Automatic flow system for sequential determination of $ABTS^{\bullet+}$ scavenging capacity and Folin-Ciocalteu index: A comparative study in food products

Luís M. Magalhães [a], Marcela A. Segundo [a,*], Salette Reis [a], José L.F.C. Lima [a], Ildikó V. Tóth [b], António O.S.S. Rangel [b]

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

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Abstract

In the present work, an automatic flow procedure for the sequential spectrophotometric determination of Folin-Ciocalteu reducing capacity (FC assay) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) scavenging capacity expressed as the trolox equivalent (TEAC assay) is proposed for a comparative study of antioxidant properties in food products. Exploiting the flexibility of flow management associated to the computer control offered by multisyringe flow injection analysis, both methodologies were carried out in the same manifold using gallic acid and trolox as standard compounds. The proposed system configuration allowed the performance of each method separately or in tandem, providing 24 determinations per hour, which accounts for its application in routine analysis.

The present methodology was applied to a large number of beverages ($n = 72$), namely red and white wines, herbal and tea infusions, juices and beers. The results obtained showed that FC reducing capacity and TEAC values of red wines were significantly different from those obtained for the other beverages, while tea infusions provided significantly higher TEAC values when compared to white wines, herbal infusions, juices and beers. A good correlation was found between TEAC and FC reducing capacity ($R > 0.9$) for red wines, herbal and tea infusions, and beers. For these beverages, similar slope values were found (106–140 mg L^{-1} of gallic acid per mM of Trolox), except for beers that showed a higher response for FC assay. These results provided evidence that the correlation between these assays vary according to the type of sample, reinforcing the idea that more than one method should be used for evaluation of antioxidant capacity.

Introduction

Nowadays, the development and validation of analytical methods for assessment of antioxidant capacity in food products are an increasing area of research [1]. These methods are of great interest not only to the food industry but also to medical and nutritional researchers as active dietary constituents including phenolic compounds, vitamins C and E, and carotenoids, are capable of preventing free radical-induced reactions [2,3]. These reactions are implicated in the oxidative rancidity of food products and also in the development of several human pathologies such as cardiovascular diseases, diabetes, neurological degeneration, and certain types of cancer [4,5]. However, the methods currently used to assess this property differ from each other in terms of substrates, probes, reaction conditions, and in the form that results are expressed. Even when only one of these assays is considered, different antioxidant standard compounds, solvents, reaction time and pH value are frequently applied [6], which makes the comparison of results from different studies difficult. This situation stresses the importance to standardize analytical methods for application in routine assessment of antioxidant capacity.

In this context, the Folin-Ciocalteu reducing capacity (FC assay) and Trolox equivalent antioxidant capacity (TEAC assay) have been recently proposed as standardized methods for measurement of antioxidant capacity of food products and dietary supplements [7]. These methods are based on electron-transfer donation from the antioxidant to the oxidant probe. The degree of color change of the probe is proportional to the antioxidant concentration. For the FC assay, the oxidant is a

* Corresponding author. Tel.: +351 222078994; fax: +351 222078961.
E-mail address: msegundo@mail.ff.up.pt (M.A. Segundo).

molybdotungstophosphoric heteropolyanion and the absorbance increase is measured at 750 nm, whilst for the TEAC assay the oxidant is the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) and the absorbance decrease at 734 nm is measured [1,6]. Nevertheless, for the implementation of standardized methods exhaustive studies are required for reliable comparison of data generated by different assays. Moreover, the application of batch methodologies for research tasks and/or routine analysis is time-consuming, tedious and laborious.

The drawbacks outlined above could be circumvented by the implementation of automatic analytical methodologies based on flow analysis, which are characterised by high throughput, reduced intervention of operator, reagent and sample saving, reduced production of residues and improved reproducibility [8,9]. Moreover, owing to its higher versatility, recent computer-controlled automatic methods are capable to accommodate a wide variety of assays without the need for system reconfiguration, allowing simultaneous and/or sequential determinations, which makes them especially suitable to establish comparisons between methods [10–12].

Therefore, the objective of the present work was the development of an automatic flow procedure for the sequential determination of FC reducing capacity and TEAC intended for a comparative study of the antioxidant capacities of several food products. The automation of these two methodologies was based on multisyringe flow injection analysis (MSFIA) [13,14], which combines the multichannel operation of flow injection analysis and the flexibility of flow management offered by the multicommutation technique. The proposed method was applied to a large number of beverages with recognised antioxidant properties and the results were compared within and between methods.

Experimental

Reagents, standards and samples

For the preparation of all solutions, water from Milli-Q system (resistivity > 18 MΩ cm) and ethanol absolute pro analysis were applied. All chemicals used were of analytical-reagent grade with no further purification.

Folin-Ciocalteu reagent (FCR), gallic acid and horseradish peroxidase (HRP) from type VI-A (EC 1.11.1.7, 1280 units mg^{-1}) were purchased from Sigma (St. Louis, MO, USA). ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] in the crystallized diammonium form and trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were obtained from Fluka (Buchs, Switzerland). H_2O_2 (30%, v/v) was obtained from Riedel-de Haën (Seelze, Germany). Sodium hydroxide and sodium acetate were purchased from Merck (Darmstadt, Germany).

