JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Automatic Method for the Determination of Folin−Ciocalteu Reducing Capacity in Food Products

LUÍS M. MAGALHÃES,† MARCELA A. SEGUNDO,*,† SALETTE REIS,† JOSÉ L. F. C. LIMA,† AND ANTÓNIO O. S. S. RANGEL‡

REQUIMTE, Serviço de Química-Física, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4099-030 Porto, Portugal, and Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

In the present work, an automatic flow procedure based on multi-syringe flow injection analysis was developed for the assessment of Folin−Ciocalteu reagent (FCR) reducing capacity in several types of food products using gallic acid as the standard. Different strategies for mixing of sample and reagent were tested (continuous flow of FCR, merging zones, and intercalated zones approaches); lower reagent consumption and higher determination throughput were attained for the merging zones approach (100 μL of sample + 100 μL of FCR). The application of the proposed method to compounds with known antioxidant activity (both phenolic and nonphenolic) and to samples (wines, beers, teas, soft drinks, and fruit juices) provided results similar to those obtained by the conventional batch method. The detection limit was 0.6 mg L^{-1}, and the determination frequency was about 12 h^{-1}. Good repeatability was attained (RSD < 1.3%, $n = 10$).

KEYWORDS: Multi-syringe flow injection; Folin−Ciocalteu reducing capacity; phenolic compounds

INTRODUCTION

Free radicals, reactive oxygen species (ROS), and reactive nitrogen species (RNS) derived either from normal metabolic processes or from external sources are directly related with oxidation in food and biological systems. They are also implicated in the oxidative rancidity, which is one of the most critical factors affecting the shelf life of processed food, and in the development of several human diseases such as neurological degeneration, cataracts, diabetes, cardiovascular diseases, and certain types of cancer (*1*, *2*). Interest in antioxidant nutrients has increased in the light of recent evidence regarding their protective effects against these free radical-induced reactions (*3*, *4*). With a few exceptions (such as carotenoids, vitamin C, and vitamin E), the most important dietary antioxidants are the phenolic compounds (*5*). For this reason, the assessment of total phenolic content has gained enormous attention in the last few years, especially within the food, biological, and agrochemical fields.

Many analytical procedures have been developed for quantification of total phenolic content in foods (*6*, *7*). Although separative methods such as capillary electrophoresis and high-performance liquid chromatography with diode array detection are powerful techniques for the isolation and identification of phenolic compounds in complex samples, their application to estimate the total phenolic content may be inaccurate (*8*). Moreover, the separative techniques are time-consuming, expensive, and often not suitable for routine determinations.

For quantification of total phenolic content, most of the available methods are based on the reaction of phenolic compounds with a colorimetric reagent, thus allowing their measurement in the visible region of the spectra (*7*). Among these methods, the Folin−Ciocalteu assay (FC assay) is frequently applied (*9*, *10*), and recent studies have shown that total phenols determined by this method can be correlated to antioxidant activity determined by different methods ($ABTS^{+\bullet}$ and $DPPH^{\bullet}$ assays, for instance) (*11*). For this reason, the method described by Singleton and Rossi (*9*) has been proposed recently as a standardized method for use in the routine quality control and measurement of antioxidant capacity of food products and dietary supplements (*12*). Moreover, the novel designation "FC reagent reducing capacity" was suggested (*13*).

For routine analysis, the automation of FC assay has been described using flow injection analysis (FIA) (*14*−*17*) and sequential injection analysis (SIA) (*18*) for the determination of total polyphenols index of wine and beer samples. However, these methodologies replaced the recommended gallic acid reference standard with oenological tannin (*14*), coumaric acid (*15*), or tannic acid (*16*−*18*).

Therefore, the objective of the present work was the development of an automatic flow procedure based on multi-syringe flow injection analysis (MSFIA) (*19*, *20*) for the assessment of FC reagent reducing capacity using gallic acid as standard. MSFIA was introduced in 1999 in order to combine the multichannel operation of flow injection analysis to the flexible

* Corresponding author. Telephone: +351 222078994. Fax: +351 222004427. E-mail: msegundo@mail.ff.up.pt.
† Universidade do Porto.
‡ Universidade Católica Portuguesa.

10.1021/jf060324s CCC: $33.50 © 2006 American Chemical Society
Published on Web 06/23/2006

flow management offered by the multi-commutation technique. These features were exploited in the present work for evaluation of different strategies for mixing of sample and reagent. Furthermore, the application of the proposed method to samples and compounds with known antioxidant activity (both phenolic and nonphenolic) was also evaluated. The results were compared with the conventional batch method proposed for standardization.

