Eur Food Res Technol (2003) 216:445–448
DOI 10.1007/s00217-003-0671-x

ORIGINAL PAPER

Ángeles M. Alonso · Dominico A. Guillén · Carmelo G. Barroso

Development of an electrochemical method for the determination of antioxidant activity. Application to grape-derived products

Received: 14 October 2002 / Revised: 5 December 2002 / Published online: 13 February 2003

Abstract A new method, rapid and simple to apply, has been developed to measure antioxidant activity. It is based on the electrochemical oxidation of 2,2́–azino–bis(3–ethylbenzthiazoline–6–sulfonic acid) (ABTS) and gives reliable results, with the monitoring of only absorbance and time. It has been compared with the total antioxidant status test and gives similar but more repeatable results. The method has been applied to wines, brandies and vinegars. It has been confirmed that the antioxidant activity of these products is highly correlated with their polyphenolic content, and that other majority components in the samples, such as ethanol, SO_2 or acetic acid, do not interfere in the method.

Keywords Antioxidant activity · Electrochemical method · ABTS · Wine · Brandy · Vinegar

Introduction

In recent years, many published studies have stated that a diet rich in fruit, vegetables, cereals, and grains has a preventive effect against cardiovascular diseases, cancers, and other diseases related to ageing. This beneficial action in respect to health has been attributed to the phenolic compounds present in these foods, and largely to the antioxidant capability possessed by these compounds [1, 2, 3]. A notable case, widely studied, is that of the potential protective effect against cardiovascular diseases provided by a moderate consumption of wine [4, 5].

This situation has encouraged the development of numerous methods for measuring antioxidant activity, both in foods and drinks and in body fluids [6, 7, 8, 9, 10]. The most commonly used methods in food technology are those in which free radicals are generated; these are then easily detectable using fluorimetric or photometric techniques. However, there are considerable methodological and instrumental differences between these different methods (manipulation of reagents, processing of data, automation, possible interferences, etc.). Comparative studies of the methods of antioxidant activity have been published, and all conclude that each methodology gives different responses for the same compounds or samples [11, 12, 13, 14]. For this reason, there is a clear requirement for a simple, rapid, and reliable method.

Our research group has taken a previous design [15] as a basis and has developed a new method based on the electrochemical oxidation of 2,2́–azino–bis(3–ethylbenzthiazoline–6–sulfonic acid) (ABTS). This is a compound that, when oxidized, generates a colored cation-radical, easily detectable by UV–visible spectrophotometry. When an antioxidant compound or sample is added to the solution, oxidation of the ABTS is delayed, and the reaction consumes a greater quantity of electrical current, which is related to the degree of antioxidant activity of the sample added. The electrochemical reaction avoids the need to use oxidizing reagents, with the result that the principal factor influencing the response is the structure of the compounds oxidized, thus avoiding any possible interference and providing a more reliable result than most of the methods published to date.

Materials and methods

Reagents and samples

In the electrolytic system were used a saturated solution of $Zn(CH_3COO)_2$ (Panreac, Barcelona, Spain) and a solution of 2,2́–azino–bis(3–ethylbenzthiazoline–6–sulfonic acid) (ABTS) (Sigma–Aldrich, Madrid, Spain) in a phosphate buffer medium (pH 6). 6–hydroxy–2,5,7,8–tetramethylchroman–2–carboxylic acid (Trolox) supplied by Sigma–Aldrich was used to construct the calibration curve.

The results obtained using this proposed method were compared with those obtained by means of the total antioxidant status (TAS)

Á. M. Alonso (✉) · D. A. Guillén · C. G. Barroso
Departamento de Química Analítica, Facultad de Ciencias,
Universidad de Cádiz, C/ República Saharaui s/n,
11510 Puerto Real, Cádiz, Spain
e-mail: angeles.alonso@uca.es
Tel.: 34 956 016 363
Fax: 34 956 016 460

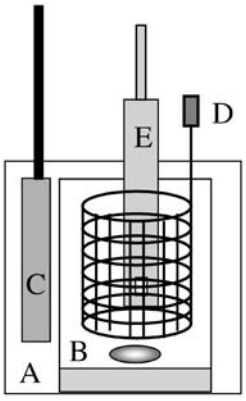

Fig. 1 Scheme of the device constructed for the measurement of antioxidant activity

kit for the measurement of antioxidant power, from Randox (United Kingdom) [16].

The Folin reagent (Sigma–Aldrich) and sodium carbonate (Panreac) were employed for the measurement of the Folin–Ciocalteau total polyphenolic index [17].