For the determination of FC reducing capacity, FCR was diluted 1:10 (v/v) with water. Sodium hydroxide solution (0.25 mol L^{-1}) was also prepared. For the determination of Trolox equivalent antioxidant capacity (TEAC), ABTS, H_2O_2, and HRP stock solutions of 18 mM, 20 mM, and 3.2×10^6 units L^{-1}, respectively, were prepared in 0.060 mol L^{-1} acetate buffer pH 4.6. These stock solutions were stable for over a month when stored at 4 °C. $ABTS^{\bullet+}$ working solution in 0.060 mol L^{-1} acetate buffer pH 4.6 was obtained by mixing appropriate volumes of the following solutions at the indicated final concentrations: ABTS (0.80 mM), H_2O_2 (0.080 mM), and HRP (3 units mL^{-1}). This solution was prepared 12 h before analysis and protected from light. It was stable for at least 2 days at room temperature.

For the determination of FC reducing capacity and TEAC, gallic acid and trolox were used as antioxidant standard compounds, respectively. Gallic acid (500 mg L^{-1}) and trolox (2.0 mM) stock solutions were prepared by dissolving the appropriate amount of the respective solid in water and in ethanol solution 50% (v/v), respectively. These stock solutions were found to be stable for at least 1 week when stored at 4 °C. The working solutions containing either gallic acid (5.0–75 mg L^{-1}) or trolox (0.020–0.20 mM) were prepared daily by dilution in water.

All samples were purchased at local markets. Herbal and tea infusions were prepared by pouring 200 mL of deionized water at 90 °C into a glass with herbal or tea bag and by brewing for 5 min. Carbon dioxide from white wines and beers was completely removed by magnetic stirring. All beverages were diluted with water just before measurement. The dilutions performed for red wines, herbal infusions, and tea infusions (green and black) were 1:200, 1:4 to 1:20, and 1:40, respectively. White wines, juices and beers (blond and dark) were diluted 1:20. Samples were analysed in a randomised way.

Apparatus

Solutions were propelled through the flow network by means of a multisyringe piston pump (Crison Instruments, Allela, Spain) equipped with syringes of 5 mL (Hamilton, Switzerland). Each syringe is connected to a three-way commutation valve (N-Research, Caldwell, NJ, USA) that allows the access to two different channels (solution flasks or flow network). Two extra commutation valves were included for introduction of standard/sample (V5) and FCR (V6). The flow assembly also includes two 8-port multiposition selection valves, disposed in the same module (Crison Instruments, Allela, Spain) that accommodated six reaction coils (RC*i*). The three and four-way connectors (T1 and T2, respectively) were used as confluences. All tubing connecting the different components of MSFIA was made from polytetrafluoroethylene (Omnift, Cambridge, U.K.) of 0.8 mm i.d., except for tubes between flasks and syringes, which were of 1.5 mm i.d. in order to avoid back pressure or vacuum at high flow rates. End-fittings and connectors from Gilson (Villiers-le-Bel, France) were also used.

To perform the FC and TEAC assays, the analytical absorbance measurements should be carried out at 750 and 734 nm, respectively [7]. For this, an Ocean Optics PC 2000-ISA (Winter Park, FL) spectrophotometer connected to 200 μm fiber optic cable and a DH-2000 deuterium-halogen light source (Top Sensor Systems, Eerbeek, The Netherlands) were used. Facing the fiber optic, a Hellma (Müllheim/Baden, Germany) 178.710-QS flow-through cell (10 mm light path, 80 μL inner volume) was placed in an Ocean Optics CUV cell support.

A personal computer equipped with an Advantec PCL-711 B interface card, running homemade software written in QuickBasic 4.5 (Microsoft), controlled the multisyringe and valves operation. The data acquisition at 4 Hz, corresponding to an integration time of 0.023 s and an average scan of 11, was performed by SpectraWin (version 4.2) through an external trigger signal from the interface card.

Statistical analysis

For each sample, data are reported as mean ± standard deviation ($n = 3$) (see supplementary data, Tables S1–S6). One-way analysis of variance (ANOVA) was performed on these values to determine whether they differed significantly at a 95% level. Since the Levene test showed that there was no homogeneity of variances ($p < 0.05$), the Welch and Brown-Forsythe statistics were estimated instead of the usual F test. A post hoc comparison test (Tamhane's T2) was also applied to determine which group(s) differ from each other. Linear regression was applied for studying the possible correlation between the studied parameters. All statistical analyses were carried out using SPSS version 14.0 for Windows.

MSFIA manifold and procedure for FC reducing capacity and TEAC assays

The system components were arranged as shown schematically in Fig. 1. The connections between the multisyringe and the valves V5 and V6 were 200 cm long. The tubing length between these valves and confluence T1 were 5 cm long. The mixing coil (MC) had the same length. The connection between confluence T2 and the central channel of selection valve VA was 20 cm long, while the connection between the central channel of selection valve VB and the flow-through cell was 25 cm long. The reaction coils, where the FC reducing reaction (RC2, RC3, RC4) or the $ABTS^{\bullet+}$ scavenging reaction (RC6, RC7, RC8) took place, were 250 cm long. The washing coil L1 had the same length.