MATERIALS AND METHODS

Chemicals. All chemicals used were of analytical-reagent grade with no further purification. Folin−Ciocalteu reagent (FCR), gallic acid, ascorbic acid, resorcinol, butylated hydroxyanisole (BHA), quercetin, and ferrous sulfate were purchased from Sigma (St. Louis, MO). Caffeic acid, catechol, propyl gallate, ferulic acid, and cinnamic acid were obtained from Aldrich (Milwaukee, WI). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), β-carotene, sinapic acid, ellagic acid, and (−)-epicatechin were obtained from Fluka (Buchs, Switzerland). Pyrogallol and tannic acid were purchased from Riedel-de-Haën (Seelze, Germany). Citric acid, sodium sulfite, D-(+)-glucose, sodium carbonate, and sodium hydroxide were obtained from Merck (Darmstadt, Germany).

Reagents and Samples. Water from Milli-Q system (resistivity > 18 MΩ cm) and ethanol absolute pro analysis were used for the preparation of all solutions.

The stock solutions were prepared by dissolving gallic acid, ascorbic acid, citric acid, ferrous sulfate, sodium sulfite, and D-(+)-glucose in water. Resorcinol, catechol, BHA, trolox, pyrogallol, caffeic acid, propyl gallate, tannic acid, ferulic acid, sinapic acid, and cinnamic acid were dissolved in ethanol solution 50% (v/v). β-Carotene, ellagic acid, (−)-epicatechin, and quercetin were dissolved in ethanol. The working solutions were prepared daily in a range between 3.1 and 766 μM by rigorous dilution of the respective stock solutions in water.

For the studies concerning different strategies for mixing sample and reagent, the following solutions were prepared: NaOH, 0.25 M; HCl, 0.10 M; and working standard solutions of gallic acid (2.5−100.0 mg L^{-1}). FCR was diluted 1:20 (v/v) with water.

For the automatic determination of FCR reducing capacity of compounds and food products, FCR was diluted 1:40 (v/v) (experiments using pure compounds) and 1:10 (v/v) (experiments using samples) with water. Sodium hydroxide solution (0.25 M) and working standard solutions containing gallic acid (2.5−40.0 mg L^{-1}) were also prepared.

For the batch method, FCR was diluted 1:5 (v/v) in water. Sodium carbonate (60 g L^{-1}) and working standard solutions of gallic acid (2.5 and 25.0 mg L^{-1}) were also prepared.

All food products were purchased at local markets. The tea extracts were prepared by pouring 200 mL of deionized water at 90 °C into a glass with tea bag (1.49−1.66 g of leaves) and by brewing for 5 min. No sample treatment other than dilution using water was applied before determination. The dilutions performed for the flow system and for the batch method varied from 1:25 to 1:200.

For determination of dispersion coefficient of Ruzicka (*21*), a bromothymol blue (BTB) solution was prepared from a stock solution (0.20 g L^{-1}) by dilution in 0.1 mol L^{-1} NaOH solution in order to provide an absorbance value of about 0.646 at 620 nm.

Apparatus. Solutions were propelled through the flow system by means of a multi-syringe piston pump (Crison Instruments, Allela, Spain) equipped with syringes of 5 mL (Hamilton, Switzerland). Each syringe is connected to a three-way solenoid valve (N-Research, Caldwell, NJ) that allows the access to two different channels (solutions flask or flow network). The multi-syringe module also comprises extra commutation valves. For all valves, the exchange options were classified in on/off lines. The "off" line was assigned to the solution flasks, and the "on" line was reserved for the flow network in the valves placed at the multi-syringe. For the other valves, the positions are assigned in order to maintain the valves turned "off" most of the time to avoid over-heating problems. All tubing connecting the different components of MSFIA was made of PTFE (Omnifit, Cambridge, U.K.) of 0.8 mm i.d. with Gilson (Villiers-le-Bel, France) end-fittings and connectors.

