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Relationship between the antioxidant properties and the phenolic and flavonoid content in traditional balsamic vinegar

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Abstract

The antioxidant properties of traditional balsamic vinegar as regards its phenolic and flavonoid content comparing it to selected vinegars and red wines have been investigated. The polyphenols were separated from interfering compounds utilizing C18 columns. The polyphenolic content was determined utilizing both Folin–Ciocalteu and peroxidase assays. The antioxidant capacity was quantified using both ABTS and FRAP assays. The results show that traditional balsamic vinegar has lower antioxidant activity and phenolic and flavonoid content than Nero d'Avola but higher than the other tested products. The antioxidant capacity of wines and vinegars is highly correlated with their phenolic content, measured by peroxidase assay and it is also highly correlated with their flavonoid content while in traditional balsamic vinegar and balsamic vinegar this correlation diminishes. The study describes a simple and fast method of separating from other compounds and of measuring polyphenols in the analysis of red wines and vinegars with complex composition such as traditional balsamic vinegar.

Keywords: Antioxidant capacity; Polyphenolic content; Flavonoid content; Traditional balsamic vinegar

1. Introduction

The consumption of fruit and vegetables, and all the food and drink derived from these, has been inversely associated with morbidity and mortality from degenerative and coronary heart diseases (Block, Patterson, & Subar, 1992; Gillman et al., 1995). The protection that fruit and vegetables provide against diseases has been attributed to the various antioxidants contained in them (Gey, 1990). Particularly polyphenols show protective effects on brain degenerative processes (Conte, Pellegrini, & Tagliazucchi, 2003) and have antiinflammatory (Subbaramaiah et al., 1998), anticarcinogenic (Kuroda & Hara, 1999), antiatherogenic (Dell'Agli, Buscialà, & Bosisio, 2004) and cardioprotective (Visioli, Borsani, & Galli, 2000) effects.

Not all vegetables, fruit and their derivatives have the same phenolic composition and not all phenolics have the same antioxidant capacity (Rice-Evans, Miller, & Paganga, 1996; Velioglu, Mazza, Gao, & Oomah, 1998). Hence, it is the quality and not the quantity of polyphenols that determines the antioxidant capacity of food. For example, cabbage and green chiles have the same phenolic content (~100 mg/100 g of product) but for cabbage over 50% are flavonoids, whereas in green chiles only 9% are flavonoids (Ninfali, Mea, Giorgini, Rocchi, & Bacchiocca, 2005). Moreover, cabbage has an antioxidant capacity that is 5-fold higher than that of green chiles. Spinach has a slightly lower total phenolic content than green chiles but a 4-fold higher flavonoid content and an antioxidant capacity that is 5-fold higher (Ninfali et al., 2005).

The presence of phenolics in red wine and vinegar has positive health effects because these products maintain a good portion of the phenolics present in the grapes, expressing a significant antioxidant capacity. The most

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abundant phenolic compounds in red wine are phenolic acids and flavonoids. The most abundant non-flavonoid phenolic compound in red wine is resveratrol (Arnous, Makris, & Kefalas, 2001; Lopez, Martinez, Del Valle, Orte, & Miro, 2001). White wine contains more phenolic acids (mainly hydroxycinnamic acids) but less flavonoids and resveratrol with respect to red wine (Benassi & Cecchi, 1998). The concentration of total phenols ranges from 800 to 4000 mg/l for red wines and from 200 to 1000 mg/l for white wines (Ronca, Palmieri, Maltinti, Tagliazucchi, & Conte, 2003). The wine vinegars are also rich in polyphenols (Andlauer, Stumpf, & Fürst, 2000; García-Parrílla, León Camacho, Heredia, & Troncoso, 1994), being derivative products of wine, and also exhibit potential health benefits, such as the antihypertensive effects observed in rats (Dávalos, Bartolomé, & Gómez-Cordovés, 2005). It has been demonstrated that wine vinegars show an antioxidant capacity that is correlated with their polyphenolic content (Dávalos et al., 2005). Balsamic and traditional balsamic vinegars (BV and TBV, respectively) from Modena and Reggio Emilia are unique products, produced from the alcoholic fermentation and acetic bioxidation of cooked and concentrated locally grown grape must. The differences between balsamic and traditional balsamic vinegars are mainly due to the aging period and production procedures. For TBV the use of any extra additives is forbidden. TBV is a natural product, prepared with cooked and concentrated must in wood barrels containing aged TBV, natural yeasts providing alcoholic fermentation of sugars and acetobacters. The latter process is performed in a series of barrels, containing aged TBV, of varying capacity and made of different woods for at least 12 years. BV, on the contrary, is prepared with cooked and concentrated must, wine vinegar and flavouring like caramel, and its aging period ranges from two months to 20 years, depending on the quality.

