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Application of the 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) Radical Cation Assay to a Flow Injection System for the Evaluation of Antioxidant Activity of Some Pure Compounds and Beverages

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The 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) assay was adapted to a flow injection (FI) system to obtain a sensitive and rapid technique for the monitoring of antioxidant activity of pure compounds and complex matrixes, such as beverages and food extracts. The FI system includes a HPLC pump that flows the mobile phase (a solution of $ABTS^{\bullet+}$ in ethanol) through a 20 μL loop injector, a single bead string reactor filled with acid-washed silanized beads, a delay coil and a photodiode array UV−visible detector. The technique was very sensitive, with limits of detection and of quantification of 4.14 and 9.29 μmol of Trolox/L, respectively, and demonstrated high repeatability and reproducibility. The proposed technique was then applied to the evaluation of the antioxidant activity of some pure compounds, demonstrating good agreement with published data obtained by the original spectrophotometric $ABTS^{\bullet+}$ assay. Finally, the total antioxidant activity of 10 beverages was determined by both the proposed and the original method. The values ranged from 0.09 mmol L^{-1} for cola to 49.24 mmol L^{-1} for espresso coffee and did not result significantly different from those obtained by the original spectrophotometric $ABTS^{\bullet+}$ assay (Student's paired *t*-test: $t = 1.4074$, $p = 0.1929$). In conclusion, the proposed FI technique seems suitable for the direct, rapid and reliable monitoring of total antioxidant activity of pure compounds and beverages and, due to the ability to operate in continuous, it allows the analysis of about 30 samples h^{-1} making the assay particularly suitable for large screening of total antioxidant activity in food samples.

KEYWORDS: Antioxidant activity; ABTS radical cation assay; antioxidant compounds; beverages; method

INTRODUCTION

Due to their chemical reactivity, free radicals and other reactive oxygen species, derived either from normal metabolic processes or from external sources, can damage all types of cellular macromolecules. These effects have been implicated in the causation of some degenerative diseases, such as cataracts, atherosclerosis, and certain types of cancer (*1*−*4*).

The consumption of antioxidant-rich foods might play an important role in the maintenance of health and in disease prevention (*5*). Epidemiological studies have demonstrated an inverse association between the intake of antioxidants from fruits and vegetables and the morbidity and mortality from coronary heart diseases (*6*, *7*) and cancer (*8*−*10*). Nevertheless, many clinical studies have not shown direct beneficial effects of individual antioxidant molecules, such as vitamin E and β-carotene, on various chronic diseases (*11*−*13*) suggesting that the functionality of dietary antioxidants might be strongly linked to cooperative mechanisms among different antioxidant molecules present in the matrix. On the basis of these observations, a number of assays have been introduced in the past decade for determining the total antioxidant activity (intended as the cumulative capacity of food components to scavenge free radicals) of food extracts and beverages (*14*−*16*). These assays diverge in that they relate to the generation of different radicals, often acting through different mechanisms and/or target molecules, and the measures are made on a range of different endpoints (*17*). In general, two types of approach have been taken: (1) inhibition assays, for which the extent of the scavenging of a preformed free radical by hydrogen or electron donation is the marker of antioxidant activity; (2) assays involving the presence of antioxidant systems during the generation of the radical, for which the activity is measured on the rate of oxidation of a target molecule.

The quenching of the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) forms the basis of a spectrophotometric method that, thanks to a recent improvement in its chemistry, allows the evaluation of both water-soluble and lipid-soluble antioxidants (*18*). The method is a decolorization assay that, after the addition of an antioxidant, measures

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the reduction of absorbance at 734 nm of the $ABTS^{\bullet+}$ solution, which in turn is proportional to the antioxidant concentration and activity calculated in relation to the reactivity of a standard of Trolox analyzed under the same conditions.

Even though spectrophotometric techniques, this included, are simple procedures, these require time, extreme accuracy, and good manual skill to give consistent results.