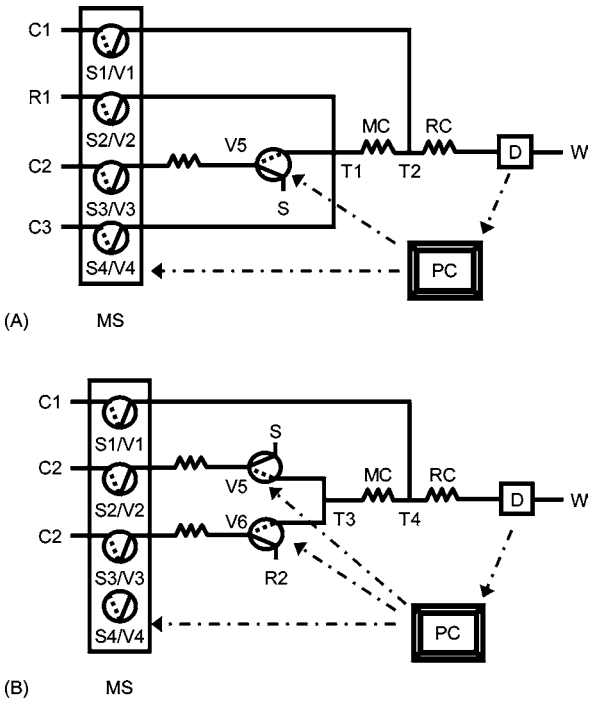

Figure 1. MSFIA manifolds for evaluation of different mixing strategies (**A**) and determination of FCR reducing capacity (**B**): MS, multi-syringe; S*i*, syringe; V*i*, commutation valves (solid and dotted lines represent the position on and off, respectively); MC, mixing coil; RC, reaction coil (100 cm); D, detector; T*i*, confluences; C1, NaOH 0.25 M; C2, water; C3, HCl 0.10 M; R1 and R2, Folin–Ciocalteu reagent diluted at 1:20 and 1:10 (v/v), respectively; S, standard solution or sample; PC, personal computer; W, waste.

A personal computer, running homemade software written in QuickBasic 4.5, controlled the multi-syringe operation (number of steps and direction of piston displacement) and the position of all commutation valves.

As detection system, a Jenway 6100 (Essex, U.K.) UV−vis spectrophotometer equipped with a flow-through cell from Hellma (internal volume = 80 μL, ref 178.710-QS, Mullheim/Baden, Germany) was used, and the wavelength was set at 750 nm. The data acquisition was performed through a PCL-711 B interface card at 3 Hz, using the same software developed for controlling the flow system. Furthermore, the analytical signals were also recorded in a Kipp & Zonen (Delft, The Netherlands) BD 111 strip chart recorder.

MSFIA Manifold and Procedure for Evaluation of Different Mixing Strategies. The system components were arranged as shown schematically in **Figure 1A**. The connection between the multi-syringe and the valve V5 was 200 cm long. The tubing length between valve V5 and confluence T1 was 20 cm long while the mixing coil (MC) was 100 cm long. The reaction coil (RC) had the same length.

These components constituted a flow network, where the management of solutions was defined through software control. This aspect allowed the implementation of different strategies for mixing sample and reagent (gallic acid−FCR) without physical reconfiguration of the manifold. Therefore, five different strategies were implemented based on continuous flow of FCR or on merging or intercalation of segments of sample and reagent. The protocol sequence was similar for each strategy adopted. Initially, the syringes were filled with solutions from the respective reservoirs (1650 μL) with all valves at off position. Then, 100 μL of gallic acid standard solution was aspirated by activating valves V3 and V5. After a dummy step (250 μL), applied to change the flow direction (*22*), the different mixing strategies were applied as described below in detail. Thereafter, at confluence T2 an alkaline solution was added, and the mixture was further propelled toward the detection system (total flow rate = 3 mL min^{-1}).

The first approach tested was similar to a conventional FIA procedure (*23*), involving the continuous flow of FCR. In this case, the sample

Table 1. Protocol Sequence for the Determination of FCR Reducing Capacity

step	description	position of the commutation valves[a] 1	2	3	4	5	6	volume[b] (μL)	time (s)
1	syringes are filled with the respective solutions	F	F	F	F	F	F	2300	6.90
2	sample and FCR are aspirated	F	N	N	F	N	N	200	12.00
3	dummy step to change the flow direction	F	F	F	F	F	F	500	1.50
4	sample, FCR, and NaOH are sent toward detection system	N	N	N	F	F	F	600	18.00
5	flow stop	F	F	F	F	F	F		240.00
6	carrier and NaOH are sent to wash the system	N	N	N	F	F	F	1400	21.00

[a] N and F represent the positions on and off, respectively. [b] The indicated values for volume refer to syringe 10 mL.

segment was merged at confluence T1 with reagent stream (valves V1, V2, and V3 were in position on).

