JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 4341–4346 **4341**

Rapid, Fully Automated Flow Injection Antioxidant Capacity Assay

ELENI P. LABRINEA AND CONSTANTINOS A. GEORGIOU*

Chemistry Laboratory, Agricultural University of Athens, 75 Iera Odos, 11855 Athens, Greece

A flow injection method for antioxidant capacity assessment based on a low-cost laboratory-made analyzer is reported. A sample of 30 μL is injected in acetate buffer stream, pH 4.6, that converges with ABTS•$^+$ reagent stream. Detection is achieved by monitoring absorbance at 414 nm. The proposed method achieves a sample throughput of up to 120 samples h^{-1}, the detection limit being 1.3 μM trolox. Precision was better than 5% relative standard deviation ($n = 4$) and the linear range was 4−100 μM, expanded to 250 μM trolox utilizing concentration gradients formed along the injected sample bolus. Information on reaction kinetics is obtained through a single injection. The method was applied to pure compounds and wine and honey samples. Good correlation was found between antioxidant capacity assessed through the proposed method and phenolic content: $r = 0.94$ for red wines, $r = 0.96$ for white and rose wines, and $r = 0.89$ for honeys.

KEYWORDS: Total antioxidant capacity; ABTS•$^+$; flow injection; gradient dilution

INTRODUCTION

Free radicals and other reactive oxygen species can be formed in human body and food systems and have been implicated in the pathology of several diseases (*1*, *2*). Antioxidant compounds protect against these radicals, and their role in nutrition and human health has come under attention (*3*). The effect of antioxidants in complex mixtures where various interactions may take place (*4*) and unidentified antioxidants could be present is better described by antioxidant activity rather than concentrations of specific antioxidants. On this basis, many assays have been introduced for determining total antioxidant capacity, a widely used parameter to characterize different pure compounds, body fluids, plant materials, and food extracts (*5*, *6*).

Among the methods that have been developed for the assessment of antioxidant activity, the ABTS assay, based on scavenging of the colored ABTS radical cation, is one of the most popular (*6*−*8*). The stability of ABTS•$^+$, the applicability in both aqueous and ethanolic solutions, and the wide spectral and pH working range resulted in the use of ABTS•$^+$ for the estimation of the antioxidant activity of pure compounds and complex matrixes. The original ABTS assay is based on the inhibition of ABTS•$^+$ formation while in modified versions; ABTS•$^+$ is pregenerated chemically or enzymatically and then allowed to react with antioxidant compounds (*8*, *9*). Consumption of ABTS•$^+$ is measured spectrophotometrically at a fixed time. Comparison with the trolox induced consumption provides the trolox equivalent antioxidant capacity (TEAC) value.

Flow injection (FI) is a very efficient technique for automation of wet chemical assays. Several FI methods for the evaluation of TAC have been developed on the basis of chemiluminescent (*10*) and electrochemical (*11*) detection, while recently two FI methods based on spectrophotometric detection have been reported (*12*, *13*). These methods are based on single-line manifolds and achieve sample throughputs up to 30 samples h^{-1}. This study presents a modified FI assay that exploits the inherent advantages of FI technique concerning high analysis rates and concentration gradients formed along the injected sample bolus (*14*). Strict control of mixing and timing in FI allows assays to be carried out reproducibly, even when chemical reactions involved do not reach completion (*15*). Readouts in FI are the outcome of two simultaneously occurring kinetic processes: physical dispersion and chemical reaction. Physical dispersion results in reproducible concentration gradients formed during mixing of the injected sample zone with the flowing reagent streams (*15*, *16*). Beyond utilization of FI as an automation tool, this work includes an approach using concentration gradients for the assessment of TAC at different end point times.

The proposed FI assay was applied to pure compounds and food matrixes. Compounds studied are representative of different classes of antioxidants widely spread in foods and biological material. In addition, synthetic antioxidants commonly used in the food industry were studied. For validation of the proposed methodology, wines and honeys that contain various phenolic antioxidants were assessed for TAC. Wines have been extensively studied with various TAC assays (*17*) while there is a increased interest in the beneficial health effects of honey (*18*).

MATERIALS AND METHODS

Chemicals. ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] in the crystallized diammonium form, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), caffeic acid, ferulic acid, gallic

* Corresponding author: Tel: +30-210-5294248; fax: +30-210-5294265, e-mail: cag@aua.gr.

10.1021/jf050106j CCC: $30.25 © 2005 American Chemical Society
Published on Web 04/26/2005

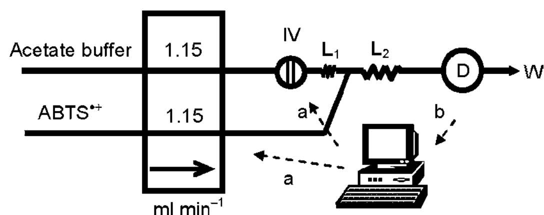

Figure 1. Laboratory-made flow injection analyzer for the determination of antioxidant capacity. Acetate buffer: 0.020 M, pH 4.6; ABTS•+: 6.4×10^{-5} M in acetate buffer; L_1 3-cm mixing and L_2 50-cm reaction coil; IV: injection valve; D: spectrophotometer, $\lambda = 414$ nm; W: waste; a: digital control signals; and b: analogue data.