The purpose of our study was to adapt the original spectrophotometric assay (*18*) to a flow injection (FI) system to obtain a sensitive and rapid technique based on direct injection of the sample in a flow of $ABTS^{\bullet+}$ solution. This technique was applied for determining the antioxidant activity of some pure water-soluble and lipid-soluble antioxidants. Moreover, total antioxidant activity of some common beverages was determined using both the proposed and the original spectrophotometric method.

MATERIALS AND METHODS

Beverages. All the beverages (juices of orange, peach, pear, apricot, and tropical fruit; cola, lemon ice tea, and beer) were purchased in local supermarkets. Black tea was prepared by 3-min infusion of a tea bag (about 2 g) in 250 mL of boiling water. Espresso coffee was prepared in local coffee shops. Four different brands of each beverage were mixed in equal amounts and adequately diluted with pure deionized water (between 10- and 200-fold, depending on their activity). Carbon dioxide from cola and beer was completely removed by magnetic stirring. Diluted beverages were centrifuged 5 min at 1000*g*, and the supernatant was collected and analyzed without further preparation.

Chemicals. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 4-hydroxy-3-methoxycinnamic (ferulic), 3,4-dihydroxycinnamic (caffeic), 3,4,5-trihydroxybenzoic (gallic), and 4-hydroxy-3-methoxybenzoic (vanillic) acids, quercetin, naringenin, and α-tocopherol were purchased from Sigma-Aldrich (Sigma-Aldrich srl, St. Louis, MO). L-Ascorbic acid was obtained from Merck (Merck, Darmstadt, Germany). All chemicals and solvents used were HPLC-grade and purchased from Carlo Erba (Carlo Erba, Milan, Italy).

Preparation of Standard Solutions. Stock solutions (10 mmol L^{-1}) of flavonoids, hydroxycinnamates, hydroxybenzoates, and α-tocopherol were prepared in ethanol and stored at −20 °C for a maximum of 1 month. Working solutions were prepared daily in ethanol at five different concentrations (between 10 and 400 μmol L^{-1}) within the range of the dose−response curve of Trolox. A stock solution of ascorbic acid (10 mmol L^{-1}) was prepared daily in deionized ultrapure water and then diluted with water to obtain appropriate concentrations (between 10 and 300 μmol L^{-1}). Finally, a 5 mmol L^{-1} stock solution of Trolox was prepared in ethanol and stored at −20 °C for a maximum of 6 months. The stock solution of Trolox was diluted daily in ethanol at different concentrations (see below) to obtain working solutions for building the dose−response curve.

Flow Injection Method. *Principle*. The method is based on the ability of antioxidant molecules to quench the long-lived $ABTS^{\bullet+}$, a blue-green chromophore with characteristic absorption at 734 nm, in comparison of that of Trolox, a water-soluble vitamin E analogue. The addition of antioxidants to the preformed radical cation reduces it to ABTS, determining a decolorization.

Apparatus. The flow injection system consisted of a Hewlett-Packard 1100 pump, a reohdyne injection valve equipped with a 20-μL loop, a single bead string reactor (250 μm × 30 cm × 0.5 mm i.d.) filled with acid-washed silanized beads (Supelco, Bellefonte, PA), a delay coil (0.5 mm i.d. x 1.58 mm o.d. x 306 cm) (Supelco), and a photodiode array UV−visible detector (Waters Corporation, Milford, MA). The flow of the mobile phase was set to 0.8 mL min^{-1} at room temperature, and the acquisition, to 734 nm. A schematic configuration of the system is shown in **Figure 1**.

Mobile Phase. A stable stock solution of $ABTS^{\bullet+}$ was produced by reacting a 7 mmol L^{-1} aqueous solution of ABTS with 2.45 mmol L^{-1} potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12−16 h before use (*18*). The solution was stable for at least 48 h. At the beginning of the analysis day, an $ABTS^{\bullet+}$ working solution was obtained by dilution in ethanol of the stock solution to reach an absorbance of 0.70 (± 0.02) AU at 734 nm, checked by a Hewlett-Packard 8453 diode array spectrophotometer (HP, Waldbronn, Germany), and used as mobile phase.