The merging zones strategy (*24*) was also implemented, using equal (experiment merging zones I) or different volumes (experiments merging zones II and III) of FCR and sample. In the experiment merging zones I (100 μL of sample + 100 μL of FCR), the sample was pushed by carrier until confluence T1 (valves V1, V3, and V4 were in position on). After that, the sample and reagent segments were simultaneously sent to MC by activating valves V1, V2, and V3. In the experiment merging zones II, 100 μL of sample was merged with 300 μL of FCR. For this, sample and reagent zones were sent into the MC during a single forward displacement of the piston driver bar by activating valves V1, V2, and V3. The experiment merging zones III was performed using 100 μL of FCR and 300 μL of sample. After the sampling step, sample was pushed by carrier into the MC creating a front zone of sample of 100 μL (valves V1, V3, and V4 were in position on). After that, by activating valves V1, V2, and V3, 100 μL of sample and 100 μL of FCR were merged at confluence T1. Finally, in all these experiments, the final mixture was further propelled to the detector by activating valves V1, V3, and V4.

The last approach tested was implemented by sequential introduction of reagent and sample segments into MC channel, creating a plug of intercalated zones. The sample was placed between two segments of reagent and the mixture took place at the boundaries of each segment, as occurs in sequential injection analysis (SIA) (*25*). In this case, each segment had 100 μL of volume. The flow protocol was applied by sequentially activating valves V1, V2, and V3 (insertion of reagent) or valves V1, V3, and V4 (insertion of sample). For each experiment, the dispersion coefficient of the sample was calculated as recommended by Ruzicka and Hansen (*21*).

MSFIA Manifold and Procedure for Determination of FCR Reducing Capacity. The system components were arranged as shown schematically in **Figure 1B**. The connections between the multi-syringe and the valves V5 and V6 were 200 cm long. The connections between these valves and confluence T3 were 5 cm long. The mixing coil (MC) had the same length. The reaction coil (RC) was 100 cm long.

The following modifications were performed in the manifold presented in **Figure 1A**. The Folin−Ciocalteu reagent was introduced into the flow system by aspiration through one extra commutation valve (V6) instead of direct introduction through syringe 2. Moreover, the mixing coil was reduced from 100 to 5 cm in order to minimize the dispersion of standard/sample and FCR.

The protocol sequence for the determination of FCR reducing capacity is summarized in **Table 1**. Before starting the analytical cycle, syringe 1 was filled with NaOH solution while the other two syringes were filled with water. After flow reversal, these carriers were propelled toward the detection system. Thus, the flow-through cell was filled, and the absorbance signal was adjusted to zero.

In the first step of the analytical cycle, syringes were filled with the respective solutions. Then, 100 μL of standard/sample and 100 μL of FCR were aspirated. After a dummy step applied to change the flow direction (*22*), the standard/sample and FCR plugs were propelled through confluence T3 and MC up to confluence T4, where NaOH solution was added. After passing through RC, this mixture was propelled until it reached the flow-through cell. Then, the flow was stopped, and the absorbance at 750 nm was monitored during 240 s at room temperature. After the last step, in which carrier and NaOH were sent to wash the flow-through cell, the flow system was ready for a new analytical cycle. All experiments were performed in triplicate.

For the analysis of pure compounds, the same flow procedure was performed without flow stop. The reactivity of each compound was estimated through the establishment of linear calibration curves by plotting the absorbance as a function of concentration of testing compound (μM). Under these conditions, the slope of the calibration curve for testing compound was compared to the slope of the calibration curve for the standard compound (gallic acid). This ratio (%) reflected the FCR reducing capacity of the testing compound.

Folin−Ciocalteu Batch Method. The Folin−Ciocalteu method described by Singleton and co-workers (*9*, *10*) was adapted to a microplate reader (Synergy HT, Bio-Tek, Winooski, VT). Hence, 50 μL of gallic acid standard solution or food sample and 50 μL of FCR were placed in each well. After that, 100 μL of sodium carbonate solution was added. The absorbance of the blue complex formed was monitored at 760 nm every 60 s during 2 h. All experiments were performed in quadruplicate, and the temperature was kept at 25.0 ± 0.1 °C.

RESULTS AND DISCUSSION

Evaluation of Different Mixing Strategies. The chemistry behind the FC assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that can be detected spectrophotometrically (*9*). In this case, the sequence of mixture is of utmost importance, especially in order to avoid premature alkaline destruction of the FCR (*10*). This aspect was considered when developing the automatic flow system. Therefore, the reagent solution (FCR) was placed in syringe 2, and it was mixed with gallic acid standard solution in mixing coil (MC) after confluence T1. This mixture was further merged at confluence T2 with NaOH solution propelled by syringe 1. As the FC reagent contains acid, HCl solution was placed in syringe 4 as the carrier in order to maintain the pH value through flow system.

As MSFIA systems are based on a flow network relying on computer-controlled solenoid valves, different strategies for mixing sample and reagent after confluence T1 were evaluated. This was performed through software control without manifold reconfiguration.

