Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 68 (2005) 207–213
Talanta
www.elsevier.com/locate/talanta

A new approach for the sequential injection spectrophotometric determination of the total antioxidant activity

M.J. Reis Lima [1], Ildikó V. Tóth, António O.S.S. Rangel *

Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

Available online 8 August 2005

Abstract

A sequential injection system based on the ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic-acid) methodology was developed. The proposed method, incorporating a mixing chamber in the side port of the selection valve, was evaluated to measure the total antioxidant activity of several beverages and foods.

The $ABTS^{\bullet+}$ is generated by oxidation of ABTS with potassium persulfate and is reduced in presence of hydrogen-donating antioxidants converting into a colourless product. The applicability of the developed method was tested by measurement of the antioxidant activity of pure compounds as well as by analysing complex food and beverage samples. The antioxidant activity was presented as L(+) ascorbic acid equivalence. The values obtained by this methodology were not significantly different from the results obtained by the original spectrophotometric ABTS assay. For most of the studied antioxidants, antioxidant activity varied with pH and dilution. The proposed SIA system is suitable for screening direct or diluted total antioxidant activity of pure compounds or food samples.

Keywords: Antioxidant activity; ABTS; Sequential injection; Mixing chamber; Single standard calibration

1. Introduction

The activity of antioxidants is widely used as a parameter directly related to enhance their protective role in human organism. Antioxidants minimize the cellular damage from reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1,2]. Since free radicals are toxic to cells, body has developed a sophisticated antioxidant system that essentially relies upon antioxidant nutrients in order to protect our health from oxidative stress [2]. The identification and measurement of the concentration of each potential antioxidant compound of a sample is possible but can be time consuming and expensive. Moreover, there might be a synergistic effect between the antioxidants resulting in an over or under estimation of their combined action [3,4]. The total antioxidant capacity (TAC) is accomplished as a result of the synergetic redox interactions between the proportions of the totality of the molecules present in food [5]. The quantification of this synergetic action can be based on electrochemical or spectrophotometric detection. A wide range of spectrophotometric methods, namely diphenylpicrylhydrazyl radical (DPPH) [6], *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) [7], ferric reducing ability (FRAP) [8], desoxiribose assay [9] and ABTS [10] have been tested for the study of the antioxidant activity of pure compounds. Various limitations have been encountered for these assays [11], like slow reaction rates and solubility problems [6], possible interferences from organic acids [7] or from any reducing compounds [8] and elevated assay temperatures [9].

The most widely used spectrophotometric method for measuring antioxidant activity in biological samples is the ABTS assay [3,4]. This methodology is based on the capacity of antioxidant molecules to quench the $ABTS^{\bullet+}$ (blue-green chromophore with characteristic absorption at 414, 734 and 842 nm) converting it into a colourless product. The degree

* Corresponding author. Tel.: +351 22 5580064; fax: +351 225090351.
E-mail address: aorangel@esb.ucp.pt (A.O.S.S. Rangel).
[1] Permanent address: Escola Superior Agrária de Viseu, Instituto Superior Politécnico de Viseu, Campus Politécnico, Repeses, 3504-510 Viseu, Portugal.

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.06.058

of this decolourisation reflects the amount of $ABTS^{\bullet+}$ that was scavenged and can be directly monitored by spectrophotometric detection.

This methodology has suffered various modifications since its first appearance, namely the selection of the adequate monitoring wavelength (734 nm, far from visible region, is recommended) [11]. Additionally, direct production of the $ABTS^{\bullet+}$ through the reaction with potassium persulfate was introduced. This way, the interference of the antioxidant and pro-oxidant compounds in the process of generation of the radical cation was avoided [10].

The application of flow analysis techniques to this particular determination presents some important advantages. Characteristics like the reproducible timing of the whole analytical procedure are important when reactions with differences in kinetics are involved in the determination. Another feature to be exploited is the computer control that makes the systems flexible, and easy to adjust the flow parameters without physical manipulation in the manifold. Additionally, the flow circuits are closed, excluding the potential prejudicial effects of oxygen from the ambient air in the determinations.

Nevertheless, the implementation of flow methodologies for the determination of the antioxidant activity in food is not yet very exploited. Few works describe the application of flow injection analysis (FIA) for the study of antioxidant activity using different detection techniques, like amperometry [12,13], chemiluminometry [14,15], spectrofluorimetry [16], electron spin resonance spectrophotometry [17] and UV/Vis spectrophotometry [18–21]. The use of sequential injection analysis (SIA), introduced by Ruzicka and Marshall in 1990 [22], presents greater potentialities, as it allows more versatile sample manipulation inside the flow tubes with possible stop-and-go periods as well as possibility of adaptation for multi-reagent techniques and multi-detection systems without the need of manifold reconfiguration. This methodology has been tested for evaluating the antioxidant activity against hypochlorite ion by luminol chemiluminescence detection [23], for the evaluating of relative antioxidant capacity of wine samples by spectrophotometric and fluorimetric detection [24] and for routine screening tests of the antioxidant activity of mushrooms extracts by spectrophotometric detection [25].

