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⁺ and DPPH⁻ 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
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 sulfate, 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 (Crisson 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; Si, syringe; Vi, commutation valves (solid and dotted lines represent the position on and off, respectively); MC, mixing coil; RC, reaction coil (100 cm); D, detector; Ti, 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.
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 \( \mu \)L of sample + 100 \( \mu \)L 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 \( \mu \)L of sample was merged with 300 \( \mu \)L 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 \( \mu \)L of FCR and 300 \( \mu \)L of sample. After the sampling step, sample was pushed by carrier into the MC creating a front zone of 200 cm long. The reaction coil (RC) was 100 cm long. The connections between the multi-syringe manifold and procedure for determination of FCR reducing capacity was adapted to a microplate reader (Synergy HT, Bio-Tek, Winooski, VT). Hence, 50 \( \mu \)L of gallic acid standard solution or food sample and 50 \( \mu \)L of FCR were placed in each well. After that, 100 \( \mu \)L 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 triplicate, 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. The flow rate was 50 mL min−1, which is sufficient to ensure the flow through all the components of the manifold. The flow rate was determined by the capacity of the injection valve and the carrier flow rate. The flow rate was kept constant throughout the experiment.

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 \( \mu \)L, 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 \( \mu \)L 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.

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

<table>
<thead>
<tr>
<th>step</th>
<th>description</th>
<th>position of the commutation valves</th>
<th>volume</th>
<th>time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>syringes are filled with the respective solutions</td>
<td>F F F F F F</td>
<td>2300</td>
<td>6.90</td>
</tr>
<tr>
<td>2</td>
<td>sample and FCR are aspirated</td>
<td>F N N F N N</td>
<td>200</td>
<td>12.00</td>
</tr>
<tr>
<td>3</td>
<td>dummy step to change the flow direction</td>
<td>F F F F F F</td>
<td>500</td>
<td>1.50</td>
</tr>
<tr>
<td>4</td>
<td>sample, FCR, and NaOH are sent toward detection system</td>
<td>N N N F F F</td>
<td>600</td>
<td>18.00</td>
</tr>
<tr>
<td>5</td>
<td>flow stop</td>
<td>F F F F F F</td>
<td>2400.00</td>
<td>21.00</td>
</tr>
<tr>
<td>6</td>
<td>carrier and NaOH are sent to wash the system</td>
<td>N N N F F F</td>
<td>1400</td>
<td>2.00</td>
</tr>
</tbody>
</table>

* N and F represent the positions on and off, respectively. The indicated values for volume refer to syringe 10 mL.
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\(^{-1}\), respectively. Moreover, the time necessary to reach the maximum absorbance value was 2.5, 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, \(\beta\)-carotene, sodium sulfite) and other compounds which are known to react with FCR but are not effective as antioxidant (citric acid, ferrous sulfate, \(\alpha\)-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, \(\beta\)-carotene, cinnamic acid, citric acid, \(\alpha\)-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
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: 

\[ A = 0.0132 (± 0.0002) \times C + 0.001 (± 0.001) \text{ and } R^2 = 0.9998 \]

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 (\(C_{\text{MSFIA}}\)) and by the conventional batch method (\(C_{\text{batch}}\)) 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 (\(C_{\text{MSFIA}} = C_0 + S \times C_{\text{batch}}\)) was established (\(n = 15\)), and the values for intercept (\(C_0\)), slope (S), and correlation coefficient were 13.5 (± 8.4), 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 \(t\) value of 2.5; 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.

The detection limit was calculated as the concentration corresponding to the intercept value plus three times the statistic \(s_y/S_y\) (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


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