The analytical features of different mixing strategies are summarized in **Table 2**. The sensitivity estimated through the slope of calibration curve was inversely related to the dispersion coefficient of sample. Thus, for the larger sample volume tested (300 μL, experiment merging zones III), the sensitivity is about twice that obtained for other experiments. However, the linear range decreased, and the determination frequency also decreased from 27 to 21 determinations h^{-1}. For experiments using 100 μL of sample, the sensitivity was similar (in the range 8.88−9.28 mAU mg^{-1} L). However, as the reaction on the intercalated zones approach took place at the boundaries of each segment, the linear range decreased from 5 to 100 to 5−40 mg L^{-1}. On

Table 2. Analytical Features of Different Strategies for Mixing Sample and Reagent in MSFIA

mixing strategy	slope[a] (mAU mg^{-1} L)	linear range (mg L^{-1})	D_{sample}	determination frequency (h^{-1})	FCR consumption[b] (μL/determination)	NaOH consumption (mg/determination)
continuous flow of FCR (100 μL of sample)	8.88	5–100	7.83	27	75	15
merging zones I (100 μL of sample + 100 μL of FCR)	9.21	5–80	7.60	25	5	15
merging zones II (100 μL of sample + 300 μL of FCR)	8.90	5–100	7.83	26	15	15
merging zones III (300 μL of sample + 100 μL of FCR)	17.43	2.5–40	3.96	21	5	15
intercalated zones (100 μL of FCR/100 μL of sample/100 μL of FCR)	9.28	5–40	7.60	24	10	15

[a] For all calibration curves $R \geq 0.9997$, $n \geq 5$. [b] Values refer to the commercial solution.

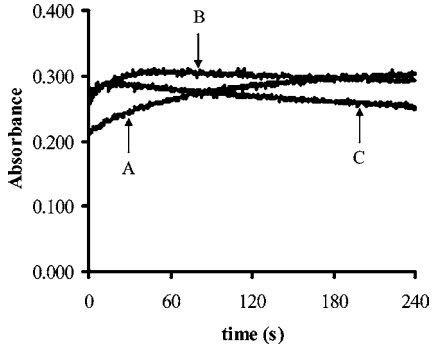

Figure 2. Reaction monitoring during 4 min after flow halting for different NaOH concentrations (M): A, 0.10; B, 0.25; C, 0.50. Other conditions: gallic acid concentration, 25 mg L^{-1}; FCR concentration, 1:20 (v/v).

the other hand, in the experiments with continuous flow of FCR, merging zones II, and intercalated zones approaches, the consumption of FCR was 15, 3 and 2 times of that verified on the experiment merging zones I. Therefore, this approach (100 μL of standard/sample and 100 μL of FCR) was chosen as it provided good linear range (5−80 mg L^{-1}), low reagent consumption (5 μL of commercial FCR per determination), and determination frequency similar to that obtained in the other experiments.

Study of Chemical Aspects. Studies concerning the reaction time, Folin−Ciocalteu reagent, and NaOH concentration were carried out using a univariate approach. A preliminary study on the effect of reaction time was carried out using FCR diluted 1:20 (v/v) and NaOH 0.25 M. The flow was stopped in the reactor (RC) during 0, 30, 60, and 90 s before the detection step; the slope of calibration curves obtained was 10.5, 11.2, 11.4, and 11.4 mAU mg^{-1} L, respectively. The sensitivity increased with time of flow stop up to 60 s; this value was chosen for the next experiment.

The Folin−Ciocalteu reagent concentration was evaluated at 1:40, 1:20, 1:10, and 1:5 (v/v) using the experimental conditions described above. The sensitivity obtained was 10.6, 11.2, 13.1, and 10.7 mAU mg^{-1} L, respectively. Thus, the FCR concentration 1:10 (v/v) was chosen as it provided the highest sensitivity.

To evaluate the influence of NaOH concentration, the kinetic of the reaction was also considered. For this, the flow was stopped when the sample segment reached the flow-through cell (18 s after the solutions were sent toward the detector) and the absorbance was monitored during 240 s (**Figure 2**). For 25 mg L^{-1} gallic acid, it was observed that higher alkali levels accelerated the color development and its fading. For that reason, it is important to have enough but not excessive alkalinity because it affects the kinetic of the reaction and also the stability of the complex formed (*10*).