To our knowledge, no data about the flavonoid content of BV and TBV are present in the literature.

Red wines and vinegars have a lot of substances (such as sulphur dioxide, ascorbic acid, ethanol, and reducing sugars) that can interfere with the total phenol, flavonoid, and antioxidant activity assays. The high concentrations of sugars (mainly glucose and fructose) and Maillard reaction products present in TBV and BV could also interfere with these assays. The aim of this work is to study the antioxidant properties of TBV and BV as regards their phenolic and flavonoid content after separation of polyphenols from other water-soluble compounds that potentially interfere with the assays.

2. Materials and methods

2.1. Materials

Folin–Ciocalteu reagent, catechin, quercetin, resveratrol, epigallocatechin gallate (EGCG), gallic acid, cinnamic acid, 2,4,6-tripyridyl-*S*-triazine (TPTZ), 5-hydroxymethyl-furfural (HMF), ascorbic acid (vitamin C), 4-aminoantipyrine (4-AP), and horseradish peroxidase (HRP), type II, were supplied from Sigma (Milan, Italy). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was supplied from Calbiochem (La Jolla, CA). The enzymatic kit for D-glucose and D-fructose determination was from Roche (Darmstadt, Germany). The colorimetric kit Fructosamines NTB assay was from FAR (Verona, Italy). All the other chemical reagents were from Carlo Erba (Milan, Italy). Sephadex C-18 columns (quantity of sorbent 500 mg, volume above packing 6 ml, catalogue number 205350) were supplied from Alltech (Deerfield, IL). Traditional balsamic vinegar (TBV) and balsamic vinegar (BV) were kindly supplied by the "Consorzio fra produttori di Aceto Balsamico Tradizionale di Reggio Emilia" (Reggio Emilia, Italy). The red wine vinegar (RWV) with 7.5% of acidity was supplied by a local producer. Red wines (Nero d'Avola, Dolcetto d'Alba, and Lambrusco) were purchased from a local supermarket. The absorbance was read using a Jasco V-550 UV/Vis spectrophotometer.

2.2. Determination of D-glucose/D-fructose and Maillard reaction products

The content of D-glucose and D-fructose in the samples was assayed using a hexokinase and glucose-6-phosphate dehydrogenase method. The Maillard reaction products (MRP) content in the samples was determined using a colorimetric method based on the nitroblue tetrazolium (NTB) reduction by hydroxilamine group and expressed as μM of glycosilate albumin. Glycosilate albumin prepared in accordance with Dolhofer and Wieland (1979) and a MRP mixture prepared from glucose-histidine in accordance with Yilmaz and Toledo (2005) were utilized as MRP standards. With both standards the NTB assay was linear up to 1000 μmol/l of glycosilate albumin.

2.3. Phenolic extraction and recovery

Because red wines and vinegars contain a lot of compounds that potentially interfere with the common assays used to determine phenolic content and antioxidant activity, Sephadex C-18 columns were used to separate the phenols from these compounds (Robards & Antolovich, 1997). Briefly, 0.2 ml of red wine or vinegar was passed through the column. Columns were washed 3 times with 2 ml of water and adsorbed phenols were eluted 3 times with 3 ml of methanol (HPLC grade) on the basis of UV monitoring. At the end of the separation, the aqueous (W1, W2 and W3) and methanolic fractions (M1, M2 and M3), were separately analyzed. Different standards (polyphenols, glucose, fructose, MRP standards, ascorbic acid, and 5-hydroxymethyl-furfural) were passed through the Sephadex C-18 columns to test the recovery percentage in the aqueous and methanolic fractions. Briefly, 1 ml of standard solution was loaded into the columns. Each standard solution contained a concentration of standard compound such that present in red wine or vinegar (Table 1). The

Table 1
Percentage of recovery in the aqueous and methanolic fractions of different compounds after passage through Sephadex C-18 column