The antioxidant activity of some compounds was studied: Malvin supplied by EGA–Chemie (Germany), quercetin, gallic acid, and acetic acid supplied by Merck (Darmstadt, Germany), syringic acid from Eastman–Kodak (Rochester, NY, USA), ethanol from Panreac, 2[3]–t–butyl–4–hydroxylanisol (BHA), catechin, mirycetin, caffeic acid, ferulic acid and ascorbic acid supplied by Sigma–Aldrich, and SO_2 (prepared from sodium bisulfite) from D'Hemio Laboratories (Madrid, Spain).

Also, some samples related to wine and grapes were studied: 9 brandies, 7 vinegars and 1 white wine from Jerez (Spain), 2 red wines from La Rioja (Spain) and 2 red wines from Oporto (Portugal). All samples were supplied by wine companies.

Apparatus

The device constructed for the measurement of the antioxidant activity (scheme in Fig. 1) consists of: an 80 mL beaker (A, cathode) inside of which a 30 mL filtering crucible, pore size 4, is set (B, anode). A flat platinum electrode (C, 30×60 mm) is introduced in the cathode and a cylindrical platinum mesh (D, h=22 mm, d=22 mm) is introduced in the anode The feed source used (FAC–307C from Promax) allows the working conditions to be set in constant intensity mode. An UV–visible transmission probe (E) coupled to a PC2000 miniaturized spectrophotometer from Ocean Optics (Eerbeek, The Netherlands) with a DH–2000 halogen–deuterium light source from Top Sensor Systems (Eerbeek, The Netherlands) is used to monitor the reaction.

Electrochemical antioxidant test

The test consists of the oxidation, by means of the electrolytic system described, of a solution of ABTS 50 μM, to which the sample to be tested is added. The procedure is as follows: First, the platinum electrodes have to be washed with nitric acid 60% and flame-calcined (every 5–10 assays). Then they are placed, one in the cathode immersed in 30 mL of a saturated solution of $Zn(CH_3COO)_2$, and the other in the anode with 25 mL of a solution of ABTS 50 μM (pH 6). Aliquots of the samples are added to the anode, which is continuously agitated by a magnetic agitator. To start the experiment, a constant intensity of 2 mA is applied, while the spectrophotometer is continuously recording the absorbance at 414 and 734 nm (the two wavelengths at which $ABTS^{-+}$ presents its principal maximum values) and the time.

The final point of the assay is taken to be the moment when the ABTS begins to oxidize, considering that this marks the termination of the oxidation of the sample. At this moment, the spectrum of the cation-radical begins to appear, with the absorbance at 414 and 734 nm increasing, and the quotient Abs 414/Abs 734 becoming constant. When the variation between two consecutive measurements of this quotient falls below 10%, it is considered that the oxidation of the ABTS has begun and therefore that the oxidation of the antioxidant or sample added has concluded. Depending on the degree of antioxidant power of the sample added, the formation of the cation-radical is delayed, resulting in the consumption of a greater quantity of coulombs. This quantity of coulombs is then compared with a calibration curve previously obtained from solutions of known concentration of Trolox.

Total antioxidant status test

The TAS test is performed in accordance with the supplier's instructions. The measurement of the antioxidant activity is made by pipetting into 3 cuvettes (of 1 cm light step) 20 μL of blank (bi-distilled water), standard (Trolox), and sample, respectively, together with 1 mL of chromogene (metmyoglobin and ABTS). The initial absorbance is read (A_1), taking care that the temperature is kept constant at 37 °C. Then, a volume of 200 μL of substrate (H_2O_2) is added to each cuvette and the absorbance (A_2) is read again precisely 3 min later. The antioxidant activity is calculated as follows:

$$Factor = \frac{conc.\ standard}{(A_2 - A_1)_{blank} - (A_2 - A_1)_{standard}} \quad (1)$$

$$mmol/L = Factor * \left[(A_2 - A_1)_{blank} - (A_2 - A_1)_{sample}\right] \quad (2)$$

Total polyphenolic index: Folin–Ciocalteu method

Observing the sequence specified here, the following are introduced into a calibrated 25 mL flask: 250 μL of wine, 12.5 mL of distilled water, 1250 μL of Folin–Ciocalteu reagent, 5 mL of a solution of sodium carbonate at 20% and distilled water to make up the total volume to 25 mL. The solution is agitated to homogenize it and left to stand for 30 min for the reaction to take place and stabilize. The absorbance at 750 nm is determined in a cuvette of 1 cm.

The calibration curve was prepared with gallic acid solutions ranging from 0 to 1000 mg/L, and the results are given as gallic acid equivalents (GAE).

