance can be measured at 593 nm. The FRAP reagent was prepared using 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride and 10 mM TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) made up in 40 mM HCl. All three solutions were mixed together in the ratio 10:1:1. To 1.5 mL of the FRAP reagent was added *x* ml standard or sample and (50-*x*) µL water. The final volume was 1.55 ml. The increase of the absorbance was measured after 5 minutes at 593 nm against a FRAP reagent blank. Ascorbic acid (1mM) was used for standard. The results were expressed in mmol ascorbic acid equivalent/ L.

Determination of total antioxidant capacity by TEAC method

The TEAC or ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) assay was described by Miller et al. (1993). It is based on scavenging of the $ABTS^{\bullet+}$ radical cation by the antioxidants. The $ABTS^{\bullet+}$ radical has a dark green colour. When there are antioxidants in the reaction medium they react with the free radicals. Therefore the reaction medium loses the colour and the absorbance decreases. The reaction is traceable with spectrophotometer at 734 nm.

ABTS (7 mM) and potassium persulfate (125 mM) were mixed in the ratio 50:1 and allowed to stand for 12-16 hours at room temperature in the dark while free radicals formed. Then the solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.7±0.02 at 734 nm. To 1960 µL of the $ABTS^{\bullet+}$ reagent was added *x* ml standard or sample and (40-*x*) µL of 5 mM phosphate buffer. The final volume was

2 ml. The blank was phosphate buffer (5 mM). Trolox was used as standard. The results were expressed in mmol trolox equivalent/ L.

Determination of total polyphenolic content (TPC)

The total phenolic content was determined with Folin-Ciocalteu reagent (Singleton et al., 1999). The reagent is a mixture of tungsten and molybdenum oxides. The product of the metal oxide reduction has a blue colour. The change of the absorbance is measured at 760 nm. To 1250 µL of the freshly prepared Folin-Ciocalteu reagent (1:10 with water) *x* µL sample or standard and (250-*x*) µL methanol-water (80:20 v/v %) was added. After 1 min, 1 ml sodium carbonate (0.7 M) was added to the solution. The mixture was incubated for 5 minutes at 50 ºC. The absorbance was measured at 760 nm using the Folin-Ciocalteu Reagent as blank. The soluble polyphenol content was calculated from a standard curve based on the gallic acid concentration. The results were expressed in mmol gallic acid equivalent/ L.

Determination of total monomeric anthocyanin content (TMA)

The pH differential method was applied to quantify the total monomeric anthocyanin content (TMAC) as described by Lee et al., (2005). The sample was extracted with 50% v/v aqueous ethanol and then placed into a chilled ultrasound bath for 1 hour. The extract of the sample was mixed with buffer at pH 1.0 and pH 4.5 (25 mM potassium potassium chloride and 400 mM sodium acetate, respectively). The sample should not exceed 20 % of the total volume because of the buffer's capacity. The dilutions were equilibrated for 15 min. Absorbances were measured at 520 and 700 nm. The monomeric anthocyanin content was expressed as mg cyanidin-3-glycoside / g fresh weight (ε = 26900, molecular weight=449.2 g/mol).

Determination of total anthocyanin content (TA)

The total anthocyanin content was determined a method described by Iland et al., (1996). The sample was extracted with 50% v/v aqueous ethanol and it was then placed into chilled ultasound bath for 1 hour. After this incubation, 200 µL of the extract was added to 3.8 mL of 1.0 M HCl. It was then covered with Parafilm and incubated at room temperature for at least 3 hours, but for no longer than 24 hours. The blank was 1.0 M HCl. The absorbance was measured at 520 nm. The total anthocyanin content was expressed as mg malvidin-3-glycoside / g fresh weight (ε = 26 455, molecular weight=529 g/mol).

All spectrophotometric measurements were done with a Nicolet Evolution 300 BB spectrophotometer (Thermo Electron Corporation, Cambridge, UK).

Statistica-l analysis

Statistical analysis of the data using a paired sample test was done with SPSS (version 14) statistical software with a significance level of $p < 0.05$.

Result and discussion

Efficiency of the homogenization (visual and temperature)

Results of visual and temperature examinations are summarized in Table 2.

Table 2. Effect of processing method on sample homogenity and temperature

Method	Scores of visual observations		Temperature (ºC)	Comment
	skin	seed		
A	2[1]	2	35 ±8	skin and seed parts remained
B	2	2	33 ±7	skin and seed parts remained
C	2	2	38 ±4	skin parts and whole seed remained
D	1	2	<0	whole seed remained
E	1	1	<0	visually homogenized sample