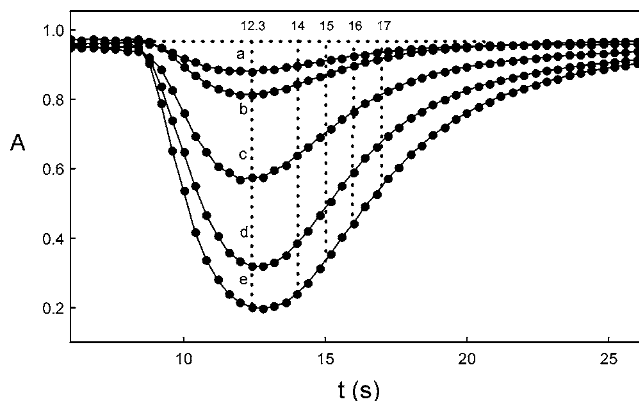

Figure 2. Flow injection peaks acquired through injections of gallic acid solutions of 5 (a), 10 (b), 25 (c), 50 (d), and 75 (e) μM. Readings at 12.3, 14, 15, 16, and 17 s were used for gradient calibration.

acid, uric acid, L-ascorbic acid, rutin, quercetin, BHA (butylated hydroxy-anisole), *t*-BHQ (*tert*-butylhydroquinone), (+)-catechin, and (−)-epicatechin were purchased from Sigma. HRP (horseradish peroxidase) was obtained from Serva. H_2O_2 (30% v/v) and other chemicals of analytical grade were purchased from Merck. Distilled water was used throughout.

Reagents and Samples. ABTS, H_2O_2, and HRP stock solutions of 20 mM, 20 mM, and 3.0×10^6 units L^{-1}, respectively, prepared in 0.020 M acetate buffer, pH 4.6, were stable for over a month when stored at 0−4 °C. ABTS•+ working solution was prepared by mixing appropriate volumes of the ABTS, H_2O_2, and HRP stock solutions in 0.020 M acetate buffer, pH 4.6, and allowing 2 h for reaction. To check ABTS•+ formation, the absorbance at 414 nm was monitored. Working solution was stable for at least 2 days at 0−4 °C.

The stoichiometry of ABTS oxidation by H_2O_2 is 2 ABTS:1 H_2O_2. To obtain an ABTS•+ working solution of 0.9−1.1 absorbance units and prevent possible reactions between antioxidants and unreacted hydrogen peroxide, a H_2O_2 concentration of 32 μM and a 5-fold excess of ABTS, 0.32 mM, were chosen.

To prepare 2.0 mM antioxidant stock solutions, compounds were dissolved in appropriate solvents: trolox and uric acid in 0.020 M phosphate buffer, pH 7.4; L-ascorbic acid, (+)-catechin, (−)-epicatechin, and glutathione in distilled water; ferulic acid, caffeic acid, BHA, and *t*-BHQ in ethanol; and quercetin and rutin in methanol. Stock solutions were found to be stable for at least 1 week when stored at 0−4 °C. Working solutions of 2−100 μM in 0.020 M acetate buffer, pH 4.6, were prepared daily.

Twenty-seven wines from different origins and vintages and 18 honey samples of several floral sources were supplied from local producers.

Flow-Injection Assay. The laboratory-made FI analyzer depicted in **Figure 1** consists of an Ismatec IPC-8 peristaltic pump, a Milton Roy Spectronic-20 spectrophotometer equipped with a Helma 18 μL, 1-cm path length flow cell, a VICI Valco C22Z-3186E cheminert low-pressure six-port valve with electric actuator, and an Advantech PCL-818 interface card for data acquisition and control. PTFE tubing, 0.8-mm i.d., was used for the construction of mixing and reaction coils. The laboratory-made software package for data acquisition and control provided modules for FI experiments; data treatment, measurement of peak height and correction of drifting baseline; and data export to text files. The MS-DOS based software was developed in C language and provided for fast, up to 60 kHz, data acquisition rates by using direct memory access data transfer from the AD converter. Control of the pump, start and stop, and injection valve, load and inject, were achieved through C language functions. The software developed has low system requirements and was used with an 80486 personal computer. A C language function was incorporated in the data treatment module to overcome possible problems due to bubble formation. After bubble detection, the function either replaces fault data points by fitting previous and next points to a third-degree polynomial or marks the injection as unusable.

Honeys and red, rose, and white wines are diluted 1:20, 1:250, 1:100, and 1:50, respectively, with 0.020 M acetate buffer, pH 4.6. Standards and samples are injected in the acetate buffer stream (**Figure 1**) that converges with the ABTS•+ reagent stream while flowing to the detector. The absorbance at 414 nm is continuously monitored resulting in negative absorbance peaks (**Figure 2**). The reagent blank is the baseline signal. Measurements are made on peak maximum at 12.3 s and on the tail of the dispersed sample zone at 14, 15, 16, and 17 s. The antioxidant capacity of the samples, expressed in TEAC units, is calculated using the corresponding calibration curve of trolox.