Results

Calibration

For calibration of the method, solutions of Trolox in methanol of between 0 and 25 mM were prepared, covering the range of response expected for the phenolic compounds, according to the bibliography. Taking aliquots of 150 μL, 6 points were measured in triplicate: 0, 2.5, 5, 10, 15, and 25. The results were introduced into the ALAMIN program [18], which provided the calibration curve (Coulombs=0.0334*[Trolox]+0.0979), together with the analytical parameters: limit of detection LOD=3.295, linearity LOL=95.630% and coefficient of correlation R=0.9851.

Table 1 Antioxidant activity of some pure compounds, measured by the electrochemical method and the TAS method

Compound	$[Trolox]_{eq}$ (mM)	TAS (mM)
Quercetin	6.20	20.60
Mirycetin	4.14	9.48
Gallic acid	2.67	3.02
Syringic acid	2.30	1.82
Catechin	2.10	2.60
Ferulic acid	1.59	2.59
BHA	1.40	1.00
Malvin	1.12	2.99
Caffeic acid	0.89	1.78
Ascorbic acid	nd	0.62

Repeatability study

To study the repeatability of the method, five aliquots of 150 μL of a solution of Trolox 5 mM were measured, giving the result of $[Trolox]_{eq}$=5.1 mM and a relative standard deviation (RSD) of 11.4%. Also measured were five aliquots of 150 μL of a red wine, giving the result of RSD=8.3%.

To compare the proposed electrochemical method with another widely used method, the TAS test was selected. This is an adaptation of the well-known method of Miller [19]. The repeatability of the TAS method, calculated by applying it manually to 5 aliquots of sample, was 14.8%.

Application to standards and samples

To complete the validation of the method, measurements were made of the antioxidant activity of a series of pure compounds, principally polyphenols, and samples of products derived from grapes, rich in those compounds that have been attributed with significant antioxidant power and are considered to give potential health protection in foods such as fruits, vegetables, grains, tea, and wines.

Table 1 shows the antioxidant activity found for 10 pure compounds (8 polyphenols and 2 known antioxidants, ascorbic acid and BHA). The experiments were carried out in triplicate. All the results obtained refer to a concentration of 1 mM of the compounds. In some cases, the results are approximately the same for the two methods and in others they differ. Nevertheless, constructing a regression curve with these results, it is demonstrated that a high correlation exists between the two methods (R=0.9009). Furthermore, comparing the results with the Student's *t* test applied to pairs of values, it is found that there are no significant differences at a 95% confidence level.

To study the application to samples of products derived from grapes, samples of wines, brandies and vinegars were measured. In these cases repetitions were not made, considering that the method was already sufficiently validated. Using the electrochemical method, aliquots of different volumes were measured according to the type of sample, and the results were referred to the volume that had been calibrated. Using the TAS method, a volume of 20 μL was always employed, in accordance with the protocol for the application of the test. The index of total polyphenols was also measured to test whether there was any correlation between this and the antioxidant activities measured. All the results are given in Table 2.

Table 2: Antioxidant activity (measured by the electrochemical method and the TAS method) and total polyphenolic index of some brandies, vinegars and wines

Sample	$[Trolox]_{eq}$ (mM)	TAS (mM)	GAE (mg/L)
Brandy 1	2.92	3.13	423.91
Brandy 2	2.56	2.71	300.27
Brandy 3	2.17	3.27	406.64
Brandy 4	2.38	2.32	278.46
Brandy 5	1.58	1.31	211.18
Brandy 6	1.08	1.19	177.55
Brandy 7	1.09	1.04	108.46
Brandy 8	0.41	0.63	60.27
Brandy 9	0.25	0.36	76.64
Vinegar 1	5.66	3.72	978.50
Vinegar 2	1.30	0.69	378.50
Vinegar 3	0.71	1.01	383.00
Vinegar 4	3.84	2.01	982.10
Vinegar 5	1.10	1.59	543.90
Vinegar 6	0.39	nd	377.50
Vinegar 7	0.19	nd	217.50
Rioja red wine 1	15.53	14.42	1504.09
Rioja red wine 2	8.45	13.96	1390.45
Port red wine 1	5.14	10.24	1133.64
Port red wine 2	2.92	5.51	760.64
Sherry white wine	nd	4.17	563.45

As before, some samples present an antioxidant power very similar for both methods, and others not. Performing a linear regression analysis between the results of the electrochemical method and the TAS test for all the samples, a coefficient of correlation R=0.8653 is found. Using the Student's *t* test, it is again found that there are no significant differences at a 95% confidence level. Studying the various samples separately, coefficients of correlation of 0.9398, 0.9064, and 0.9056 are found for brandies, vinegars, and wines, respectively. These data indicate that the results obtained from both methods are similar. The apparent differences in some compounds and samples between the results of the electrochemical method and the TAS method could be explained by the great inaccuracy of the manual application of the TAS method, that in fact presents a worse repeatability. This problem of the TAS test has been commented on in previous publications, ours and of other authors [14, 20]. Also, as stated in the introduction, each methodology gives different responses for the same samples or has a different response scale.