[1] 1= non observable; 2 = observable

Method "A" and "B", based on Heidolph Diax 600 homogenizer, did not adequately prepare single berry samples. The homogenate contained observable parts (>1mm) of berry skins and seeds. The size of the homogenizer head (10mm in method "A", and 18mm in method "B") had no effect on clarity of the sample. Efforts to better homogenize the sample with longer grinding times were not successful because of the generation of excess heat and subsequent degradation of antioxidant molecules. Method "C" did not completely homogenize the one berry samples either. The glass head of Warszawa homogenizer left untouched seeds, and observable (>1mm) skin parts. In addition, the temperature of the final homogenate occasionally reached 42 ºC. Method "D" adequately homogenized the skin of the whole berry, and no visible skin parts reminded in the mortar. However the seed appeared untouched. Thus, method "D" did not adequately prepare berry samples for antioxidant and polyphenol measurements, although it might be useful for determining anthocyanin concentration from a single berry without peeling its skin. Our aim was to select a method capable of antioxidant and anthocyanin assays from the same berry. The presence of unprocessed seed in the sample will not allow these concurrent measures as the polyphenol content and antioxidant capacity of a berry is highly effected by seeds. Therefore once the seeds appeared while berries were being homogenized under liquid nitrogen, they were removed from the mortar and crushed between steel plates. The crushed seeds were returned to the same mortar for further homogenization with the skin and pulp. This method, method "E" was considered to be the most suitable for one berry homogenization based on visual examination. Our

Table 3. Statistical analysis of the results the total antioxidant capacity by FRAP and TEAC, the total polyphenol content by TPC and the total anthocyanin content by TMA and TA

Method	Paired Differences			95% Confidence Interval of the Difference		t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	Lower	Upper			
FRAP	-,013	,377	,084	-,189	,164	-,152	19	,881
TEAC	,190	,572	,128	-,078	,457	1,484	19	,154
TPC	,083	1,356	,303	-,552	,717	,272	19	,788
TMA	1,920	6,896	1,542	-1,307	5,147	1,245	19	,228
TA	2,753	14,420	3,225	-3,996	9,502	,854	19	,404

FRAP= Ferric Reducing Ability of Plasma , TEAC= Trolox Equivalent Antioxidant Capacity, TPC= Total Polyphenol Content, TMA= Total Monomeric Anthocyanin, TA= Total Anthocyanin

results show that not all the homogenization methods are usable for further analysis of a single berry. The results of the total antioxidant capacity shows (Table 3.) that in general FRAP is hardly can be used with most of the homogenization process. Also important which method we use for homogenization of total polyphenols content measurements. The mean of our data indicate that some of the methods are not suitable. The best result for all analysis was obtained by the application of method "E".

Efficiency of the homogenization (chemical examination)

We then used Method "E" to process 100 individual berries. Two subsamples were taken from the homogenate of each berry. The subsamples were analyzed in pairs by five different analytical methods to determine antioxidant capacity, total polyphenols and total anthocyanins. Results of the paired sample test are presented in Table 3. Based on the T-test analysis of paired sample statistics the values of the two subsamples did not differ significantly. The results demonstrate that single berries could be processed and that the homogenates were suitable for taking chemically uniform subsamples. The procedure is summarized in Figure 1.

References

Apak, R., K. Güclü, B. Demirata, M. Özyürek, S.E. Celik, B. Bektasoglu, K.I. Berker, D. Özyurt. (2007): Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. Molecules. 12: 1496-1547.