Compound	Quantity (μmol)	Percentage of recovery in the aqueous fractions (W1 + W2 + W3)	Percentage of recovery in the methanolic fractions (M1 + M2 + M3)
Catechin	0.10	0 ± 0	100 ± 0
EGCG	0.10	0 ± 0	97 ± 1
Quercetin	0.10	0 ± 0	55 ± 2
Cinnamic acid	0.10	0 ± 0	98 ± 2
Gallic acid	0.10	70 ± 5	30 ± 5
Resveratrol	0.20	0 ± 0	97 ± 1
Total phenolics (Lambrusco)	0.65	4 ± 1	93 ± 2
Total phenolics (Nero d'Avola)	2.12	2 ± 1	96 ± 1
HMF 1:300	2.38	0 ± 0	98 ± 1
Ascorbic acid	2.27	100 ± 0	0 ± 0
D-Glucose	302.77	100 ± 0	0 ± 0
D-Fructose	302.77	100 ± 0	0 ± 0
Glu–His mixture	7.00	60 ± 4	3 ± 2
Glycosyl–albumin	7.00	74 ± 5	26 ± 4

columns were washed 3 times with 2 ml of water and the compounds were eluted 3 times with 3 ml of methanol (HPLC grade). The percentage of recovery of the different compounds was assayed in the aqueous and methanolic fractions. D-Glucose and D-fructose were dissolved in water and determined by utilizing the enzymatic method described above. MRPs were prepared as described above and determined with fructosamine NTB assay and at 420 nm. Quercetin was dissolved in a 5% DMSO solution and determined at 370 nm; resveratrol was dissolved in a 1% DMSO solution and determined at 305 nm; catechin, gallic acid and EGCG were dissolved in water and determined at 280 nm; cinnamic acid and HMF were dissolved in methanol and determined at 280 nm. Ascorbic acid was dissolved in water and determined at 252 nm. The wavelength used for each standard was the λ of maximum absorption as spectrophotometrically determined.

2.4. Determination of total phenolic content

The total phenolic content was determined with the Folin–Ciocalteu reagent, in accordance with the method of Singleton, Orthofer, and Lamuela-Raventos (1999), adapted to a microscale. In a 1.5 ml Eppendorf tube 790 μl of distilled water, 10 μl of appropriately diluted aqueous or methanolic fractions or catechin standard solutions and 50 μl of the Folin–Ciocalteu reagent were added and mixed. Water (10 μl) or methanol were used as blank. After exactly 1 min, 150 μl of 20% aqueous sodium carbonate was added, the mixture was mixed and left to stand at room temperature in the dark for 120 min. Detection was achieved at 760 nm. In some experiment, 10 μl of HMF standard solution 0.7 M (5.5 g/l) dissolved in methanol or 10 μl of MRP standard solutions (ranging from 5 to 100 mM), prepared as described above, were utilized. Catechin standard solutions were prepared by dissolving catechin in water at a concentration ranging from 5 to 50 mg in 100 ml.

The total phenolic content was also determined using an enzymatic method, in accordance with Stevanato, Fabris, and Momo (2004). In a 3 ml spectrophotometric cell, 0.1 ml of appropriately diluted aqueous or methanolic fractions or catechin standard solutions was added to 3 ml of 0.1 M potassium phosphate-buffered solution, pH 8, containing 3 mM 4-AP, 2 mM H_2O_2, and 0.33 μM horseradish peroxidase (HRP). Water (0.1 ml) or methanol were used as blank. The absorbance value was read at 500 nm at the endpoint of 10 min. In some experiment, 0.1 ml of HMF standard solution or 0.1 ml of MRP standard solutions or 0.1 ml of glucose 0.3 M and fructose 0.3 M solutions were utlilized. Catechin standard solutions were prepared by dissolving catechin in water at a concentration ranging from 0.1 to 3 mg in 100 ml. In both the assays the total phenolic content was expressed in milligrams of catechin equivalents per 100 ml of product.

2.5. Determination of flavonoids

The total flavonoid concentration was measured using a colorimetric assay in accordance with the method of Zhishen, Mengeheng, and Jianming (1999). Briefly, 1 ml of appropriately diluted aqueous or methanolic fractions or catechin standard solutions or water or methanol as blank was added to 4 ml of distilled water. At time zero, 0.3 ml of 5% $NaNO_2$ was added. Ten percent of $AlCl_3$ (0.3 ml) was added 5 min later. At 6 min, 2 ml of 1 M NaOH was added and the solution was made up to 10 ml with distilled water and mixed. The absorbance was determined at 510 nm against an appropriate blank. The total flavonoid content was expressed in milligrams of catechin equivalent per 100 ml of product. Catechin standard solutions were prepared by dissolving catechin in water at a concentration ranging from 2.5 to 25 mg in 100 ml.