As regards the comparison with the index of total polyphenols, it is confirmed that both the electrochemical and the TAS methods are very well correlated with this index, for the three types of sample: brandies (0.9257 and 0.9755), vinegars (0.9418 and 0.8727), and wines (0.8439 and 0.8768).

The measurement of antioxidant activity by means of the method proposed is based on the electrochemical oxidation of the samples. In the grape-derived products tested, of complex composition, it is possible that there were other compounds susceptible to being oxidized, as well as the polyphenols. For this reason, a study was conducted of the antioxidant activity possessed by the majority of substances present in these samples, such as ethanol (11% in wines and 40% in brandies), acetic acid (60 g/L in vinegars) or SO_2 (100 mg/L in wines). For this, standard solutions of these compounds were prepared, at the concentrations in which they are normally found in real samples, and the antioxidant activity was measured. In all cases the results were very low, below the detection limit of the method, which would indicate that these compounds contribute very little to the antioxidant activity of the samples.

Conclusions

For the purposes of measuring antioxidant activity, an electrochemical method has been devised that is simple to apply and provides reliable results in two minute assays. The results require only the monitoring of absorbance and time, without the need for oxidizing reagents nor the control of other variables, which are features of most of the methods published.

In comparisons made with the TAS test, the electrochemichal method gives similar but more repeatable results. It requires fewer reagents and avoids the need to control the temperature.

From its application to samples of products derived from grapes, it has been demonstrated that it is the polyphenols present in the samples, and not other majority components, that are mainly responsible for the antioxidant activity in these products.

Acknowledgments This study was supported by the CICYT of the Spanish Ministry of Education and Science and FEDER of U.E. (Project 1FD97-0683-C05-01).

References

1. Rice-Evans C, Packer L (1998) Flavonoids in health and disease. Marcel Dekker, New York
2. Pratt DE (1992) Phenolic compounds in food and their effects on health. American Chemical Society, Washington DC
3. Duthie GG, Duthie SJ, Kyle JAM (2000) Nutr Res Resv 13(1):79–106
4. Kinsella JE, Frankel E, German B, Kanner J (1993) J Food Technol April:85–89
5. Renaud S, De Lorgeril M (1992) The Lancet 339:1523–1526
6. De Gaulejac N S-C, Vivas N, De Freitas V, Bourgeois G (1999) J Sci Food Agric 79:1081–1090
7. Ou B, Hampsch-Woodill M, Prior RL (2001) J Agric Food Chem 49:4619–4626
8. Bartosz G, Janaszewska A, Ertel D, Barstoz M (1998) Biochem Mol Biol Int 46(3):519–528
9. Arnao MB, Cano A, Acosta M (2000) Span. ES 2151401 A1 16 Dec 2000, 9 pp
10. Shanbrom E (2000) Int. Appl. WO 2000072024 A1 30 Nov 2000, 17 pp
11. Pérez DD, Leighton F, Aspee A, Aliaga C, Lissi E (2000) Biol Res 33(2):71–77
12. Schwarz K, Bertelsen G, Nissen LR, Gardner PT, Heinonen MI, Hopia A, Huynh-Ba T, Lambelet P, McPail D, Skibsted LH, Tijburg L (2001) Eur Food Res Technol 212(3):319–328
13. Baderschneider B, Luthria D, Waterhouse AL, Winterhalter P (1999) Vitis 38(3):127–131
14. Arnao MB, Cano A, Acosta M (1999) Free Rad Res 31:S89–96
15. Alonso AM, Domínguez C, Guillén DA, Barroso CG (2002) J Agric Food Chem 50:3112–3115
16. Soleas GJ, Diamandis EP, Goldberg DM (1997) J Clin Lab Anal 11:287–313
17. Singleton VL, Rossi JAJ (1965) Am J Enol Vitic 16:144–158
18. García AM, Cuadros L, Alés F, Román M (1997) Trends in Anal Chem 16(7):381–385
19. Miller NJ, Rice–Evans CA, Davies MJ, Gopinathan V, Milner A (1993) Clin Sci 84:407–412
20. Alonso AM, Guillén DA, Barroso CG (2000) Bulletin O.I.V. 73(837–838):794–808