Determination of Phenolic Content. The determination of total phenolics was carried out by Folin−Ciocalteu method as described by Singleton et al. (*19*). Honeys and red, rose, and white wines were diluted 1:20, 1:100, 1:20, and 1:10, respectively, with distilled water. Absorbance measurements were carried out with a Jasco V-550 spectrophotometer.

RESULTS AND DISCUSSION

Optimization. It is well-known that tests for antioxidant activity are pH dependent (*20*, *21*). In this context, as most plant extracts are acidic, to measure antioxidant activity at pH relative to the food systems tested, the pH was set at 4.6. The thermal stability of ABTS•+ was assessed in the range of 15−50 °C by monitoring the absorbance at 414 nm. It was found that over 40 °C the radical cation was not stable, so experiments were performed at 25 ± 1 °C. ABTS•+ is stabilized in the presence of unreacted ABTS (*7*). Although the ratio [ABTS]/[ABTS•+] at pH 4.6 could be just 1 (*20*), to ensure that all H_2O_2 used for the formation of the radical cation has been consumed, a 5-fold excess of ABTS was chosen. The usually used wavelength for the ABTS assay is 734 nm where the molecular absorption coefficient of ABTS•+ is 1.33×10^4 M^{-1} cm^{-1}. However, to achieve higher sensitivity, the wavelength was set at 414 nm where a maximum with higher molecular absorption coefficient, 3.1×10^4 M^{-1} cm^{-1}, exists (*7*).

A single-line manifold (*12*, *13*) in comparison to this two-line manifold method is simpler. However, the two-line manifold allows complete mixing of sample with reagent and widens the dynamic range of the method by maintaining excess of reagent throughout the sample zone (*14*). This manifold also eliminates the sample blank that is a potential problem with single-line manifolds. The mixing coil before the confluence point (L_1, **Figure 1**) was made as short as possible to prevent high sample dispersion (*22*).

As antioxidant activity depends on a variety of antioxidant compounds that show different kinetic behavior toward ABTS•+ (*20*), the determination of TAC by the ABTS assay is influenced by end point time. End points between 0.1 s (*23*) and 20 min (*7*) have been reported and make almost impossible the comparison of results obtained through different variants of the assay described in the literature. Therefore, considering that antioxidant activity is a relative property and a main advantage

Table 1. Effect of Sample Volume on the Characteristics of the Calibration Curve (A vs C, $n = 7$) for Trolox and (–)-Epicatechin[a]

antioxidant	sample volume (μL)	slope ± SD ($A \times mM^{-1}$)	r	%RSD[b] ($n = 4$)	detection limit (μM)	linear range (μM)
trolox	15	3.5 ± 0.1	0.9990	0.1–5	2.6	8.6–200
	30	6.8 ± 0.2	0.9992	0.2–4	1.3	4.4–100
	60	11.4 ± 0.4	0.998	0.2–5	0.8	2.6–75
	100	15.9 ± 0.5	0.996	0.5–6	0.6	1.9–50
(–)-epicatechin	15	4.5 ± 0.2	0.994	0.2–5	2.0	6.7–150
	30	9.3 ± 0.4	0.996	0.2–4	1.0	3.2–75
	60	13.2 ± 0.7	0.994	0.2–5	0.7	2.2–50
	100	17.6 ± 0.8	0.994	0.5–7	0.5	1.7–35

[a] Total flow rate: 2.3 mL min^{-1}, reaction coil: 50 cm. [b] Range for calibration standards.

Table 2. Effect of the Reaction Coil on the Characteristics of the Calibration Curve (A vs C, $n = 7$) for Trolox and (–)-Epicatechin[a]

antioxidant	reaction coil (μL)	slope ± SD ($A \times mM^{-1}$)	r	%RSD[b] ($n = 4$)	detection limit (μM)	linear range (μM)
trolox	25	8.1 ± 0.2	0.993	1–7	1.1	3.7–60
	50	6.7 ± 0.2	0.9997	0.2–5	1.3	4.5–100
	75	5.6 ± 0.4	0.9999	0.6–6	1.6	5.4–120
	100	4.4 ± 0.1	0.996	0.1–4	2.0	6.8–150
(–)-epicatechin	25	8.9 ± 0.4	0.994	0.6–6	1.0	3.4–50
	50	9.3 ± 0.3	0.997	0.5–5	1.0	3.2–75
	75	9.1 ± 0.3	0.996	0.7–5	1.0	3.3–75
	100	8.9 ± 0.4	0.994	1–5	1.0	3.4–50

[a] Sample volume: 30 μL; other conditions as in Table 1. [b] Range for calibration standards.