2.6. ABTS radical scavenging activity

The ABTS was dissolved in distilled water to 14 mM concentration. ABTS radical cation ($ABTS^{\cdot+}$) was produced by reacting to the ratio of 1:1 ABTS stock solution with 4.9 mM potassium persulphate solution and leaving the mixture to stand in the dark at room temperature for 12–16 h before use (Re et al., 1999). The concentration of the resulting blue-green ABTS radical solution was adjusted to an absorbance of 0.650 ± 0.020 at 734 nm. Appropriately diluted aqueous (40 μl) or methanolic fractions or HMF standard solution 0.7 M dissolved in methanol or MRP standards at a concentration ranging from 5 to 100 mM, prepared as described above, were added to 1960 μl of the resulting blue-green $ABTS^{\cdot+}$. The mixture, protected from the light, was incubated in the Jasco V-550 spectrophotometer at 37 °C for 10 min; the decrease in

absorbance at 734 nm was measured at the endpoint of 10 min.

2.7. Measurement of the reducing power

For the determination of the reducing ability of wines and vinegars, a protocol based on the ferric reducing/antioxidant power (FRAP) assay was utilized (Benzie & Strain, 1999). Briefly, 0.1 ml of appropriately diluted aqueous or methanolic fractions or HMF standard solution 0.7 M dissolved in methanol were added to 3 ml of FRAP reagent that was freshly prepared by mixing 300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at a ratio of 10:1:1. After exactly 6 min, the absorbance was read at 593 nm.

2.8. Antioxidant capacity calculation

FRAP and ABTS units of the samples were measured and calculated as vitamin C equivalent antioxidant capacity (VCEAC) in accordance with the method described by Kim, Lee, Lee, and Lee (2002). Vitamin C standard curves that correlate the concentration of vitamin C (ranging from 0.1 to 15 mg/100 ml) and the amount of absorbance reduction (ABTS scavenging assay) or absorbance increase (FRAP assay), caused by vitamin C, were obtained. The results were calculated as milligrams of VCEAC per 100 ml of product.

2.9. Statistics

All data are presented as means ± SD for at least five replications for each prepared sample. The statistical analysis and regression analyses were performed using GraphPad Instat (GraphPad Software, San Diego, CA). Differences of $P < 0.05$ were considered significant. Correlation between the antioxidant capacity, the total phenolic content and flavonoid content were established using regression analysis at a 99% significance level.

3. Results

Table 1 shows the recovery of the different compounds after passage through Sephadex C-18 columns. The flavonoids catechin and EGCG and the stilbene resveratrol are totally recovered in the methanolic fractions. For quercetin only about 55% is recovered in the methanolic fractions; the remaining percentage remains bound to the column. Among the phenolic acids, cinnamic acid is totally recovered in the methanolic fractions, while 70% of gallic acid is recovered in the aqueous fractions. Table 1 also shows that more than 90% of the polyphenols contained in the red wines Lambrusco and Nero d'Avola, determined using the peroxidase enzymatic assay, were recovered in the methanolic fractions. When the Folin–Ciocalteu method was utilized the percentage of recovery in the methanolic fractions was 86% and 92%, respectively for Lambrusco and Nero d'Avola. The remaining percentage was recovered in the aqueous fractions. D-glucose, D-fructose, and ascorbic acid were totally recovered in the aqueous fractions, while HMF standard is totally recovered in the methanolic fractions. MRP standards show different behaviour in the recovery test. More than 70% of glycosylate albumin is recovered in the aqueous fractions while the remaining percentage is recovered in the methanolic fractions. Glucose-histidine standard mixture recovery was 60% in the acqueous fractions, 3% in the methanolic fractions while the remaining percentage remains bound to the column.

The total phenolic content in the methanolic fractions was determined using two different assays. As shown in Fig. 1, the concentration of phenolics in the methanolic fractions of wines and RWV is almost the same as for the Folin–Ciocalteu and peroxidase assays: 325.26 and 305.79 mg catechin/100 ml, respectively, for Nero d'Avola; 91.12 and 91.13 mg catechin/100 ml, respectively, for Lambrusco; 212.60 and 201.75 mg catechin/100 ml, respectively, for Dolcetto d'Alba; 71.17 and 64.95 mg catechin/100 ml, respectively, for RWV. Instead, the concentration of phenolics determined in TBV and BV is different for the Folin–Ciocalteu and peroxidase assays. As shown in Fig. 1, in TBV and BV the polyphenol concentration determined by the Folin–Ciocalteu assay is higher than that determined by peroxidase assay: 372.00 and 272.45 mg catechin/100 ml, respectively, for TBV; 199.53 and 139.48 mg catechin/100 ml, respectively, for BV. Because the results obtained in wines and RWV with the peroxidase assay coincide with the value obtained with the Folin–Ciocalteu assay, the presence of peroxidase inhibitors in the methanolic fraction of TBV and BV was verified by carring out the assay using variable amounts of samples. The results show that polyphenols concentration in TBV and BV determined by peroxidase assay is linear with the samples dilutions