These components constituted the flow network for the sequential spectrophotometric determination of FC reducing capacity and TEAC of several food products. Nevertheless, as the FC and TEAC assays are standardized for different antioxidant compounds [7], namely gallic acid and trolox respectively, the calibration procedures were performed separately.

The protocol sequence adopted for the FC and TEAC calibration is described in Table 1. Before starting the calibration procedures, all syringes were filled with the respective solutions. For the FC procedure, syringes S1, S2 and S3 were simultaneously activated and depending on the position of selection valves, the NaOH solution and carrier were propelled through reaction coils RC2, RC3 or RC4 towards the detector and the absorbance signal was adjusted to zero. For the TEAC procedure, carrier and $ABTS^{\bullet+}$ solution (delivered by syringes S2, S3, and S4) were simultaneously propelled through reaction coils RC6, RC7 or RC8. The absorbance value measured (0.760 ± 0.010) was due to the $ABTS^{\bullet+}$ radical solution from syringe S4 after dilution inside the flow system.

Briefly, the calibration protocol can be divided in the following stages: antioxidant standard solution uptake and delivery to the reaction coil (steps 1–4); flow stop for reaction development (steps 5, 6); and spectrophotometric measurement (steps 7, 8).

In particular, for FC calibration 100 μL of gallic acid standard solution and 100 μL of FCR were aspirated into the flow system through commutation valves V5 and V6, respectively. After that syringes S1, S2, and S3 were simultaneously activated and the antioxidant plug pushed by carrier was sequentially merged with FCR plug and NaOH stream at confluence T1 and T2, respectively. This mixture was directed to reaction coil RC2. These operations were repeated twice more, in order to fill the other two reaction coils (RC3 and RC4). After a waiting period of 250 s, the reaction product stored in each RC was successively

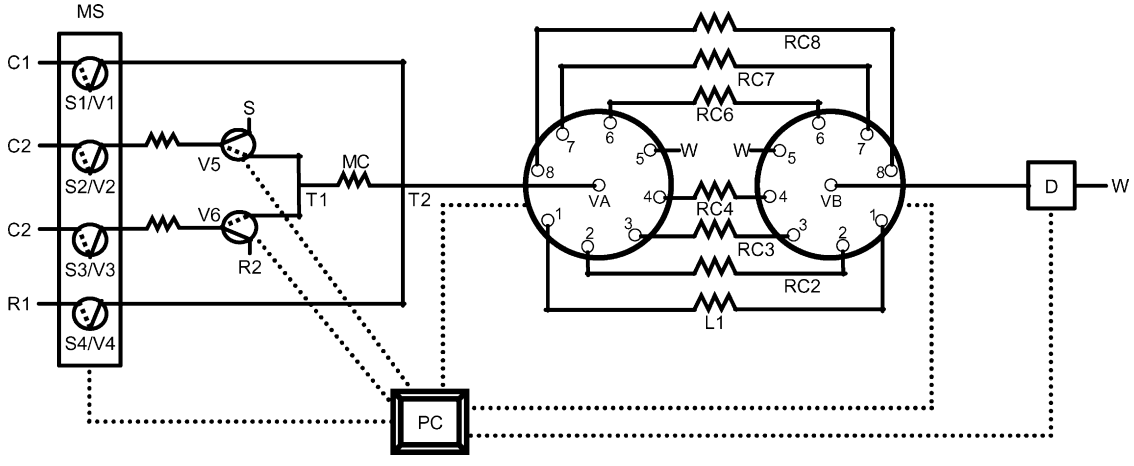

Fig. 1. MSFIA manifold used for the sequential determination of FC reducing capacity and TEAC in food products: MS, multisyringe; Si, syringes; Vi, commutation valves (solid and dotted lines represent the position on and off, respectively); T1 and T2, confluences; MC, mixing coil (5 cm); VA and VB, 8-port multiposition selection valves; L1, washing coil (250 cm); RC*i*, reaction coils (250 cm); D, detector; C1, NaOH 0.25 mol L^{-1}; C2, water; R1, $ABTS^{\bullet+}$ in 0.060 mol L^{-1} acetate buffer pH 4.6; R2, Folin-Ciocalteu reagent diluted at 1:10 (v/v); S, standard solution or sample; PC, personal computer; W, waste. The exchange options of the commutation valves 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 multisyringe, while 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.

Table 1
Protocol sequence for the Folin-Ciocalteu and TEAC calibration using the MSFIA method