Therefore, the influence on the sensitivity and on the time necessary to attain the maximum value of absorbance was assessed. For NaOH concentrations of 0.10, 0.20, 0.25, 0.30, and 0.40 M, the sensitivity obtained was 10.0, 14.1, 13.7, 13.1, and 12.6 mAU mg^{-1} L, respectively. Moreover, the time necessary to reach the maximum absorbance value was 2.5, 5, 4, 2.5, and 1.5 min, respectively. Although the highest sensitivity was obtained with 0.20 M NaOH, the time required to reach a stable absorbance value increased to 5 min. Thus, the concentration chosen was 0.25 M since the sensitivity was similar and the time of stopped flow was reduced to 4 min.

Application to Pure Compounds. Several phenolic and nonphenolic compounds were tested, including phenolic antioxidants as propyl gallate and BHA that are frequently used as additive in foods. Moreover, nonphenolic compounds with known antioxidant properties (ascorbic acid, β-carotene, sodium sulfite) and other compounds which are known to react with FCR but are not effective as antioxidant (citric acid, ferrous sulfate, D-glucose) were also evaluated. Cinnamic acid was chosen as negative control.

The FCR reducing capacity, expressed as the ratio between the slopes of the calibration curves determined for pure compounds and for gallic acid, are presented in **Table 3**. The values obtained for the MSFIA system were in agreement with those obtained using the conventional batch procedure; they are also similar to those described by other authors (*9*, *10*).

Some exceptions were observed, as occurred for resorcinol that originated a lower ratio value for MSFIA when compared to the batch procedure employing carbonate buffer solution for pH adjustment. When performing the batch procedure using NaOH solution, results similar to MSFIA were attained. For (−)-epicatechin, a lower ratio value was also found for the MSFIA procedure when compared to the batch method. Nevertheless, when the reaction conditions in the MSFIA system were changed (flow stop during 4 min and FCR 1:10 (v/v)), similar results were observed (RD = +2.3%).

Some nonphenolic substances, such as ascorbic acid and ferrous ion, also reacted with FCR. On the other hand, β-carotene, cinnamic acid, citric acid, D-glucose, and sodium sulfite did not react with FCR (the upper limits of concentration tested were 0.005, 1.00, 5.01, 11.2, and 16.0 mM, respectively). Therefore, the present method is not suitable for determination of total phenolic content unless interfering substances are considered or removed. Moreover, the application of this method for determination of antioxidant capacity in food samples is

Table 3. Relative FCR Reducing Capacity Obtained for Phenolic and Nonphenolic Compounds Determined by the Proposed MSFIA and Batch Method[a]

compounds	linear range[b] (μM)	MSFIA	batch	RD[c] (%)
	Phenolic and Polyphenolic			
BHA	25–126	88	37	+138
caffeic acid	15–150	103	104	–1.0
catechol	22–93	107	108	–0.9
ellagic acid	10–50	202	196	+3.1
(–)-epicatechin	13–64	169	214	–21
ferulic acid	31–123	79	80	–1.3
propyl gallate	15–150	98	94	+4.3
pyrogallol	15–150	109	108	+0.9
quercetin	14–58	191	195	–2.1
resorcinol	22–354	58	83	–30
sinapic acid	29–116	88	137	–36
tannic acid	3.1–11	762	789	–3.4
trolox	29–353	58	39	+49
	Nonphenolic			
ascorbic acid	28–224	63	64	–1.6
ferrous sulfate	153–766	8.4	16.9	–50

[a] The results are expressed as the ratio (%) between the slope of the calibration curves obtained for the testing compound and for gallic acid. [b] For all calibration curves $R \geq 0.9995$, $n \geq 4$. [c] RD = relative deviation.

proposed for evaluation of the contribution from phenolic and other reducing substances (as ascorbic acid, for instance). The contribution from other antioxidant compounds with different mechanism of action (such as β-carotene) may not be considered.

Application to Food Samples. The assessment of FCR reducing capacity of several food products including wines, beers, teas, soft drinks, and juices was performed using the proposed MSFIA system. The absorbance value obtained for samples was interpolated in the following calibration curve: $A = 0.0132\ (\pm 0.0002) \times C + 0.001\ (\pm 0.001)$ and $R^2 = 0.9998$, where A is the absorbance and C is the concentration of gallic acid (mg L^{-1}). Thus, the FCR reducing capacity was expressed as gallic acid equivalents (mg L^{-1}). This result was multiplied by the respective dilution factor.

The results obtained by the proposed methodology (C_{MSFIA}) and by the conventional batch method (C_{batch}) for the analysis of the samples are presented in **Table 4**. The FCR reducing capacity values obtained for wines were in agreement with those reported by other authors (*26*, *27*) that have also found values about 10 times higher for red wines in comparison to white wines.