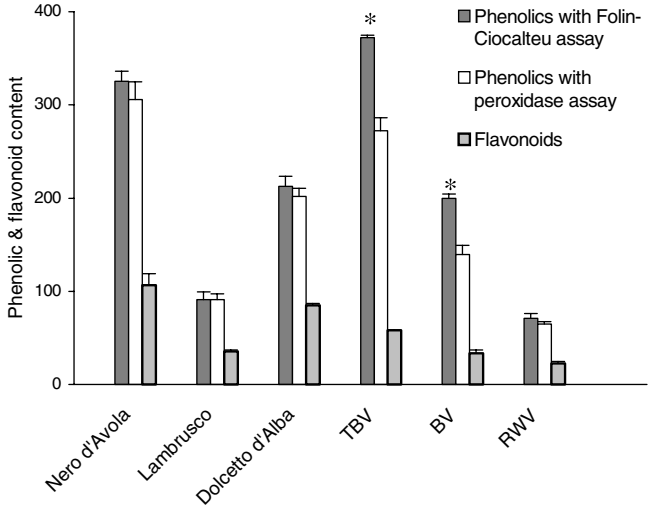

Fig. 1. Total phenolic and flavonoid contents of Nero d'Avola, Lambrusco, Dolcetto d'Alba, TBV, BV, and RWV expressed as milligrams of catechin equivalent per 100 ml of product. Data are means ± SD ($n = 5$). * denotes $P < 0.01$ with respect to the enzymatic peroxidase assay.

(data not shown), demonstrating the absence of inhibitors in the samples.

HMF standard does not react with the Folin–Ciocalteu and the peroxidase assays. MRP standards react in a concentration dependent manner in the Folin–Ciocalteu assay while no reaction were observed with peroxidase assay. Also reducing sugars such as glucose and fructose do not react with peroxidase assay while they react with the Folin–Ciocalteu assay. Because the higher values obtained with the Folin–Ciocalteu assay could be due to the presence of these interfering compounds in the methanolic fraction of TBV and BV, the glucose, fructose and MRPs content in the aqueous and methanolic fractions of BV, TBV, red wines and RWV were determined. Glucose and fructose, according to the recovery tests, were totally recovered in the aqueous fractions (W1 + W2 + W3) and the sum of the concentrations of glucose and fructose was 1.89 and 3.44 M in BV and TBV, respectively. Sugars were not found in the aqueous fractions of red wines and RWV. MRPs are present both in the methanolic and aqueous fractions of BV and TBV with a concentration of 8.85 and 33.35 mM in the aqueous fractions (W1 + W2 + W3) and 0.45 and 2.25 mM in the methanolic fractions (M1 + M2 + M3) of BV and TBV, respectively. The concentrations of MRPs in the aqueous fractions decrease, passing from W1 to W3 and an increase in the number of washings with water did not change the amount of compounds recovered in the methanolic fractions, suggesting that some MRPs are adsorbed at the hydrophobic stationary phase of the columns. MRPs were not found in the aqueous and methanolic fractions of red wines and RWV. The presence, in the methanolic fractions, of Maillard products that react with Folin–Ciocalteu but not with the enzymatic method could explain the higher polyphenol concentration values determined by Folin–Ciocalteu assay respect to peroxidase assay in TBV and BV.

Because some phenolic acids are recovered in the aqueous fractions, we have tested the presence of phenolic compounds in these fractions with the peroxidase assay. The results show that the concentration of phenolics was 5.02 mg catechin/100 ml for Nero d'Avola, 4.69 mg catechin/100 ml for Dolcetto d'Alba and 4.31 mg catechin/100 ml for Lambrusco. Phenolics were not found in the aqueous fractions of the vinegars when measured with the peroxidase assay because their concentration was under the detection limit of the assay (detection limit $\leqslant$4 mg/l with gallic acid as standard).

The flavonoid content in the methanolic fractions is highest for Nero d'Avola (106.50 mg catechin/100 ml), followed by Dolcetto d'Alba (84.83 mg catechin/100 ml), TBV (58.06 mg catechin/100 ml), Lambrusco (35.36 mg catechin/100 ml), BV (33.47 mg catechin/100 ml), and RWV (22.90 mg catechin/100 ml) (Fig. 1). About 1/3 of phenolics in red wines and RWV are flavonoids while in TBV and BV the flavonoids:phenolics ratio is about 1/4 (Fig. 1).