Step	Instrumentation	Protocol[a]	Description
Folin-Ciocalteu calibration			
		start loop: standard injection	
1	Multiposition valve	Valve VA and VB move to position X	X (FC calibration) = position 2 to 4
2	Multisyringe Piston	Pick up 1362 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Loading syringes with carrier/reagents
3	Multisyringe piston	Pick up 100 μL at 1.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 off, V5 on, and V6 on)	Aspiration of gallic acid standard solution and FCR
4	Multisyringe piston	Dispense 362 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Merging gallic acid with FCR and then with NaOH stream towards RC X
		End loop: standard injection	Repeat 3 times
5	Multiposition valve	Valve VA and VB move to position 1	
6	Stopped-flow	Wait 250 s	Development of FC reducing reaction
		Start loop: standard measurement	
7	multiposition valve	Valve VA and VB move to position X	X (FC calibration) = position 2 to 4
8	Multisyringe piston	Dispense 1100 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Propulsion of RC X content to detector (λ = 750 nm); signal acquisition; system clean-up
		End loop: standard measurement	Repeat 3 times
TEAC calibration			
		start loop: standard injection	
1	Multiposition valve	Valve VA and VB move to position X	X (TEAC calibration) = position 6 to 8
2	Multisyringe piston	Pick up 1362 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Loading syringes with carrier/reagents
3	Multisyringe piston	Pick up 100 μL at 1.0 mL min^{-1} (V1 off, V2 on, V3 off, V4 off, V5 on, and V6 off)	Aspiration of trolox standard solution
4	Multisyringe piston	Dispense 362 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	Merging trolox with carrier and then with $ABTS^{\bullet+}$ stream towards to RC X
		end loop: standard injection	Repeat 3 times
5	Multiposition valve	Valve VA and VB move to position 1	
6	Stopped-flow	Wait 295 s	Development of $ABTS^{\bullet+}$ scavenging reaction
		Start loop: standard measurement	
7	Multiposition valve	Valve VA and VB move to position X	X (TEAC calibration) = position 6 to 8
8	Multisyringe piston	Dispense 1100 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	Propulsion of RC X content to detector (λ = 734 nm); signal acquisition; system clean-up
		End loop: standard measurement	Repeat 3 times

[a] The indicated values of volume and flow rate are referred to syringe 1 (5 mL).

propelled towards the detector and the absorbance increase at 750 nm was measured.

For the TEAC calibration, after loading the trolox standard solution (100 μL), the antioxidant plug was sequentially merged with carrier and $ABTS^{\bullet+}$ stream at confluence T1 and T2, respectively, and propelled further up to RC6. These steps were repeated twice more and, depending on the position of selection valves, the mixture was directed to reaction coils RC7 or RC8. After a flow stop period of 295 s, the content of each reaction coil was directed to the detector and the absorbance decrease due to radical scavenging was measured at 734 nm.

Noteworthy, both determinations started with the measurement of a blank signal through aspiration of water as sample. The obtained signals correspond to the absorbance values in the absence of reducing or scavenging compounds.

The MSFIA procedure for the sequential determination of FC reducing capacity and TEAC of food products is summarized in Table 2. In the first commands (steps 1–4), sample and FCR were aspirated and further propelled to reaction coils RC2–RC4, similar to FC calibration procedure. Then, carrier and $ABTS^{\bullet+}$ radical solution were dispensed in order to clean the manifold line between confluence T2 and selection valve VA (steps 5, 6). After loading syringes with carrier/reagent, sample was aspirated and further sent with carrier and $ABTS^{\bullet+}$ solution to reaction coils RC6–RC8, similar to TEAC calibration procedure (steps 7–10). Next, NaOH solution and carrier were dispensed through L1 towards the detector in order to establish the absorbance baseline for the FC assay (steps 11, 12). Following the piston bar adjustment (step 13), the reaction product of the FC assay stored in RC2–RC4 was propelled through the detector (steps 14, 15), providing three replicate measurements of FC capacity. Afterwards, with selection valves in position 1, the $ABTS^{\bullet+}$ radical solution and carrier were sent to the detector in order to establish the absorbance baseline for TEAC assay (steps 16, 17). Thereafter, the contents of RC6–RC8 were sent to the detector and the $ABTS^{\bullet+}$ scavenging capacity was measured ($n = 3$, steps 19, 20). Finally, the NaOH solution and carrier were propelled through L1 toward the detector in order to re-establish the initial conditions, rendering the system for analysis of the next sample.

For the determination of intrinsic absorption of sample, the same flow procedure was applied (Table 2). However, in these experiments acetate buffer solution (0.060 mol L^{-1}) at pH 4.6 was placed in syringe S4 instead of $ABTS^{\bullet+}$ radical solution,

Table 2
Protocol sequence for the automatic sequential determination of FC reducing capacity and TEAC using the MSFIA method