Regarding the functionality of the original spectrophotometric ABTS assay [10], the control of the degree of mixing is critical. To accomplish this requirement in an automatic flow system, a sequential injection incorporating a well-stirred mixing chamber in the side port of the selection valve was explored in this work. This approach seemed very adequate, due to the possibility to achieve a perfect mixture [26] with a straightforward in-line manipulation of solutions. This incorporation was also considered positive, due to the possibility of using one single standard to perform the calibration procedure and simplify manipulation without the necessity of increasing the analytical cycle. The decrease of sample and standard manipulation outside the manifold also avoids the excessive exposure of the sensitive compounds to ambient air.

This new approach for the determination of the TAC was applied to pure substances and commercial food samples of diverse origin, presented as L(+) ascorbic acid equivalence.

2. Experimental

2.1. Reagents, standards and samples

All chemicals used were of analytical reagent grade and water from a Milli-Q (Millipore Corporation, Bedford, MA, USA) plus system was used throughout. In order to minimize bubble formation inside the tubes, solutions were prepared with previously boiled deionised water.

The ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) solution was obtained by the mixture of equal volume of a 2.45 mmol L^{-1} ABTS (Fluka 11557, Steinheim, Germany) solution with a 7 mmol L^{-1} potassium peroxodisulfate (Merck 5091, Darmstadt, Germany) solution, both in water. This solution stands for 12 h in dark in the fridge before use and a diluted 1:2 fresh solution was prepared daily.

The phosphate buffer solution pH 7.4 was prepared by dilution of 17.8 g of $Na_2HPO_4{\cdot}2H_20$ in 500 mL of deionised water by adjusting pH with HCl 2 mol L^{-1}. The sodium acetate buffer solution pH 5.4 was obtained by dissolving 8.20 g of CH_3COONa (Merck, Darmstadt, Germany) in 500 mL of deionised water by adjusting pH with HCl 2 mol L^{-1}.

To prepare antioxidants stock solutions, compounds were dissolved in appropriate solvents: α-tocopherol (Sigma Ref: 89550, Dorset, UK) and caffeic acid (Sigma Ref: C0625, Dorset, UK) in ethanol and trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid) Sigma Ref: 56510 Dorset, UK), L(+) ascorbic acid (VWR 20150.231 Fontenay-sous-Bois, France), gallic acid (Sigma Ref: G7384, Dorset, UK) and catechin hydrate (Sigma Ref: 22110, Dorset, UK) in deionised water. These solutions were prepared daily.

Samples for analysis were purchased on a local supermarket, and included fruit juices (apple, mango, pumpkin), refreshing drinks (tea based), teas (black and green), beverages (different type of beers) and dairy products (milk and yoghurt). Tea infusions were prepared from 0.3 g of dry tea leaves with 200 mL of boiling water, and were filtered. No previous sample treatment other than filtration and dilution was applied.

2.2. Instrumentation

A Gilson Minipuls 3 (Villiers-le-Bel, France) peristaltic pump, equipped with Gilson PVC pumping tubes, was used to propel the solutions. This pump was connected to the central channel of an eight port electrically actuated selection valve (Valco VICI C15-3118E, Switzerland). The tubes

connecting the different parts of the sequential injection system were made of PTFE (Omnifit, Cambridge, UK) with 0.8 mm i.d. A 386 personal computer Samsung SD 700 (Korea) equipped with an Advantec PCL 818 L (Taipei, Taiwan) interface card, running a homemade software written in QuickBasic (Microsoft) was used to control the selection valve position, the rotation sense and speed of the peristaltic pump.

A mixing chamber (MC) with an internal volume of *ca*. 750 μL made of acrylic containing a magnetic bar was used and placed over a magnetic stirrer (Metrohm AG E649, Herisau, Switzerland).

For the spectrophotometric detection, a Unicam (Cambridge, UK) 5625 UV–VIS spectrophotometer (λ = 734 nm) equipped with a Hellma (178.713-QS, Mullheim/Baden, Germany) flow-through cell with 8 μL of internal volume was used.

The detection system was connected to a Metrohm E586 Labograph (Herisau, Switzerland) chart recorder.