The flavonoid content in the aqueous fractions was zero for all of the samples.

The total antioxidant capacity of the different samples was also tested, using two different methods: FRAP assay and ABTS assay. Table 2 shows that the total antioxidant capacity in the methanolic fractions of the samples measured with the ABTS assay is as follows in decreasing order: Nero d'Avola > TBV > Dolcetto d'Alba > BV > Lambrusco > RWV. In contrast, when the antioxidant activity was measured in the methanolic fractions with the FRAP assay the order of effectiveness is: TBV > Nero d'Avola > Dolcetto d'Alba > BV > Lambrusco > RWV. The ABTS and FRAP assays are highly correlated ($r = 0.937$) in spite of the different VCEAC values, indicating that the FRAP assay underestimates the antioxidant capacity by ~45% compared with the ABTS assay. The antioxidant activity in the methanolic fractions of all the tested samples are dose-dependent in both assays (data not shown) and is highly correlated with their phenolic content measured using the peroxidase assay (Fig. 2). The total antioxidant capacity of red wines and RWV methanolic fractions is also highly correlated (Fig. 3) with their flavonoid content ($r = 0.994$ and $r = 0.988$ when the antioxidant activity was assayed with the ABTS and FRAP assay, respectively). The correlation between total antioxidant capacities and flavonoid content (Fig. 3) diminishes when TBV and BV methanolic fractions are also considered ($r = 0.914$ and $r = 0.728$ with the ABTS and FRAP assay, respectively).

The antioxidant activity in the aqueous fractions was also assayed. The VCEAC values were higher with the ABTS assay than the FRAP assay: 13.77 and 6.60 VCEAC, respectively for Nero d'Avola; 10.58 and 4.35 VCEAC, respectively for Dolcetto d'Alba; 5.35 and 2.50 VCEAC, respectively for Lambrusco; 5.23 and 2.71 VCEAC, respectively for RWV; 66.42 and 15.40 VCEAC, respectively for BV; 76.47 and 32.04 VCEAC, respectively for TBV.

The antioxidant activity of HMF standard and of the two MRP standards was assayed with the ABTS method because it proved more complete and sensible than the FRAP assay. At 3 mM concentration, that is the concentration of MRPs found in the methanolic fraction of TBV, the glucose-histidine standard showed an antioxidant

Table 2
Antioxidant capacity of red wines and vinegars measured by FRAP and ABTS assays

Sample	FRAP VCEAC value[a]	ABTS VCEAC value[a]
Red wines		
Nero d'Avola	196.03 ± 5.70	363.20 ± 10.23
Lambrusco	61.83 ± 3.60	137.50 ± 5.65
Dolcetto d'Alba	134.83 ± 5.12	266.00 ± 9.22
Vinegars		
TBV	218.85 ± 6.86	298.10 ± 6.25
BV	106.38 ± 4.41	177.85 ± 8.01
RWV	48.18 ± 2.00	85.40 ± 1.73

[a] Value are expressed as mg of VCEAC/100 ml of product and are the mean ± SD ($n = 5$).

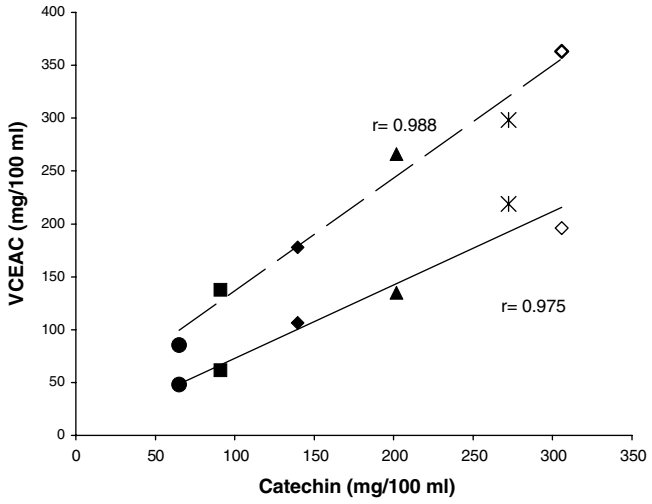

Fig. 2. Relationship between total phenolic content determined by peroxidase assay and VCEACs of (◇) Nero d'Avola, (■) Lambrusco, (▲) Dolcetto d'Alba, (✳) TBV, (◇) BV, and (●) RWV evaluated by FRAP (solid line) and ABTS (dotted line) assays. Data are means ± SD ($n = 5$).