Step	Instrumentation	Protocol[a]	Description
		Start loop: sample injection for FC assay	
1	Multiposition valve	Valve VA and VB move to position X	X (FC assay) = position 2 to 4
2	Multisyringe piston	Pick up 1150 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Loading syringes with carrier/reagents
3	Multisyringe piston	Pick up 100 μL at 1.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 off, V5 on, and V6 on)	Aspiration of sample and FCR
4	Multisyringe piston	Dispense 362 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Merging sample with FCR and then with NaOH stream towards RC X
		End loop: sample injection for FC assay	Repeat 3 times
5	Multiposition valve	Valve VA and VB move to position 5	
6	Multisyringe piston	dispense 1000 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	Dispense $ABTS^{\bullet+}$ solution and carrier to clean the manifold lines
		start loop: sample injection for TEAC assay	
7	Multiposition valve	valve VA and VB move to position X	X (TEAC assay) = position 6 to 8
8	Multisyringe piston	Pickup 1150 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Loading syringes with carrier/reagents
9	Multisyringe piston	Pick up 100 μL at 1.0 mL min^{-1} (V1 off, V2 on, V3 off, V4 off, V5 on, and V6 off)	Aspiration of sample
10	Multisyringe piston	Dispense 362 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	merging sample with carrier and then with $ABTS^{\bullet+}$ stream towards RC X
		End loop: sample injection for TEAC assay	Repeat 3 times
11	Multiposition valve	Valve VA and VB move to position 1	
12	Multisyringe piston	Dispense 1525 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Dispense NaOH solution and carrier to clean the manifold lines
13	Multisyringe piston	Pick up 2000 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Piston bar adjustment
		Start loop: sample measurement for FC assay	
14	Multiposition valve	valve VA and VB move to position X	X (FC assay) = position 2 to 4
15	Multisyringe piston	Dispense 1100 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Propulsion of RC X content to detector (λ = 750 nm); signal acquisition; system clean-up
		End loop: sample measurement for FC assay	Repeat 3 times
16	Multiposition valve	Valve VA and VB move to position 1	
17	Multisyringe piston	Dispense 1500 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	Dispense $ABTS^{\bullet+}$ solution and carrier to clean the manifold lines
18	Multisyringe piston	Pickup 4800 μL at 10.0 mL min^{-1} (V1 off, V2 off, V3 off, V4 off, V5 off, and V6 off)	Piston bar adjustment
		Start loop: sample measurement for TEAC assay	
19	Multiposition valve	Valve VA and VB move to position X	X (TEAC assay) = position 6 to 8
20	Multisyringe piston	Dispense 1100 μL at 2.0 mL min^{-1} (V1 off, V2 on, V3 on, V4 on, V5 off, and V6 off)	propulsion of RC X content to detector (λ = 734 nm); signal acquisition; system clean-up
		End loop: sample measurement for TEAC assay	repeat 3 times
21	Multiposition valve	Valve VA and VB move to position 1	
22	Multisyringe piston	Dispense 1500 μL at 2.0 mL min^{-1} (V1 on, V2 on, V3 on, V4 off, V5 off, and V6 off)	Dispense NaOH solution and carrier to re-establish the initial conditions

[a] The indicated values of volume and flow rate are referred to syringe 1 (5 mL).

while HCl 0.20 mol L^{-1} was aspirated through valve V6 instead of FCR. All measurements were carried out at room temperature (25 ± 2 °C).

Results and discussion

Development of MSFIA system for the sequential determination of FC reducing capacity and TEAC

The objectives of the present work were (i) the development of an automatic flow method for the sequential determination of FC reducing capacity and TEAC; (ii) the study of reducing and antioxidant capacities of food products, and (iii) the evaluation of correlation between these two methods. Therefore, taking into account the first purpose, the MSFIA system recently reported for the determination of FC reducing capacity [15] was improved to accommodate in the same manifold the FC reduction reaction and the $ABTS^{\bullet+}$ scavenging reaction (TEAC assay). For this, the flexibility of flow management offered by MSFIA was exploited by merging antioxidant standard solution/sample with FCR (FC assay) or with water (TEAC assay). Afterwards, NaOH solution (FC assay) or $ABTS^{\bullet+}$ radical solution (TEAC assay) was added to the prior mixture and directed to the reaction coil. Taking into consideration that both methodologies require time for reaction development [15,16], a stopped-flow approach was chosen and implemented before the determination. Furthermore, in order to enhance the sample throughput, the two assays were carried out in tandem. For this, sampling and reagent mixing for TEAC

assay were performed during the waiting period of FC assay, whilst the spectrophotometric measurement of FC assay was attained during the flow stop period of TEAC assay.

For this analytical procedure, four syringes were necessary: one of them containing the alkaline solution for FC assay, two syringes containing water to propel the standard/sample and the FCR, and the last one containing $ABTS^{\bullet+}$ radical solution (Fig. 1). Confluence T2 was connected to the central channel of selection valve VA, whereas the central channel of valve VB was connected to the flow cell. This arrangement allowed the establishment of six coiled reactors, three for FC assay and the other three for TEAC assay, by connecting the side ports of the selection valves. The washing coil L1 allowed the access of flow cell in order to establish the baseline, without disturbing the content of the reaction coils. The port 5 of selection valve VA was directed to waste to permit the exchange of standard/sample and also the cleaning of the tubing between confluence T2 and valve VA (steps 5 and 6, Table 2), without passing through the flow cell. Otherwise, the time of exchange and reagent consumption would be increased.

The mixing coil (MC) and the line between confluence T2 and valve VA were made as short as possible to prevent high sample dispersion. The length of the reaction coil (150–250 cm) and the volume of solution sent to the reaction coil (200–450 μL) prior to flow stop were studied in order to guarantee that all sample segments were inserted in the reaction coil. These experiments were carried out using gallic acid (40 mg L^{-1}) as sample and a procedure similar to "FC calibration". Thus, after loading the gallic acid standard solution and FCR, these segments were sent to RC2 by activating syringes S1, S2 and S3. After flow stop, the selection valves were in position 1 and the standard solution that would be outside the RC2 (before the valve VA and/or after the valve VB) was propelled towards the detector. Finally, the content of RC2 was sent to the detector. Using this procedure, it was observed that the reaction coil length of 250 cm and a volume of 362 μL allowed the accommodation of all sample within the reaction coil. Moreover, the time of stopped-flow applied in the FC and TEAC calibration procedures (step 6, Table 1) was optimized in order to guarantee that the standards were subjected to the same reaction time as samples. For the FC and TEAC calibration, the time values were 250 and 295 s, respectively.