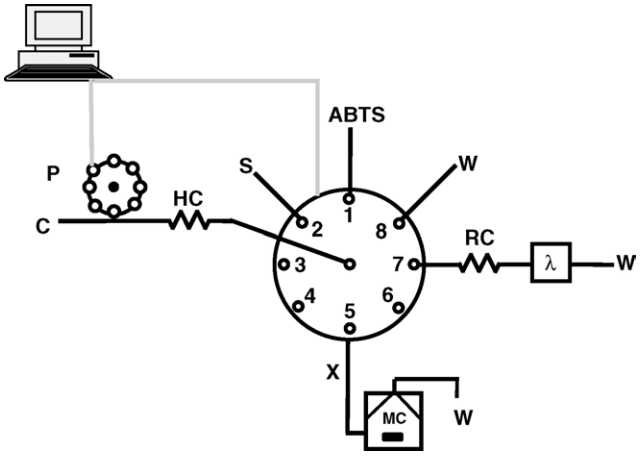

Fig. 1. Sequential injection manifold for the determination of total antioxidants in food, C, Carrier: water, acetate buffer pH 5.4 or phosphate buffer pH 7.4; P, peristaltic pump; HC, holding coil; W, waste; RC, reaction coil; S, standard or samples; λ, UV/Vis spectrophotometer; MC, mixing chamber.

2.3. Sequential injection procedure

The SIA system (Fig. 1) was designed to enable the sequential injection determination of the antioxidant activity of pure compounds as well as of food and beverage samples. The protocol sequence for these determinations is presented in Table 1.

The ABTS solution and the sample were sequentially aspirated (steps 1 and 2) to the holding coil. The flow was then reversed and the stacked zones were propelled to the mixing chamber (step 3). At this point, in order to enhance mixing in the MC, the flow was stopped for a time period between 15 and 240 s (step 4). Afterwards, when recollecting the mixture, the first part of the resulting solution was dispensed to waste (steps 5 and 6) in order to reduce bubble formation and improve repeatability. Consequently (steps 7–8), the mixing chamber content was propelled to the detector and spectrophotometric detection at 734 nm was carried out.

Three washing steps were included at the end of the analytical cycle (steps 9–11) corresponding to washing MC, empting MC and washing HC, respectively. At that point, the system was prepared for the next analytical cycle.

To assess the accuracy of the developed method, samples were also analysed by the batch procedure, based on the same spectrophotometric determination [10].

3. Results and discussion

3.1. System development

Firstly, the manifold physical characteristics and the protocol sequence were adjusted to guarantee similar conditions to the batch spectrophotometric ABTS assay [10].

The dilution of the ABTS solution was set to a value that produced a blank absorption of around 0.700 using the minimum reproducible volume for injection in the system [10]. A volume of 41.2 μL and a concentration of 0.875 mM produced a reproducible zero reading, with relative standard deviations of less than 0.33% for $n = 10$.

Table 1
Sequential injection protocol sequence for the determination of total antioxidant activity

Step	Description	Port	Time (s)	Pumping flow rate (μL s^{-1})	Flow direction	Volume (μL)
1	ABTS aspiration	1	2	20.6	Reverse	41.2
2	Sample aspiration	2/3	1–3[a]	20.6	Reverse	20.6–61.8
3	Dispense to MC	5	13	41.2	Forward	536
4	Stop period	5	15–240	0	–	–
5	Aspirate to HC	5	2	41.2	Reverse	82.4
6	Washing HC	8	10	56.4	Forward	564
7	Draw up MC content	5	7	41.2	Forward	288
8	Propel content to detector	7	35	56.4	Forward	1974
9	Washing MC	5	40	56.4	Forward	2256
10	Empty MC	5	18	56.4	Reverse	1015
11	Washing HC	8	30	56.4	Forward	1692

[a] Time was varied from 1 to 3 s corresponding to 20.6–61.8 μL for calibration using a single standard solution.

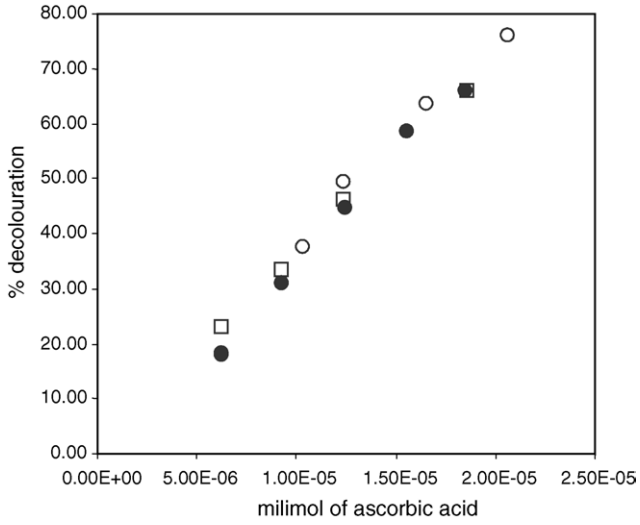

Fig. 2. Calibration curves obtained in the different calibration strategies; sampling time of 1 s or 20.6 μL (◯), 3 s or 61.8 μL (□) and variable volume (●).