Table 3. Effect of Total Flow Rate on the Characteristics of the Calibration Curve (A vs C, $n = 7$) for Trolox and (–)-Epicatechin[a]

antioxidant	flow rate (mL min^{-1})	slope ± SD ($A \times mM^{-1}$)	r	%RSD[b] ($n = 4$)	detection limit (μM)	linear range (μM)
Trolox	1.8	6.2 ± 0.1	0.9992	0.3–5	2.9	9.6–100
	2.3	6.8 ± 0.1	0.9991	0.2–4	1.3	4.4–100
	2.8	6.9 ± 0.3	0.997	0.6–6	1.4	4.7–100
(–)-epicatechin	1.8	8.5 ± 0.2	0.997	0.4–5	2.1	7.1–75
	2.3	9.2 ± 0.1	0.9997	0.1–4	1.0	3.3–75
	2.8	8.8 ± 0.3	0.996	0.7–7	1.4	4.6–75

[a] Sample volume: 30 μL; other conditions as in Table 1. [b] Range for calibration standards.

of FI is speed, optimization is aimed at increasing sample throughput rather than reproducing TEAC values reported by one specific procedure. To account for different reaction rates, all optimization experiments were carried out using both trolox and (−)-epicatechin as a fast and a slow reacting antioxidant, respectively (*20*). The effect of the injected sample volume is shown in **Table 1**. The slope of the calibration curve increases while detection limit decreases by increasing the sample volume for both trolox and (−)-epicatechin. However, linear range decreases and sample throughput decreases from 120 to 80 samples h^{-1}. A sample volume of 30 μL was chosen as a compromise between sensitivity and sampling rate. Results obtained using various reaction coil lengths and flow rates are shown in **Table 2** and **3**, respectively. As the reaction coil increases and the flow rate decreases, the residence time and the detection limit increase for trolox but no significant effect is observed for (−)-epicatechin. This is due to the opposite effects of (a) longer residence time that allows the reaction to proceed and (b) higher dispersion. From these results, it is clear that the optimization of methods that assess a property that depends on a mixture of compounds demands the simultaneous use of compounds exhibiting different reaction kinetics. A reaction coil of 50 cm and a 2.3 mL min^{-1} flow rate were chosen for high sample throughput and increased sensitivity. To achieve efficient mixing, flow rates of both streams depicted in **Figure 1** were equal throughout this study.

For the used experimental setup, the dispersion coefficient was found to be 6.7. That means that this FI method is based on a medium dispersion manifold and samples, on the way to the detector, are diluted 1:5.7.

Method Evaluation. The analyzer stability was evaluated during continuous operation for 8 h. Calibration equations for trolox at the start and the end of this time period were $A = (-2 \pm 3) + (6.7 \pm 0.2) \times C_{\mathrm{trolox}}$(mM) and $A = (-4 \pm 5) + (6.8 \pm 0.1) \times c_{\mathrm{trolox}}$(mM), respectively, while the linearity was excellent, $r = 0.999$, for both.

Precision was evaluated by multiple injections of trolox standards of 10 and 50 μM. Relative standard deviations were 2.7 and 1.4% ($n = 10$), respectively. Baseline noise measured for 8 h was lower than 0.3% RSD. Sample blanks obtained by changing the $ABTS\bullet^{+}$ stream with buffer were equal to zero.

Calibration data for all studied antioxidant compounds, along their TEAC values, are shown in **Table 4**. Ranking activities are in accordance with data obtained using different versions of the $ABTS\bullet^{+}$ assay (*8*, *9*, *24*) with the exception of quercetin. However, reported TEAC values for quercetin vary from 3.1 to 6.4 (*25*). This variation could probably be ascribed not only to procedural differences but also to *different quercetin* concentrations used (*9*).

Flow Injection Gradient and TEAC. Readouts at the tail of the dispersed sample zone (**Figure 2**

) result in calibration curves of different slopes (**Figure 3**). This approach was introduced in FI by Olsen et al. (*26*) under the name of electronic dilution or gradient calibration and exploits concentration gradients formed in FI manifolds. Using this approach, that is, on the basis of the excellent reproducibility

Table 4. Characteristics of the Calibration Curve (A vs C, $n = 7$)

antioxidant	slope ± SD ($A \times$ mM^{-1})	r	linear range (μM)	TEAC[b] ± SD ($n = 4$)
L-ascorbic acid	6.7 ± 0.1	0.9992	4–100	0.99 ± 0.02
caffeic acid	7.0 ± 0.1	0.998	4–100	1.03 ± 0.02
ferulic acid	10.5 ± 0.4	0.996	3–50	1.54 ± 0.08
gallic acid	12.3 ± 0.2	0.9992	2–50	1.81 ± 0.07
(+)-catechin	8.2 ± 0.2	0.997	3–80	1.21 ± 0.03
(–)-epicatechin	9.3 ± 0.3	0.996	3–80	1.37 ± 0.09
rutin	7.5 ± 0.3	0.9995	4–80	1.10 ± 0.06
quercetin	9.1 ± 0.7	0.992	4–40	1.34 ± 0.09
glutathione	0.20 ± 0.04	0.998	50–800	0.029 ± 0.006
uric acid	7.3 ± 0.1	0.998	4–100	1.07 ± 0.02
t–BHQ	7.1 ± 0.2	0.998	4–100	1.04 ± 0.06
BHA	7.5 ± 0.3	0.997	3–80	1.10 ± 0.07

[a] Sample volume: 30 μL; total flow rate: 2.3 mL min-[1]; reaction coil: 50 cm. [b] Calculated by dividing the slope with that of trolox.