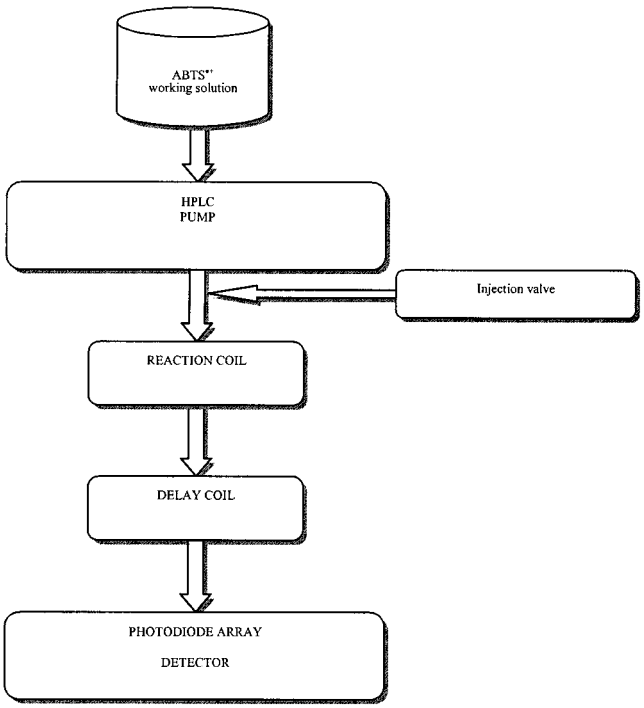

Figure 1. Schematics of the flow injection system.

Procedure. The direct injection of standards and samples through the injection valve into the FI system determined a decolorization peak whose negative area was proportional to the loss of absorbance of the $ABTS^{\bullet+}$ working solution. The quantitation of the antioxidant activity of the solutions injected was achieved by comparing the negative area of their decolorization peak to the dose−response curve obtained by injection of increasing concentrations of the Trolox standard. Results were expressed as Trolox equivalent antioxidant capacity (TEAC) for pure compounds and total antioxidant activity (TAA) for beverages. TEAC is defined as the concentration (mmol L^{-1}) of Trolox having the antioxidant activity equivalent to a 1.0 mmol L^{-1} of the compound under investigation. TAA is expressed as mmol of Trolox per liter of sample.

The activity of pure compounds was estimated in duplicate over three different days at five different concentrations ranging within the Trolox dose−response curve.

Optimization of the System. The optimization of the FI system conditions was carried out to obtain reaction times (about 1 min) and values of TEAC of pure compounds close to those obtained with the original spectrophotometric assay. To this aim, three phenolic acids (gallic, ferulic and caffeic acids, which present high, medium, and low TEAC values, respectively), were analyzed using three delay coils with the same length and different internal diameters (0.3, 0.5, and 0.8 mm) in combination with different flow rates (0.5, 0.8, 1.0, and 2.0 mL min^{-1}).

Validation of the Method. To investigate the linearity of response, ten concentrations of Trolox, ranging from 10 to 500 μmol L^{-1}, were injected over three different days.

The limits of detection and quantification were calculated from ten injections of the appropriate solvent blanks (i.e., ethanol for Trolox or deionized ultrapure water for ascorbic acid) according to EURACHEM guidelines (*19*).

Repeatability and reproducibility were evaluated by injecting five times two different concentrations of Trolox standard solutions (20 and 300 μmol L^{-1}) both in the same day and in different days by different operators.