For comparison purposes, a linear relationship ($C_{MSFIA} = C_0 + S \times C_{batch}$) was established ($n = 15$), and the values for intercept (C_0), slope (S), and correlation coefficient were 13.5 (± 18.4), 0.994 (± 0.015), and 0.9997, respectively. Considering the limits of the 95% confidence intervals presented (values in parentheses), the calculated slope and intercept do not differ significantly from the values 1 and 0, respectively. Therefore, there is no evidence for systematic differences between the two sets of results (*28*) obtained by the proposed methodology and by the conventional batch method. Furthermore, when a paired *t*-test was performed on the data obtained for all samples, a t value of 1.416 was calculated. The comparison between this value and the t ($P = 0.05$; df = 14) = 2.145 indicates no significant difference for the mean concentrations obtained by the two methods (*28*).

The repeatability of the developed method was assessed by calculating the relative standard deviation from 10 consecutive determinations of three gallic acid standard solutions (2.5, 10.0, and 40.0 mg L^{-1}) providing values of 1.33, 0.53, and 0.34%, respectively.

Table 4. Results (mg L^{-1}) Obtained for Analysis of Different Samples by MSFIA Methodology (C_{MSFIA}) and Batch Method (C_{batch}) for the Determination of FCR Reducing Capacity[a]

sample	C_{MSFIA}[b]		C_{batch}[c]		RD[d] (%)
red wine A	2422 ± 11	(1:200)	2419 ± 21	(1:100)	+0.1
red wine B	2526 ± 11	(1:200)	2490 ± 20	(1:100)	+1.4
red wine C	2278 ± 15	(1:200)	2329 ± 9	(1:100)	–2.2
red wine D	1890 ± 35	(1:200)	1889 ± 26	(1:100)	+0.1
white wine A	294 ± 8	(1:100)	305 ± 3	(1:50)	–3.6
white wine B	280 ± 2	(1:50)	282 ± 2	(1:25)	–0.7
dark beer	1073 ± 4	(1:50)	1052 ± 39	(1:100)	+2.0
lager beer	469 ± 3	(1:25)	467 ± 28	(1:100)	+0.4
green tea	768 ± 9	(1:100)	773 ± 4	(1:50)	–0.6
melissa tea[e]	623 ± 2	(1:50)	605 ± 25	(1:200)	+3.0
soft drink A	420 ± 1	(1:25)	393 ± 8	(1:100)	+6.9
soft drink B	121 ± 1	(1:50)	118 ± 4	(1:25)	+2.5
fruit juice A	312 ± 4	(1:100)	290 ± 6	(1:50)	+7.6
fruit juice B	455 ± 7	(1:25)	426 ± 12	(1:100)	+6.8
orange juice	526 ± 3	(1:25)	503 ± 9	(1:100)	+4.6

[a] Each value corresponds to the mean ± standard deviation. The values in parentheses correspond to the dilution performed prior to analysis. [b] $n = 3$. [c] $n = 4$. [d] RD = relative deviation between the two methods. [e] Honey flavor.

The detection limit was calculated as the concentration corresponding to the intercept value plus three times the statistic $s_{y/x}$ (*28*). For four different calibration curves, the calculated detection limit was about 0.6 mg L^{-1}. A complete analytical cycle (**Table 1**) took 335 s, considering the time taken for each step and also the time necessary for data transference between the computer and the multi-syringe. Therefore, the determination frequency was approximately 12 h^{-1}.

In conclusion, the present automatic methodology for the determination of FCR reducing capacity represents a suitable tool for routine determinations. It was successfully applied to food samples of diverse origin, providing results that were in agreement with those obtained by the time-consuming batch method proposed for standardization. Moreover, the strict control of reaction conditions (mixing of reagent/sample, reaction time) and the reduced intervention of operator contributed to achieving reliable results, with good repeatability.

LITERATURE CITED

(1) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*, 2nd ed.; Oxford University Press: Oxford, 1999.

(2) 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. Nutr.* **1995**, *35*, 7−20.

(3) Frankel, E. N. Antioxidants in lipid foods and their impact on food quality. *Food Chem.* **1996**, *57*, 51−55.

(4) Kaur, C.; Kappor, H. C. Antioxidants in fruits and vegetables—the millennium's health. *Int. J. Food Sci. Technol.* **2001**, *36*, 703−725.

(5) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S−2085S.

(6) Escarpa, A.; Gonzalez, M. C. An overview of analytical chemistry of phenolic compounds in foods. *Crit. Rev. Anal. Chem.* **2001**, *31*, 57−139.