The chemical conditions for the determination of FC reducing capacity were similar to those reported in previous work [15]. For the TEAC assay, the concentration of $ABTS^{\bullet+}$ solution was studied in order to provide an absorbance value near 0.8, after dilution inside the flow system. This evaluation was performed taking into account that the stoichiometry of ABTS oxidation by H_2O_2 is 2 ABTS:1 H_2O_2 and that the ABTS concentration should be in about five-fold excess. This excess, along with a 12-h preincubation period before use, guaranteed that hydrogen peroxide was exhausted, preventing the possible reaction between the antioxidant compounds present in the sample and ABTS/H_2O_2/HRP system [17]. Therefore, the HRP concentration was fixed at 3 units mL^{-1} and the following solutions of ABTS/H_2O_2 concentrations were prepared: 1.28/0.128, 0.80/0.08 and 0.64/0.064 mM, providing absorbance values of 1.190, 0.760 and 0.594, respectively. Thus, the $ABTS^{\bullet+}$ radical solution was obtained from 0.80 and 0.08 mM of ABTS and H_2O_2, respectively. The radical solution was stable during continuous operation for 8 h (R.S.D. <1.3%).

Using the described analytical procedures for FC and TEAC calibration (Table 1) and sequential determination for samples (Table 2), the analytical signals were obtained from different reaction coils ($n = 3$, per assay). Therefore, in order to evaluate if there was significant difference between them, 10 consecutive calibration procedures for FC and TEAC assays were performed using 25 mg L^{-1} of gallic acid and 0.10 mM of trolox, respectively. For each method 30 determinations were obtained, corresponding to 10 analytical signals per RC. An ANOVA test was performed and the results obtained ($p = 0.375$ for FC assay and $p = 0.363$ for TEAC assay) indicated that the analytical signals obtained by the different reaction coils were not significantly different [18].

Analytical features of the developed MSFIA system

Under the optimal conditions described above, linear calibration plots for gallic acid (5.0–75.0 mg L^{-1}) and for trolox (0.020–0.20 mM) were obtained for the FC and TEAC assay, respectively. Fig. 2 displays a typical signal output, including the FC and TEAC calibration and also the analysis of some samples. The absorbance values obtained for samples were interpolated in the following calibration curves: $A_{750} = 0.0078\ (\pm 0.0001) \times C_{\text{gallic acid}} + 0.026\ (\pm 0.005)$ ($n = 5$, $R \geq 0.9989$); $A_{734} = -3.05\ (\pm 0.05) \times C_{\text{trolox}} + 0.742\ (\pm 0.015)$ ($n = 4$, $R \leq -0.9988$); where A is the absorbance and C is the concentration of gallic acid (mg L^{-1}) or trolox (mM); values between parenthesis are the standard deviation of the parameters corresponding to twelve calibration curves performed on different days. The interpolation values were multiplied by the respective dilution factor. Furthermore, the sample blank for both methods was measured and absorbance values <0.006 were found for all samples tested. As these values represent <5% of analytical signal of samples for FC assay and <1% of initial $ABTS^{\bullet+}$ absorbance, their contribution to the absorbance measured was not significant.

The detection limit was calculated as the concentration corresponding to the intercept value plus three times the statistic $s_{y/x}$ [18]. For 12 different calibration curves, the calculated detection limit for FC and TEAC assays was about 3 mg L^{-1} and 0.008 mM, respectively. The precision of the developed method was estimated by calculating the relative standard deviation (R.S.D.) from 12 consecutive determinations of samples A11, B12, and D12 (see supplementary data). The values obtained for FC assay were 3.6, 4.0 and 3.1%, while for TEAC assay they were 3.1, 3.1, and 2.4%, respectively. The reproducibility of the methodology was assessed by the R.S.D. of calibration slopes performed in different days ($n = 12$). The results obtained were 1.3 and 1.6% for the FC and TEAC assay, respectively.

The sequential analysis of samples by the two methods allowed 24 determinations to be carried out per hour. Considering that the sample was analysed for both methodologies ($n = 3 + 3$), the present method provided FC reducing capacity and TEAC values for four samples per hour. The calibration pro-