Table 1. Trolox Equivalent Antioxidant Capacity (TEAC) Values and Acquisition Times of the Analyzed Phenolic Acids Obtained with Different Conditions of Flow Rate and Internal Diameter of the Delay Coil

compound	internal diameter (mm)	flow rate (mL min^{-1})	TEAC (mmol L^{-1})	acquisition time (s)
	0.3	0.5	1.72	42
	0.3	1.0	2.22	20
gallic acid	0.5	0.8	2.46	66
	0.5	1.0	1.92	48
	0.8	1.0	2.35	80
	0.8	2.0	2.30	42
	0.3	0.5	1.31	42
	0.3	1.0	1.14	20
ferulic acid	0.5	0.8	1.66	66
	0.5	1.0	1.63	48
	0.8	1.0	1.53	80
	0.8	2.0	1.49	42
	0.3	0.5	0.83	42
	0.3	1.0	0.92	20
caffeic acid	0.5	0.8	0.92	66
	0.5	1.0	0.92	48
	0.8	1.0	0.91	80
	0.8	2.0	0.91	42

Method Comparison. For beverages, the TAA values obtained by the proposed flow injection system were compared to those achieved by applying the original spectrophotometric assay (*18*).

Statistical Analysis. Data are reported as mean ± SD. Student's paired *t*-test analysis (two tails) was performed using a statistical package running on a PC (Statistical Statsoft Inc., Tulsa, OK); *p* values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Optimization of the Flow Injection System. The choice of the analytical conditions were based on the most recent knowledge on the chemistry of the ABTS$^{\bullet+}$ assay. Regarding the temperature of analysis, according to Cano et al. (*20*) who reported that the development of the reaction between ABTS$^{\bullet+}$ and the antioxidant is constant between 20 and 30 °C, we avoided to thermostatize the reaction coil.

With respect to the time-point of reaction, all published papers related to the determination of antioxidant activity by the ABTS$^{\bullet+}$ assay demonstrated the time dependency of the reaction between ABTS$^{\bullet+}$ and antioxidant compounds, and thus the influence of the selected time-point of measurement on the reported antioxidant activity. In the case of the complex mixtures of antioxidants such as beverages or food extracts, this influence results more evident. Thus, Arnao (*21*) determined the antioxidant activity of wine at 20 min, whereas that of juice at 5 min was determined to guarantee a complete reaction. However, Pannala et al. (*22*), studying the reaction by a stop-flow kinetic system, affirmed that the reaction between ABTS$^{\bullet+}$ and antioxidants occurs more rapidly than the first measured value (i.e., 0.1 s). On the basis of these observations, we chose 1 min as the time-point for monitoring the reaction between all reacting functional hydroxyl groups present in the antioxidant molecules and ABTS$^{\bullet+}$. This is supported by the most recent paper of the research group who first set up the method, in which the measurement of TAA of food extracts was carried out at 1 min, as in our system (*23*).

In **Table 1**, the TEAC values of the three phenolic compounds, obtained with different combinations of flow rate and volumes of the delay coil, are shown. The combination of a delay coil of 0.5 mm internal diameter with a flow rate of 0.8 mL min^{-1} gave a reaction time of about 1 min, the same time-point used by the spectrophotometric method, and provided TEAC values similar to those obtained using the original assay procedure (*24*).

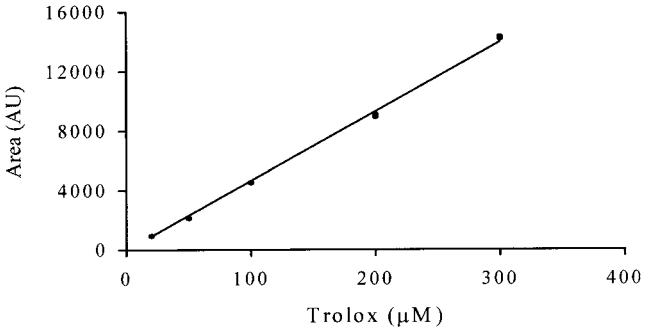

Figure 2. Dose–response curve from five Trolox concentrations (10, 50, 100, 200, and 300 μmol L^{-1}) injected in triplicate.

It is noteworthy that TEAC values of gallic and ferulic acid were strongly influenced by the different conditions of analysis. As already demonstrated by Pannala et al. (*22*), compounds containing trihydroxy B ring structures, such as gallic acid, or hindered phenols, such as ferulic acid, react very rapidly with ABTS$^{\bullet+}$, but they are followed by a slow secondary phase. Thus, for these compounds, the increasing time of acquisition determines an increase of TEAC value. Conversely, TEAC values of caffeic acid were less affected by the acquisition time, in agreement with Re et al. (*17*).