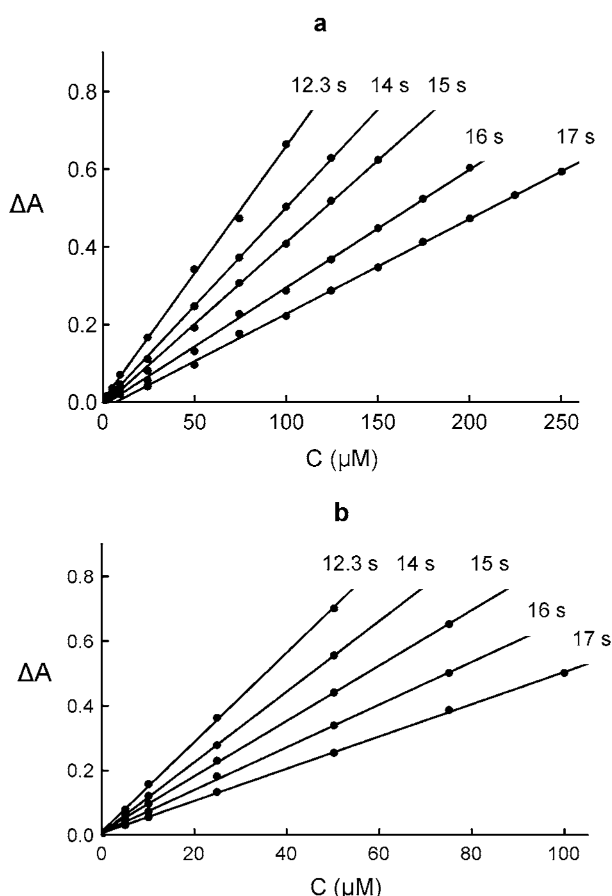

Figure 3. Calibration curves recorded on peak maximum: 12.3 s and peak tail: 14, 15, 16, and 17 s for (a) trolox and (b) gallic acid.

of FI peaks, samples that exceed the dynamic range of the detector can be analyzed without manual dilution (*27*). For trolox (**Figure 3a**), the calibration curve slope decreases from 6.7 ± 0.2 to 2.03 ± 0.07 $A \times$ mM^{-1} while the linear range is shifted from 4−100 to 10−250 μM when using readings at 12.3 and 17 s, respectively. Gallic acid shows almost the same behavior (**Figure 3b**). The calibration slope decreases from 12.3 ± 0.2 to 4.56 ± 0.06 $A \times$ mM^{-1}, while the linear range is shifted from 2−50 to 5−100 μM. The decrease of the calibration curve slope is 70% for trolox while it is 63% for gallic acid. This is due to the lower reaction rate of gallic acid. Flow injection signals are the output of two kinetic processes occurring simultaneously: physical dispersion (i.e., dilution) and chemical reaction (*15*). Physical dispersion is the only process for trolox, a fast-reacting antioxidant, and results in lower signals. However, signals from slow-reacting antioxidants, such as gallic acid, decrease to a lower extent because of the longer reaction time allowed. This is clearly shown in **Table 5** where the TEAC values for slow-reacting antioxidants, ferulic acid, gallic acid, (+)-catechin, (−)-epicatechin, *t*-BHQ, and BHA, are shown to increase using peak tail readings.

Table 5. TEAC Values Obtained along FI Peaks[b]

antioxidant	TEAC[a] ± SD ($n = 5$) 12.3 s	14 s	15 s	16 s	17 s
L-ascorbic acid	1.01 ± 0.01	0.99 ± 0.01	0.99 ± 0.02	0.99 ± 0.02	1.00 ± 0.02
caffeic acid	1.03 ± 0.02	1.00 ± 0.02	1.02 ± 0.01	1.03 ± 0.03	1.01 ± 0.02
ferulic acid	1.54 ± 0.02	1.69 ± 0.03	1.82 ± 0.02	1.94 ± 0.03	2.01 ± 0.04
gallic acid	1.81 ± 0.02	2.09 ± 0.03	2.25 ± 0.02	2.40 ± 0.03	2.53 ± 0.04
(+)-catechin	1.21 ± 0.02	1.32 ± 1.41	1.41 ± 0.02	1.53 ± 0.02	1.56 ± 0.03
(–)-epicatechin	1.37 ± 0.03	1.55 ± 0.02	1.64 ± 0.02	1.74 ± 0.02	1.79 ± 0.03
t–BHQ	1.04 ± 0.02	1.09 ± 0.02	1.14 ± 0.01	1.16 ± 0.02	1.18 ± 0.02
BHA	1.10 ± 0.01	1.21 ± 0.02	1.27 ± 0.01	1.34 ± 0.02	1.35 ± 0.02

[a] Calculated by dividing the slope with that of trolox. [b] Fifty micromolar antioxidants solutions were injected.