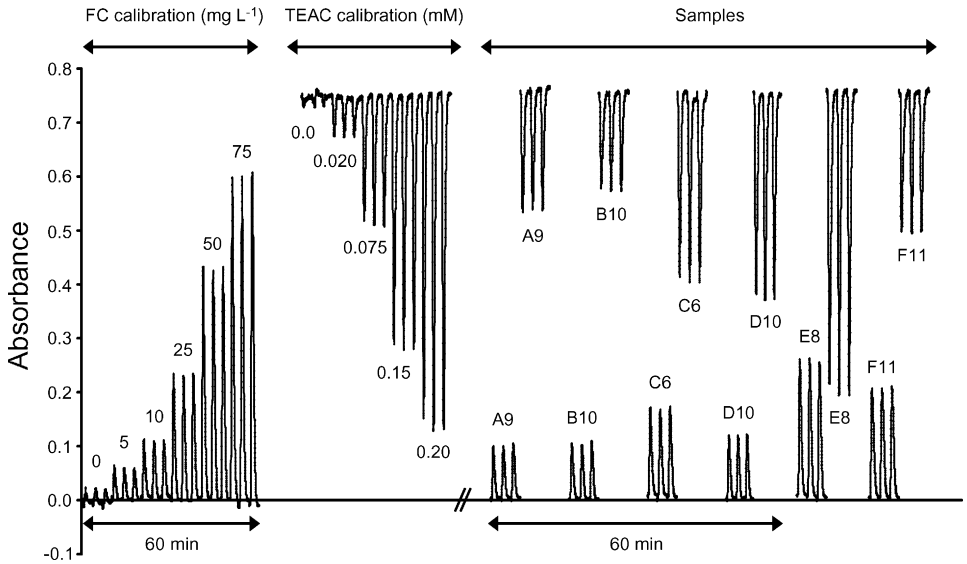

Fig. 2. Signal profile obtained for FC and TEAC assays using gallic acid (mg L^{-1}) and trolox (mM) as standard compounds, respectively. The sequential determination of FC reducing capacity and TEAC for some food samples is also presented.

cedures allowed 18 determinations per hour, corresponding to 6 standards ($n = 3$). Furthermore, it should be stressed that each method can be carried out separately, even for sample analysis. In fact, using the calibration procedure, the FC reducing capacity or the TEAC of samples can be easily assessed.

Analysis of food products and comparison of results within and between methods

The proposed automatic method was applied to a large number of beverages ($n = 72$) with recognised antioxidant capacity [19–22], namely red and white wines, herbal infusions, tea infusions (green and black), juices and beers (blond and dark). Sample details and analysis values for twelve products of each group are provided as supplementary data (Tables S1–S6). The statistical treatment of the data obtained by beverage type and assay is shown in Table 3.

The TEAC and FC reducing capacity of red wines were higher than the other beverages, ranging from 9.1 to 22.6 mM and from 1284 to 3274 mg L^{-1}, respectively. Actually, red wines showed TEAC and FC reducing capacity values approximately 10 times higher than white wines, ranging from 1.14 to 2.83 mM and from 193 to 327 mg L^{-1}, respectively. These differences between red and white wines are in agreement with results found by other authors [20,21,23]. The error plot of means with 95% confidence intervals for TEAC and FC reducing capacity of each group of beverage is presented in Fig. 3. The TEAC and FC reducing capacity of red wines were statistically different ($p < 0.05$) from the other beverages. These results are expected owing to the high content of anthocyanins and other phenolic compounds extracted from the skins and seeds during the fermentation of red wine [24]. High TEAC values were observed in tea infusions ($p < 0.05$), whereas white wines, herbal infusions, juices and beers showed no significant difference ($p > 0.05$) between them. In relation to FC reducing capacity, white wines were statistically different ($p < 0.05$) from the other beverages, with exception to herbal infusions ($p = 0.945$).

Noteworthy, the TEAC and FC reducing capacity of dark beers ($n = 6$) tested in this investigation were 2.18 ± 0.48 mM and 782 ± 212 mg L^{-1}, respectively, whilst the average values of blond beers ($n = 6$) were 1.00 ± 0.14 mM and 390 ± 69 mg L^{-1}, respectively. They were statistically different

Table 3
Statistical summary from TEAC (mM) and FC reducing capacity (mg L^{-1}) assays by beverage type

	Red wines	White wines	Herbal infusions	Tea infusions	Juices	Beers
TEAC (mM)						
Mean ± S.D.[a]	15.4 ± 3.3	1.81 ± 0.45	1.89 ± 1.16	5.49 ± 2.10	2.23 ± 0.63	1.59 ± 0.70
Max.	22.6	2.83	3.63	10.9	3.58	2.58
Min.	9.1	1.14	0.28	2.72	1.35	0.85
Interval	13.5	1.69	3.35	8.18	2.23	1.73
FCR capacity (mg L^{-1})						
Mean ± S.D.[a]	2047 ± 487	252 ± 43	325 ± 171	558 ± 228	448 ± 124	586 ± 254
Max.	3274	327	543	1081	659	962
Min.	1284	193	46	220	304	321
Interval	1990	134	497	861	355	641

[a] Results are the mean ± standard deviation (S.D.) of 12 samples.