Moreover, it is of interest to notice that at the same acquisition time (i.e., 42 s), but under different conditions of analysis (i.e., internal diameter of the delay coil and flow rate), all the pure compounds exhibited different TEAC values. This difference was related to the profile of the decolorization peaks: a slow flow rate associated with a narrow internal diameter of the delay coil produced narrow and symmetric peaks, but the system was already saturated at low standard concentrations, decreasing the response range. On the contrary, high flow rate associated with a larger internal diameter of the delay coil generated bimodal decolorization peaks, that were difficult to integrate.

Validation of Method. The dose−response curve of Trolox (**Figure 2**) was linear over a range of concentration from 10 to 300 μmol L^{-1}, and the average slope calculated from twelve calibration curves measured over seven weeks was $4.48 \times 10^4 \pm 0.2 \times 10^4$ AU μmol^{-1} L. On the basis of these results, for each day of analysis a dose−response curve was prepared by injecting five concentrations of Trolox (10−300 μmol L^{-1}) in duplicate.

The limit of detection (LOD) and the limit of quantification (LOQ), expressed as micromoles of Trolox per liter, were 4.14 and 9.29, respectively, and, expressed as micromoles of ascorbic acid per liter, were 7.90 and 17.74, respectively. Repeatability and reproducibility of Trolox standard solutions were 1.67% ($n = 5$) and 1.30% ($n = 5$) for 20 μmol L^{-1} and 1.30% ($n = 5$) and 1.90% for 300 μmol L^{-1}, respectively.

These analytical parameters demonstrate that the proposed FI technique is very sensitive and adequate to determine antioxidant activity even in solutions containing low concentrations of antioxidants. Moreover, with the proposed analytical procedure, the limit of quantification was lower by at least 2 orders of magnitude with respect to the original spectrophotometric method, possibly due to the geometry of the system.

Application of Method. In **Figure 3**, typical decolorization peaks obtained for increasing concentrations of Trolox are displayed. The peaks show good symmetry and can be accurately integrated. Moreover, the absorbance of the ABTS$^{\bullet+}$

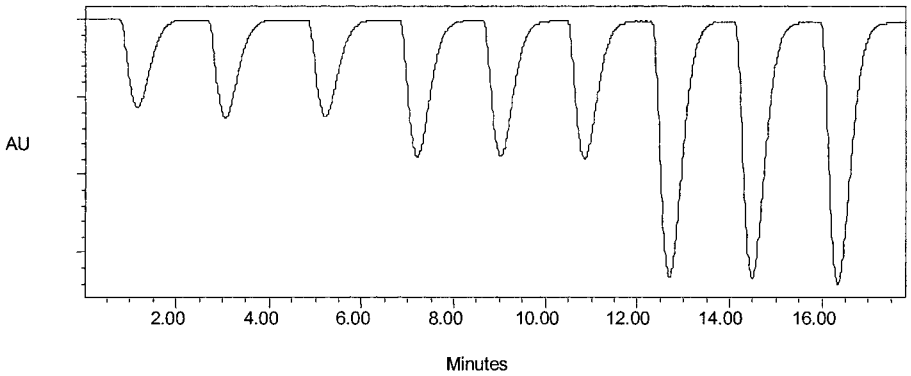

Figure 3. Typical decolorization peaks obtained by repeated injections of Trolox standard solutions (50, 100, and 200 μmol L^{-1}).