Method Application. Taking into account the variation of reported TEAC values by different procedures, results from the developed method were correlated to total phenolic content that relates to antioxidant capacity of various food matrixes (*6*).

Wine Samples. TEAC values determined by the proposed method for different types of wines along their corresponding total phenolic content are shown in **Figure 4**. Good correlation was found between total phenolic content and antioxidant capacity: $r = 0.94$ for red wines, $n = 15$; $r = 0.96$ for white and rose wines, $n = 12$. Similar correlation results have been reported in previous study utilizing different assays (*17*). Total phenolic content values ranged from 10.5 to 17.4 and from 0.96 to 2.79 mM gallic acid for red, and rose and white wines, respectively. Similar ranges for wines' phenolics have been previously reported (*17*). Antioxidant activities determined ranged from 3.57 to 6.39 and from 0.31 to 1.91 mM trolox for red, and rose and white wines, respectively. Since measurements performed at peak maximum allow reaction time of 12.3 s while the original ABTS assay allows 6 min, lower antioxidant capacity values are anticipated through the proposed assay. Values determined from readouts at 17 s were about 40% higher. However, relative activities and correlations with total phenolic content were the same.

Honey Samples. The complex composition of honey that is strongly dependent on floral source and influenced by seasonal and environmental factors makes total antioxidant capacity a useful tool for honey characterization. Results from honey analysis are presented in **Figure 5**. Antioxidant activities determined were in the range of 0.15−2.43 mmol kg^{-1} trolox while phenolic content values ranged from 1.96 to 5.0 mmol kg^{-1} gallic acid. The correlation between phenolic content and antioxidant capacity ($r = 0.89$, $n = 18$) demonstrates the contribution of phenolic compounds to antioxidant capacity although other compounds contribute (*28*). Previous study of limited numbers of honey samples showed even lower correlation between antioxidant capacity, as assessed through the DPPH assay, and phenolic content (*28*). In contrast to wines, readings at the peak tail provided the same TEAC values as those at peak maximum. This is probably due to the contribution of unknown compounds that exhibit fast reaction kinetics. The ABTS$\bullet^{+}$ assay is used for the first time for honey samples.

In conclusion, the proposed FI method provides for rapid antioxidant capacity assessment facilitating the analysis of up

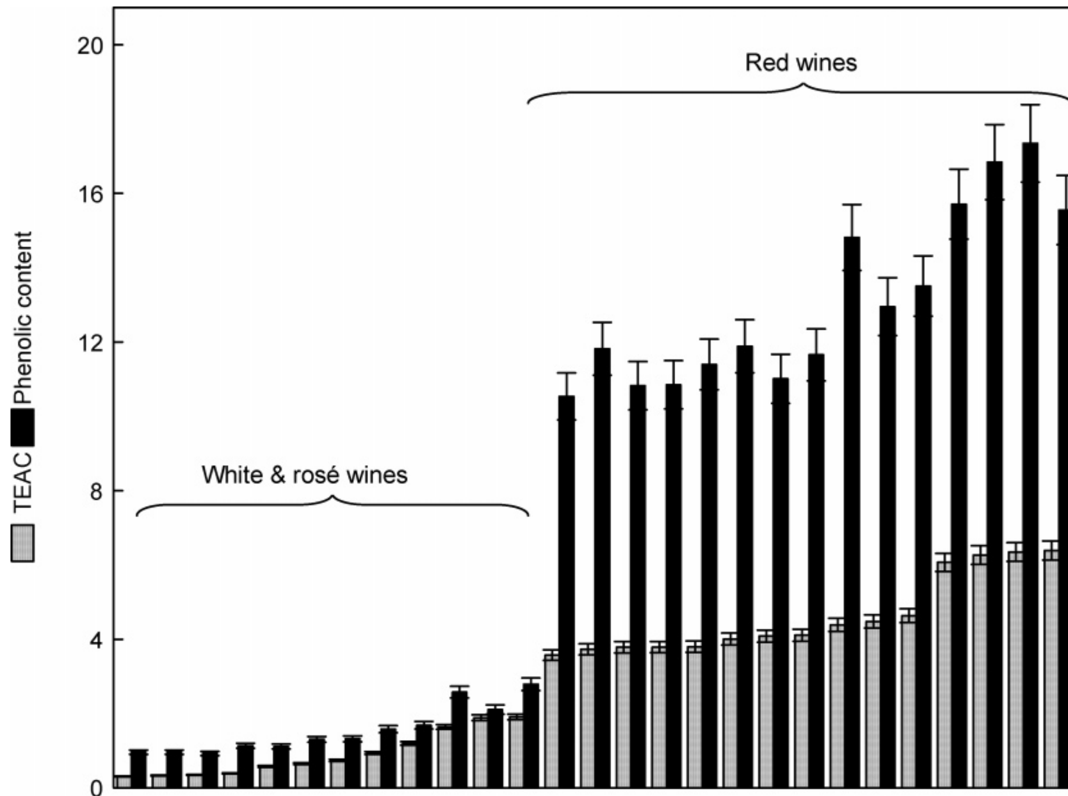

Figure 4. Results from the analysis of 27 wine samples. TEAC values expressed as mM trolox and phenolic content expressed as mM gallic acid. Values are means of triplicate measurements.