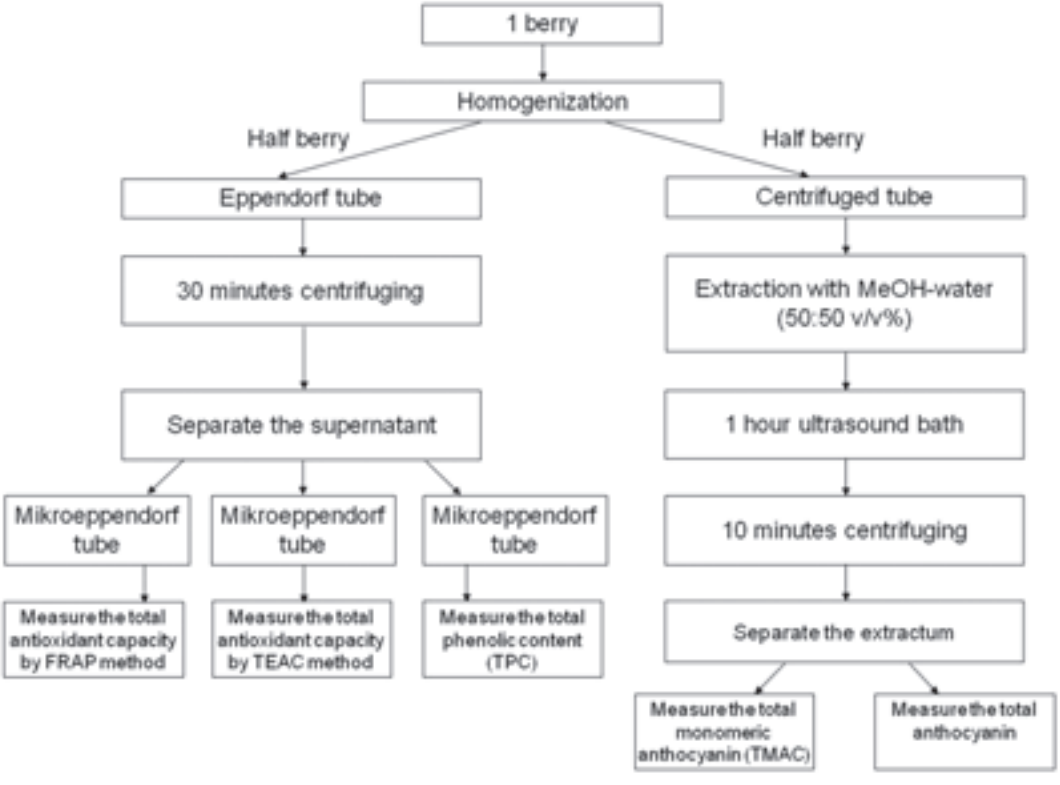

Figure 1. Sample peparation step by step

Benzie, I.F.F. & Strain, J.J. (1996): The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Analytical Bochemistry. 239:70-76.

Bertelli, A.A.A. & D.K. Das. (2009): Grapes, Wines, Resveratrol, and Heart Health. J. Cardiovasc. Pharmacol. 54: 468-476

Gómez Gallego, M.A., E. Gómez García-Carpintero, E. Sánchez-Palomo, I. Hermosín-Gutiérrez & M.A. González Viñas. (2012): Study of phenolic composition and sensory properties of red grape varieties in danger of extinction from the Spanish region of Castilla-La Mancha. European Food Research and Technology: 1-9.

Huang, D., O.U. Boxin & R.L. Prior. (2005): The chemistry behind antioxidant capacity assays. J. Agric. Food. Chem. 53: 1841-1856.

Iland, P.G., Cynkar, W., Francis, I.R., Williams, P.J. & Coombe, B.G. (1996): Optimisation of methods for the determination of total and red-free glycosyl glucose in black grape berries of *Vitis vinifera.* Australian Journal of Grape and Wine Research. 2:171-178.

Joshipura KJ, Hu F, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, Colditz G, Ascherio A, Rosner B, Spiegelman D & W. WC. (2001): Fruit and vegetable intake in relation to risk of coronary heart disease. Annals of Internal Medicine. 134: 1-9.

Kasimatis, A.N. & E.P. Vilas. (1985): Sampling for Degrees Brix in Vineyard Plots. Am. J. Enol. Vitic. 36: 207-213.

Krstic, M.P., Leamon, K., Degaris, K., Whiting, J., Mccarthy, M. & Clingeleffer, P. (2002): Sampling for wine grape quality parameters in the vineyard: Variability and post-harvest issues. [In Proceedings of the Eleventh Australian Wine Industry Technical Conference.] **R.J. Blair et al.** (eds.). Australian Wine Industry Technical Conference, Adelaide. 87-90.

Lee, J., Durst, R. W. & Wrolstad, R.E. (2005): Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborativ study. Journal of AOAC International. 88:1269-1278.

Naziroglu, M. & P.J. Butterworth. (2005): Protective Effects of Moderate Exercise With Dietary Vitamin C and E on Blood Antioxidative Defense Mechanism in Rats With Streptozotocin-Induced Diabetes. Canadian Journal of Applied Physiology. 30: 172-185.

Pagay, V. & L. Cheng. (2010): Variability in Berry Maturation of Concord and Cabernet franc in a Cool Climate. Am. J. Enol. Vitic. 61: 61-67.

Pérez-Jiménez, J., S. Arranz, M. Tabernero, M.E. Díaz-Rubio, J. Serrano, I. Goñi & F. Saura-Calixto. (2008): Updated methodology to determine antioxidant capacity in plant foods, oils and beverages: Extraction, measurement and expression of results. Food Research International. 41: 274-285.

Pezzuto, J.M. (2008): Grapes and Human Health: A Perspective. J. Agric. Food. Chem. 56: 6777-6784.

Schoonen, W.M., C.A. Salinas, L.A.L.M. Kiemeney & J.L. Stanford. (2005): Alcohol consumption and risk of prostate cancer in middle-aged men. International Journal of Cancer. 113: 133-140.

Tebib, K., J.M. Rouanet and P. Besanã§on (1997): Antioxidant effects of dietary polymeric grape seed tannins in tissues of rats fed a high cholesterol-vitamin E-deficient diet. Food Chemistry. 59: 135-141.

Trought, M.C.T. & Tannock, S.J.C. (1996): Berry size and soluble solids variation within a bunch of grapes. [In Proceedings for the Fourth International Symposium on Cool Climate Enology and Viticulture.] **T. Henick-Kling et al.** (eds.). New York State Agricultural Experiment Station, Geneva. 70-74.

van den Berg, R., G.R.M.M. Haenen, H. van den Berg & A. Bast. (1999): Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chemistry. 66: 511-517.

Waladkhani, A.R. & M.R. Clemens. (1998): Effect of dietary phytochemicals on cancer development (review). International journal of molecular medicine. 1: 747-53.

Wolpert, J.A. & E.P. Vilas. (1992): Estimating Vineyard Yields: Introduction to a Simple, Two-Step Method. Am. J. Enol. Vitic. 43: 384-388.

Zulueta, A., M.J. Esteve & A. Frígola. (2009): ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. Food Chemistry. 114: 310-316.