Table 2. Trolox Equivalent Antioxidant Capacity (TEAC) Values of Pure Compounds Obtained by the Proposed Technique in Comparison to Literature Values

compound	TEAC[a] (mmol L^{-1})	TEAC (mmol L^{-1})
hydroxybenzoates		
gallic acid	2.45 ± 0.10	2.45 ± 0.10[b]
vanillic acid	1.49 ± 0.01	1.42 ± 0.03[b]
hydroxycinnamates		
caffeic acid	0.92 ± 0.04	0.99 ± 0.02[b]
ferulic acid	1.62 ± 0.06	1.87 ± 0.06[b]
flavon-3-ols		
quercetin	2.49 ± 0.03	2.77 ± 0.02[c]
flavanones		
naringenin	0.26 ± 0.01	0.58 ± 0.09[c]
other compounds		
ascorbic acid	1.03 ± 0.04	1.05 ± 0.02[c]
α-tocopherol	1.08 ± 0.01	0.99 ± 0.02[b]

[a] Results are expressed as mean ± standard deviation ($n = 3$). [b] Data from Pellegrini et al. (*24*). [c] Data from Re et al. (*17*).

Table 3. Total Antioxidant Activity (TAA) Values of Some Beverages Obtained by the Proposed Assay Compared to Those Achieved by Applying the Original Spectrophotometric Assay (*18*)[a]

beverage	TAA[b,c] (mmol of Trolox L^{-1})	TAA[b,d] (mmol of Trolox L^{-1})
cola	0.09 ± 0.01	0.19 ± 0.06
beer	1.10 ± 0.05	0.88 ± 0.06
orange juice	3.32 ± 0.08	3.20 ± 0.27
apricot juice	3.32 ± 0.05	3.15 ± 0.20
pear juice	3.40 ± 0.01	3.70 ± 0.28
peach juice	2.88 ± 0.03	2.34 ± 0.02
tropical fruit juice	2.31 ± 0.23	2.32 ± 0.11
lemon ice tea	3.56 ± 0.18	4.81 ± 0.22
black tea	2.81 ± 0.02	3.72 ± 0.18
espresso coffee	49.24 ± 2.61	51.12 ± 1.38

[a] Student's paired *t*-test (two tails) (proposed assay versus original assay): $t = 1.4074$, $p = 0.1929$. [b] Results are expressed as mean ± standard deviation. [c] Values obtained by the proposed assay. [d] Values obtained by the original spectrophotometric assay.

working solution was stable over a sufficiently long analysis time (about 3 h) and did not significantly affect the detection limits.

The proposed FI technique was then applied for the evaluation of antioxidant activity of eight pure compounds. In **Table 2** the TEAC values are reported, compared with published data obtained using the original spectrophotometric assay (*17*, *24*). In general, the two sets of TEAC values were in good agreement, with the exception of naringenin that exhibited a TEAC value 50% lower than that reported using the original spectrophotometric assay (*17*).

Finally, the applicability of the technique was tested by measuring the TAA of ten beverages commonly consumed in Italy. The results obtained by the proposed assay are shown in **Table 3** and compared to those achieved by applying the original spectrophotometric procedure (*18*). The statistical analysis demonstrates that the TAA values obtained by the two methods are not significantly different ($t = 1.4074$, $p = 0.1929$). Among drinks, considerable differences in TAA were found; in fact, the total antioxidant activity ranged from 0.09 mmol L^{-1} for cola to 49.24 mmol L^{-1} for espresso coffee and fruit juices showed TAA values from 2.31 to 3.40 mmol L^{-1}. Finally, as already showed by Richelle et al. (*25*), the antioxidant activity of coffee was very high, more than 10 times that of black tea. This result is likely due to the method for coffee preparation used in Italy that determines a strong extraction of the coffee powder.

In conclusion, the proposed technique results suitable for the direct, rapid, and reliable analysis of total antioxidant activity of pure compounds and beverages, demonstrating wide linearity of response and a very low detection limit and giving results similar to those obtained from the original spectrophotometric method. Due to the ability to operate continuously, it is possible to analyze about 30 samples h^{-1}, making the assay particularly suitable for large screening of total antioxidant activity in food samples or for on-line quality control. Moreover, the assay is made on whole extracts without any chromatographic separation, allowing the assessment of TAA, a parameter that takes into account all the synergistic and cumulative interactions between the known and unknown antioxidants present in the sample.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic) acid; $ABTS^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; Trolox**,** 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TEAC, Trolox equivalent antioxidant capacity; TAA, total antioxidant activity.

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