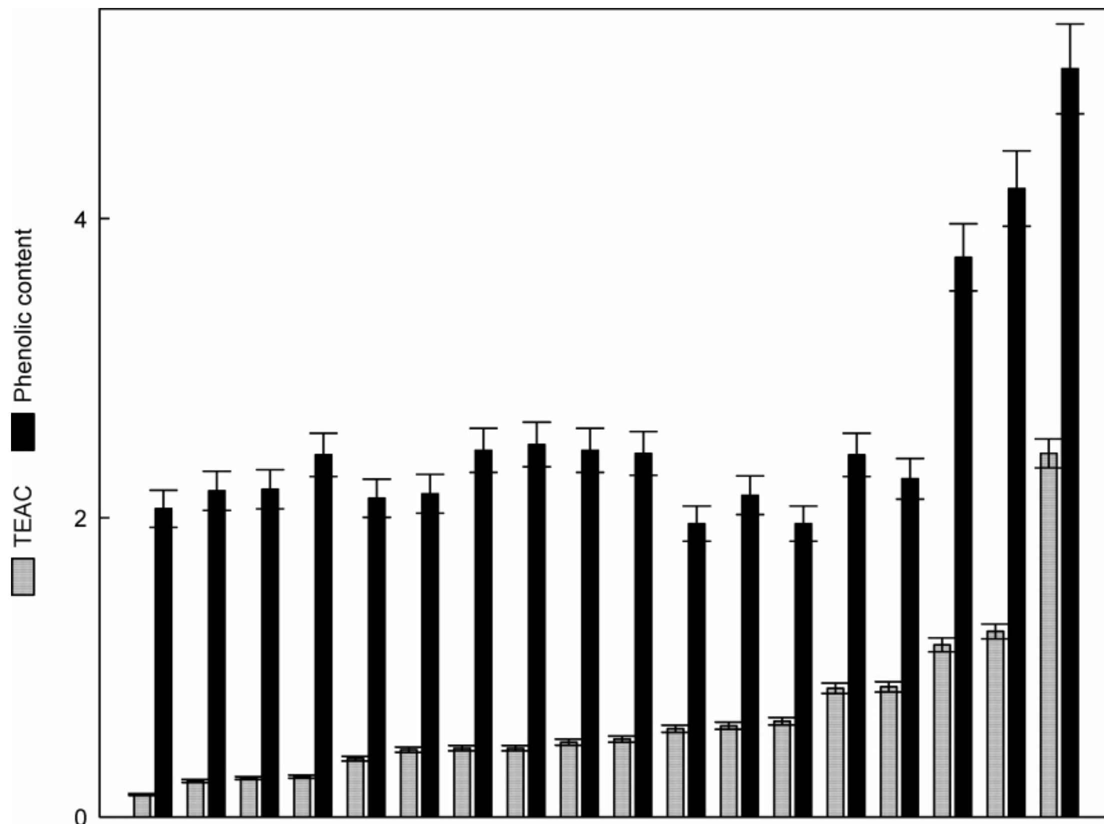

Figure 5. Results from the analysis of 18 honey samples. TEAC values expressed as mmol kg^{-1} trolox and phenolic content expressed as mmol kg^{-1} gallic acid. Values are means of triplicate measurements.

to 120 samples h^{-1}. Another advantage is the use of gradient calibration that provides automated dilution of concentrated samples and increases reaction time without loss of sampling rate. Moreover, information concerning reaction kinetics can be obtained through a single injection. It is anticipated that the proposed method will be a powerful tool for the rapid screening of pure compounds and complex materials using other chemistries beyond the ABTS assay.

ABBREVIATIONS USED

TAC, total antioxidant capacity; TEAC, trolox equivalent antioxidant capacity; FI, flow injection; 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; BHA, butylated hydroxy-anisole; t-BHQ, *tert*-butylhydroquinone; HRP, horseradish peroxidase.