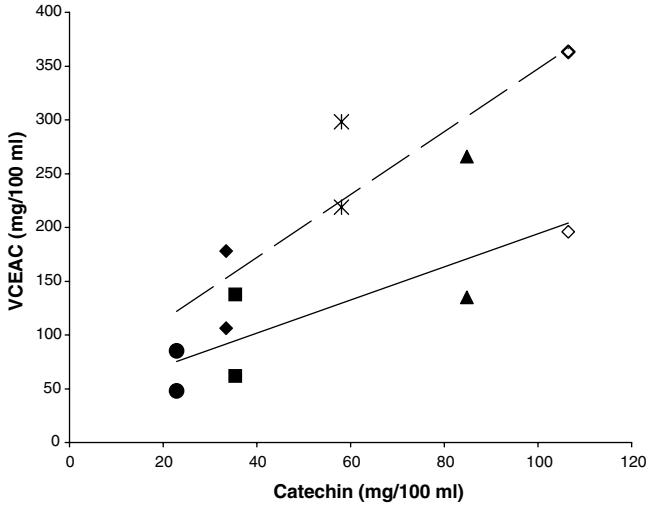

Fig. 3. Relationship between total flavonoid content and VCEACs of (◇) Nero d'Avola, (■) Lambrusco, (▲) Dolcetto d'Alba, (●) RWV, (✳) TBV, and (◇) BV evaluated by FRAP (solid line) and ABTS (dotted line) assays. Data are means ± SD ($n = 5$).

activity of 9 mg VCEAC/100 ml. The antioxidant activity was concentration dependent. Glycosilate albumin showed a greater antioxidant activity than albumin but at 3 mM concentration its antioxidant activity was too low and not detectable with ABTS assay.

HMF standard does not show antioxidant activity with ABTS method. For this reason it has been also assayed with FRAP method. No reaction were also observed with this assay.

4. Discussion

The method described in this paper represents a fast and simple method of efficiently separating polyphenols from other compounds that can interfere with Folin–Ciocalteu and antioxidant activity assays in red wine and red wine vinegar. After removal of interfering compounds with C-18 columns, the phenolic content in the methanolic fractions obtained with the Folin–Ciocalteu and peroxidase methods were the same in wine and RWV samples. In TBV and BV, the polyphenolic content was lower when determined by peroxidase assay respect to the value obtained with the Folin–Ciocalteu assay. The results obtained with the Folin–Ciocalteu assay are probably overstimated owing to interfering compounds present at high concentration in the methanolic fractions of TBV and BV that react with the Folin–Ciocalteu reagent. We have demonstrated that after separation with C-18 columns, the two MRP standards are recovered in different amounts in the methanolic fractions and react in a concentration-dependent manner with Folin–Ciocalteu reagent. The basic mechanism of the Folin–Ciocalteu assay is an oxidation/reduction reaction based on the redox properties of the compounds present in the samples and the MRPs that are antioxidants (Manzocco, Calligaris, Mastrocola, Nicoli, & Lerici, 2001), can react with the Folin–Ciocalteu reagent enhancing the measured phenolic concentration values. HMF standard that is totally recovered in the methanolic fractions, does not react with Folin–Ciocalteu and peroxidase methods therefore HMF present in the samples cannot interfere with the total phenolic determination. On the basis of our results, the peroxidase assay applied after separation with Sephadex C-18 columns seems to be the best method of determining polyphenols in wines, red wine vinegars and more complex products such as TBV and BV. With the peroxidase assay it has been shown that TBV contains less phenols than Nero d'Avola, but more phenols than the other tested products. The high phenolic content of TBV probably is due to the extraction of some phenolic compounds from the wood during aging in the barrels and to the evaporation of the water that concentrates the product. This fact was verified for sherry vinegars (Alonso, Castro, Rodríguez, Guillén, & Barroso, 2004; Tesfaye, Lourdes Morales, García-Parílla, & Troncoso, 2002) but it is reasonable to believe that it could also occur for TBV. BV is a mixture of cooked and concentrate grape must and wine vinegar and this may be the main reason of the lower polyphenolic content. RWV contains less phenolic compounds than TBV, BV and red wines. It derive from the acetification process of the red wine that is a process known (Andlauer et al., 2000) to decrease the phenolic content of RWV.