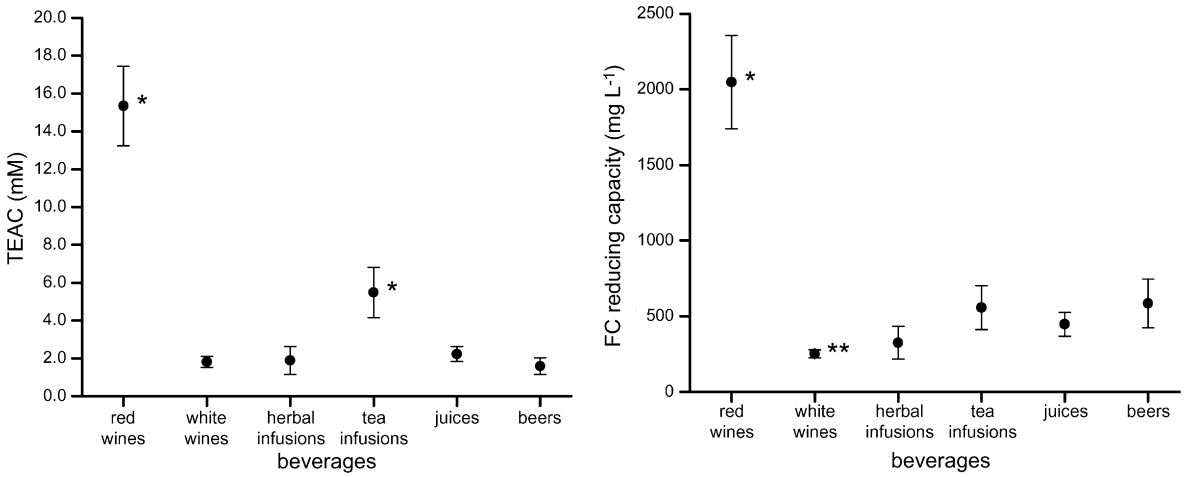

Fig. 3. Error plot of means with 95% confidence intervals for TEAC and FC reducing capacity of each group of beverage. *Significantly different from the other means ($p < 0.05$). **Significantly different from the other means ($p < 0.05$), except for herbal infusions ($p = 0.945$).

Table 4
Slope and correlation coefficients between FC reducing capacity (mg L^{-1}) vs. TEAC (mM) for the beverages analysed

	Red wines	White wines	Herbal infusions	Tea infusions	Juices	Beers
Slope (mg L^{-1} mM^{-1})	140 ± 33	57 ± 55	135 ± 41	106 ± 18	136 ± 101	349 ± 69
Correlation (R)[a]	0.947	0.590	0.918	0.972	0.690	0.962

[a] Correlation coefficients were determined by least-squares method ($n = 12$); correlation is significant at the 0.01 level (2-tailed).

for both parameters ($p < 0.05$). This higher antioxidant capacity of dark beers compared to blond beers was reported before and it may be related to their higher content in phenolic compounds and melanoidins that are formed during malting and brewing processes and that could act synergistically [22].

In the present study, the average values of TEAC and FC reducing capacity of green tea infusions ($n = 6$) were 6.78 ± 2.17 mM and 692 ± 228 mg L^{-1} respectively, while for black tea infusions ($n = 6$) the results obtained were 4.19 ± 0.96 mM and 424 ± 137 mg L^{-1}, respectively. The higher in vitro antioxidant capacity of green tea ($p < 0.05$) observed was also reported by Ivanova et al. [25] using the TEAC assay. Moreover, Serafini et al. [26] evaluated in vitro the length of the peroxyl radical induced lag-phase and observed that green tea was six-fold more potent than black tea. These differences may be attributed to the fermentation step from the black tea processing, where phenolic compounds are oxidized and polymerized enzymatically to theaflavins and thearubigens. The degradation of the major green tea catechin, epigallocatechin gallate, which is a powerful antioxidant, also takes place [27]. Nevertheless, for a better comparative assessment of the antioxidant efficiency of green and black tea infusions, other factors should be taken into consideration, such as raw material provenience, the brewing time, and the chopping grade of the tea leaves, for instance.

A good correlation was found between TEAC and FC reducing capacity when the whole set of samples was considered (FC capacity = 121.8 ± 8.6 TEAC + 127.3 ± 60.5, $R = 0.959$, $n = 72$). For each group of samples, the regression data including the slopes and the correlation coefficients established between the two methods are given in Table 4. Taking into account that these assays are based on electron transfer mechanisms [6], it is expected a significant correlation between them. The results obtained in the present study demonstrated a good correlation for red wines, herbal and tea infusions and beers ($R \geq 0.918$). Nevertheless, a lower correlation was established for white wines ($R = 0.590$) and juices ($R = 0.690$). In the case of white wines this can be an artifact caused by the low dispersion of FC capacity and TEAC values (Fig. 3). On the other hand, as juices were obtained from a variety of fruits (see supplementary data, Table S5) there was a high heterogeneity in the composition of these samples, which could explain the lower correlation obtained. Comparing the slope values obtained when a good correlation was attained (Table 4, $R > 0.9$), similar values were obtained for red wines, herbal and tea infusions, while the slope for beers was higher (about 2.5 times). This indicated a higher response for FC assay, probably due to the presence of dextrins, melanoidins, and proteins in dark beers [28].

Conclusions

The automatic method developed in the present work allowed the consecutive determination of the FC reducing capacity and TEAC, representing a useful tool for routine analysis as also for comparison purposes between these parameters. The fact of performing these determinations in parallel has also the advantage of processing the sample at the same time, avoiding errors that might arise with sample modification over time. The flexibility introduced by the proposed configuration associated to the computer control also allowed the performance of each assay separately. The present flow system was successfully applied to a large number of beverages providing reliable information about

the antioxidant properties in a simple, rapid and automatic way. The results showed that the correlation between these assays may vary according to type of sample. This observation reinforces the idea that more than one method should be used for evaluation of antioxidant capacity, especially in complex samples as food matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2007.04.028.

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