LITERATURE CITED

(1) Halliwell, B.; Murcia, M. A.; Chirico, S.; Aruoma, O. I. Free-radicals and antioxidants in food and in vivo: what they do and how they work. *Crit. Rev. Food Sci.* **1995**, *35*, 7−20.
(2) Nyska, A.; Kohen, R. Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. *Toxicol. Pathol.* **2002**, *30*, 620−650.
(3) Kaur, C.; Kapoor, H. C. Antioxidants in fruits and vegetables - the millennium's health. *Int. J. Food Sci. Technol.* **2001**, *36*, 703−725.
(4) Arts, M.; Haenen, G.; Wilms, L. C.; Beetstra, S.; Heijnen, C. G. M.; Voss, H. P.; Bast, A. Interactions between flavonoids and proteins: effect on the total antioxidant capacity. *J. Agric. Food Chem.* **2002**, *50*, 1184−1187.
(5) Antolovich, M.; Prenzler, P. D.; Patsalides, E.; McDonald, S.; Robards, K. Methods for testing antioxidant activity. *Analyst* **2002**, *127*, 183−198.
(6) Roginsky, V.; Lissi, E. A. Review of methods to determine chain-breaking antioxidant activity in food. *Food Chem.* **2005**, *92*, 235−254.
(7) Arnao, M. B. Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. *Trends Food Sci. Technol.* **2000**, *11*, 419−421.
(8) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.
(9) van den Berg, R.; Haenen, G.; van den Berg, H.; Bast, A. Applicability of an improved trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem.* **1999**, *66*, 511−517.
(10) Costin, J. W.; Barnett, N. W.; Lewis, S. W.; McGillivery, D. J. Monitoring the total phenolic/antioxidant levels in wine using flow injection analysis with acidic potassium permanganate chemiluminescence detection. *Anal. Chim. Acta* **2003**, *499*, 47−56.
(11) Buratti, S.; Pellegrini, N.; Brenna, O. V.; Mannino, S. Rapid electrochemical method for the evaluation of the antioxidant power of some lipophilic food extracts. *J. Agric. Food Chem.* **2001**, *49*, 5136−5141.
(12) Bompadre, S.; Leone, L.; Politi, A.; Battino, M. Improved FIA-ABTS method for antioxidant capacity determination in different biological samples. *Free Radical Res.* **2004**, *38*, 831−838.
(13) Pellegrini, N.; Del Rio, D.; Colombi, B.; Bianchi, M.; Brighenti, F. 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. *J. Agric. Food Chem.* **2003**, *51*, 260−264.
(14) Ruzicka, J.; Hansen, E. H. Recent developments in flow injection analysis: gradient techniques and hydrodynamic injection. *Anal. Chim. Acta* **1983**, *145*, 1−15.
(15) Hansen, E. H. Flow-injection analysis: leaving its teen-years and maturing. A personal reminiscence of its conception and early development. *Anal. Chim. Acta* **1995**, *308*, 3−13.
(16) Georgiou, M. E.; Georgiou, C. A.; Koupparis, M. A. Flow injection gradient technique in spectrophotometric determination of formation constants of micromolecule-cyclodextrin complexes. *Anal. Chem.* **1995**, *67*, 114−123.
(17) Fernandez-Pachon, M. S.; Villano, D.; Garcia-Parrilla, M. C.; Troncoso, A. M. Antioxidant activity of wines and relation with their polyphenolic composition. *Anal. Chim. Acta* **2004**, *513*, 113−118.
(18) Schramm, D. D.; Karim, M.; Schrader, H. R.; Holt, R. R.; Cardetti, M.; Keen, C. L. Honey with high levels of antioxidants can provide protection to healthy human subjects. *J. Agric. Food Chem.* **2003**, *51*, 1732−1735.
(19) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152−178.
(20) Labrinea, E. P.; Georgiou, C. A. Stopped-flow method for assessment of pH and timing effect on the ABTS total antioxidant capacity assay. *Anal. Chim. Acta* **2004**, *526*, 63−68.
(21) Lemanska, K.; Szymusiak, H.; Tyrakowska, B.; Zielinski, R.; Soffers, A.; Rietjens, I. The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxy-flavones. *Free Radical Biol. Med.* **2001**, *31*, 869−881.
(22) Ruzicka, J.; Hansen, E. H. *Flow Injection Analysis,* 2nd ed.; John Wiley: New York, 1988; p 23.
(23) Pannala, A. S.; Chan, T. S.; O'Brien, P. J.; Rice-Evans, C. A. Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 1161−1168.
(24) Pellegrini, N.; Visioli, F.; Buratti, S.; Brighenti, F. Direct analysis of total antioxidant activity of olive oil and studies on the influence of heating. *J. Agric. Food Chem.* **2001**, *49*, 2532−2538.
(25) Arts, M.; Dallinga, J. S.; Voss, H. P.; Haenen, G.; Bast, A. A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chem.* **2004**, *88*, 567−570.
(26) Olsen, S.; Ruzicka, J.; Hansen, E. H. Gradient techniques in flow injection analysis: stopped-flow measurement of the activity of lactate dehydrogenase with electronic dilution. *Anal. Chim. Acta* **1982**, *136*, 101−112.
(27) Georgiou, C. A.; Valsami, G. N.; Macheras, P. E.; Koupparis, M. A. Automated flow-injection technique for use in dissolution studies of sustained-release formulations: application to iron-(II) formulations. *J. Pharm. Biomed. Anal.* **1994**, *12*, 635−641.
(28) Aljadi, A. M.; Kamaruddin, M. Y. Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. *Food Chem.* **2004**, *85*, 513−518.

Received for review January 17, 2005. Revised manuscript received March 18, 2005. Accepted March 22, 2005.

JF050106J