The antioxidant capacity of the samples in the ABTS assay is as follows, in decreasing order: Nero d'Avola > TBV > Dolcetto d'Alba > BV > Lambrusco > RWV. The FRAP assay underestimates the antioxidant capacity of the samples with respect to the ABTS assay, even if the redox potential of Fe(III)-TPTZ (~0.70 V) is comparable with that of $ABTS^{\cdot+}$ (~0.68 V) (Prior, Wu, & Schaich, 2005). This difference could be due to the different reaction conditions: ABTS assay is carried out at neutral pH, and the FRAP assay is conducted at acidic pH 3.6 to maintain

iron solubility. Reaction at low pH decreases the ionization potential that drives electron transfer and increases the redox potential, causing a shift in the dominant reaction mechanism. Another possible explanation of the underestimation of the FRAP respect to the ABTS assay, could be that FRAP assay detects compounds that act by the single electron transfer mechanism, while ABTS assay detects compounds that act either by direct reduction via the electron transfers or by radical quenching via the hydrogen atom transfer mechanism (Prior et al., 2005).

The content of flavonoids in wines and RWV is highly related to their antioxidant capacity, while it is less correlated in TBV and BV that have a high antioxidant capacity and a lower ratio flavonoids:polyphenols. Therefore, other compounds such as phenolic compounds different from flavonoids and Maillard reaction products, contribute to the antioxidant properties of these vinegars. We have showed that MRP standards at the same concentration found in TBV methanolic fractions have an antioxidant activity ranging between 0 and 9 mg VCEAC/100 ml that is, at most, the equivalent of about 3% of the total antioxidant activity found in the methanolic fractions of TBV. It is important to underline that the MRPs present in the methanolic fractions of TBV and BV can be varied and complex because they result from a condensation reaction between amino acids (or proteins) and reducing sugars present in vinegars. These products could have very different antioxidant capacities and hence contribute in a different manner to the total antioxidant activity of TBV and BV. Because a certain amount of MRPs are collected in the methanolic fractions it is necessary to consider their contribution besides polyphenols when the total antioxidant activity must be assayed.

In the red wines aqueous fractions we found antioxidant activity that is due to the presence of phenolic compounds because sugars or MRPs was not find in these fractions. The concentration of these phenolic compounds was between 1.64% and 4.73% of the total polyphenolic content for Nero d'Avola and Lambrusco, respectively, and are probably phenolic acids since flavonoids were not find in the red wines aqueous fractions. Moreover, some phenolic acids, such as gallic acid, was recovered in the aqueous fractions with the recovery tests.

The concentration of phenolic acids in the aqueous fractions of our vinegar samples is below the detection limit of peroxidase assay in our experimental conditions. It must be considered that we have 70% of recovery for gallic acid in aqueous fractions and a dilution of 10 times after the passage through the column. Considering the dilution and the lower detection limit of peroxidase assay, the concentration of gallic acid recovered in the aqueous fractions of balsamic vinegars may be maximum 40 mg/l. At this concentration the phenolic acids may contribute to 20% of the total antioxidant activity of the TBV aqueous fraction at most. In fact 40 mg/l of gallic acid assayed with ABTS and FRAP assays have an antioxidant activity of 15.1 and 7.3 VCEAC, respectively. This activity may correspond to about 20% of the total antioxidant activity in the TBV aqueous fractions. For BV aqueous fractions reaches 47% of the antioxidant activity assayed with FRAP.

We believe that the antioxidant activity of the vinegar aqueous fractions that cannot be attributed to phenolic acids is due to the Maillard products or other antioxidant compounds.

Antioxidants in food and beverages can minimize the postprandial increase in lipid hydroperoxides generated during digestion or presents in food (Natella, Belelli, Gentili, Ursini, & Scaccini, 2002). Dietary antioxidants could protect against a built up of peroxides and their assimilitation in the digestive tract, independently of the antioxidant bioavailability. Polyphenols present in red wine and vinegar could exert some of their biological actions in the stomach, where they reach high levels following the direct consumption of red wine and tea, or fruits and vegetables. It has been demonstrated that red wine decrease the formation of hydroperoxides during the digestion of meat by pepsin in vitro (Kanner & Lapidot, 2001). We have demonstrated that the polyphenols present in red wine and vinegar are able to enhance the enzymatic activity of pepsin (Tagliazucchi, Verzelloni, & Conte, 2005). The vinegars, in particular TBV and BV, which are rich in phenolic antioxidants, are seasonings that can contribute, along with vegetables and wine, to increasing the total amount of antioxidants ingested during a meal.

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