(7) Robards, K.; Antolovich, M. Analytical chemistry of fruit bioflavonoids—a review. *Analyst* **1997**, *122*, 11R−34R.

(8) Romani, A.; Minunni, M.; Mulinacci, N.; Pinelli, P.; Vincieri, F. F.; Del Carlo, M.; Mascini, M. Comparison among differential pulse voltammetry, amperometric biosensor, and HPLC/DAD analysis for polyphenol determination. *J. Agric. Food Chem.* **2000**, *48*, 1197−1203.

(9) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.
(10) Singleton, V. L.; Orthofer, R.; Lamuela-Raventós, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin−Ciocalteu regent. *Methods Enzymol*. **1999**, *299*, 152−178.
(11) Roginsky, V.; Lissi, A. E. Review of methods to determine chain-breaking antioxidant activity in food. *Food Chem*. **2005**, *92*, 235−254.
(12) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.
(13) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.
(14) Peris-Tortajada, M.; Cerrada, M. P.; Maquieira, A. Automated determination of total polyphenols by means of the Folin−Ciocalteu reagent. *Quím. Anal*. **1989**, *8*, 211−222.
(15) Peris, M.; Müller, D.; Maquieira, A. Determination of total polyphenols in beers by flow injection analysis. *Food Chem*. **1991**, *40*, 1−8.
(16) Celeste, M.; Tomás, C.; Cladera, A.; Estela, J. M.; Cerdà, V. Enhanced automatic flow-injection determination of the total polyphenol index of wines using Folin−Ciocalteu reagent. *Anal. Chim. Acta*. **1992**, *269*, 21−28.
(17) Mataix, E.; Luque de Castro, M. D. Simultaneous (or sequential) determination of the total polyphenol index (or I_{280}) and density in wines by flow injection. *Analyst* **2001**, *126*, 251−255.
(18) Moreno, C. L.; Rudner, P. C.; García, J. M. C.; Pavón, J. M. C. Development of a sequential injection analysis device for the determination of total polyphenol index in wine. *Microchim. Acta*. **2004**, *148*, 93−98.
(19) Cerdà, V.; Estela, J. M., Forteza, R.; Cladera, A.; Becerra, E.; Altimira, P.; Sitjar, P. Flow techniques in water analysis. *Talanta* **1999**, *50*, 695−705.
(20) Segundo, M. A.; Magalhães, L. M. Multisyringe flow injection analysis: state-of-the-art and perspectives. *Anal. Sci.* **2006**, *22*, 3−8.
(21) Ruzicka, J.; Hansen, E. H. *Flow Injection Analysis*, 2nd ed.; Wiley: New York, 1988; p 301.
(22) Segundo, M. A.; Oliveira, H. M.; Lima, J. L. F. C.; Almeida, M. I. G. S.; Rangel, A. O. S. S. Sample introduction in multisyringe flow injection systems: comparison between time-based and volume-based strategies. *Anal. Chim. Acta* **2005**, *537*, 207−214.
(23) Ruzicka, J.; Hansen, E. H. Flow injection analyses. 1. New concept of fast continuous-flow analysis. *Anal. Chim. Acta* **1975**, *78*, 145−157.
(24) Bergamin, H.; Zagatto, E. A. G.; Krug, F. J.; Reis, B. F. Merging zones in flow injection analysis. 1. Double proportional injector and reagent consumption. *Anal. Chim. Acta* **1978**, *101*, 17−23.
(25) Ruzicka, J.; Marshall, G. D. Sequential injection—a new concept for chemical sensors, process analysis and laboratory assays. *Anal. Chim. Acta* **1990**, *237*, 329−343.
(26) Fernández-Pachón, M. S.; Villaño, D.; García-Parrilla, M. C.; Troncoso, A. M. Antioxidant activity of wines and relation with their polyphenolic composition. *Anal. Chim. Acta* **2004**, *513*, 113−118.
(27) De Beer, D.; Joubert, E.; Gelderblom, W. C. A.; Manley, M. Antioxidant activity of South African red and white cultivar wines: free radical scavenging. *J. Agric. Food Chem.* **2003**, *51*, 902−909.
(28) Miller, J. N.; Miller, J. C. *Statistics and Chemometrics for Analytical Chemistry*, 5th ed.; Pearson Education: Harlow, U.K.; 2005.

Received for review February 2, 2006. Revised manuscript received May 10, 2006. Accepted May 17, 2006. L.M.M. thanks FCT and FSE (III Quadro Comunitário de Apoio) for the Ph.D. Grant SFRH/BD/12539/2003.

JF060324S