By including a MC in the manifold, two objectives could be achieved. One was the employment of one single standard and different aspiration times to perform the calibration procedures. The other was to provide an efficient mixing between ABTS, the antioxidant compounds and the buffer solution, resulting in a uniform mixture where the final pH can be adjusted by appropriate selection of the carrier solution.

The evaluation of precision in using one single standard with five different aspiration times (1–3 s) instead of five different standards using fixed time (1 and 3 s) aspiration is shown in Fig. 2.

The percentage of decolouration was calculated from the absorbance values using the following equation:

$$\%_{\text{decolouration}} = \frac{A_0 - A_x}{A_0} \times 100$$

where A_0 is the absorbance for the blank assay and A_x is the absorbance for the standard or sample solutions.

The regression parameters for the percentage of decolouration as a function of the number of milimoles of ascorbic acid presented in Table 2 indicate that there is no significant difference between these three approaches [27].

Another important feature was the development of an automatic system that could concurrently perform the measurement of antioxidant content of hydrophilic and lypophilic substances, since this was one of the disadvantages presented by other methodologies [6]. The aspiration of lypophilic material dissolved in ethanol, usually leads to bubble formation due to the pressure difference. In the present system the bubbles always formed in a specific part (*x* in Fig. 1) of the manifold. By incorporating the steps 5 and 6 (Table 1) in the analytical cycle these air bubbles were removed and this way repeatability was improved.

3.2. Application to pure compounds

The measurement of the antioxidant activity at different dilutions, pH and assay time, provides information about the radical-scavenging reaction. These parameters were studied for individual substances by the $ABTS^{\bullet+}$ method.

The individual contribution of each antioxidant to the TAC is considered to be dependent on dilution [28]. For pure antioxidant compounds a linear calibration line can be established within the range of 20–80% decolouration [10]. Studies involving the pure antioxidants trolox, ascorbic acid, caffeic acid, gallic acid, catechin hydrate and α-tocopherol are depicted in Table 3. The selected concentrations indicated with 'a', correspond to better linearity with five possible calibration points within the linear range and these concentrations were adopted in further studies.

Previous studies [29] refer that antioxidant activity assessed by the ABTS assay depends on pH. In the developed SIA system, it is possible to adjust pH of the reaction by changing the carrier solution, without disturbing the sample composition. For our study, two different carriers with pH 5.4 and 7.4 were used [29] and another option without a buffer system (water) was also tested. Sensitivity was affected by pH, showing higher sensitivity with higher pHs for most of the antioxidants tasted, except for gallic acid and trolox that seemed to be not affected by changes in this parameter. It is also to note that at non-buffered pH the value of sensitivity was different from those previously mentioned.

For the reaction time study, three different times (15, 60 and 240 s) for the stop period in the MC were tested to evaluate the efficiency of the mixing and also to assess whether the reaction can be considered complete between the antioxidant and the radical cation. Previous studies indicate a time dependency of the reaction [10,28,29], resulting in different antioxidant activity for different assay times. For the majority of the antioxidants tested, it was found that assay time has an important effect in sensitivity (slope in Table 3) of

Table 2
Comparison of the regression parameters of the different strategies for calibration

Sampling volume (μL)	Intercept (% decolouration)	Slope (% decolouration × mmol AA^{-1})	*n*	*R*
20.6	−4.16 (±13.18)[a]	4.03×10^6 ($\pm 9.35 \times 10^5$)[a]	5	0.992
61.8	1.75 (±5.83)[a]	3.49×10^6 ($\pm 4.68 \times 10^5$)[a]	4	0.999
Variable[b]	−5.56 (±8.65)[a]	3.99×10^6 ($\pm 6.59 \times 10^5$)[a]	5	0.996

[a] Within brackets are the limits of the 95% confidence intervals.
[b] Calibration using a single standard solution and variable aspiration volume within the range of 20.6–61.8 μL.

Table 3
Study of antioxidant activity of pure compounds

Antioxidant	Study	Values	Slope (%dec./mmol)	Intercept	R^2
Trolox	Concentration (mM)	0.30	1.82E+06	15.9	0.987
		0.40[a]	2.37E+06	0.5	0.998
		0.50	2.65E+06	1.7	0.985
	pH	5.4	3.24E+06	−4.3	0.995
		Water	3.02E+06	−6.0	0.998
		7.4	3.58E+06	−8.8	0.992
	Time (s)	15	3.02E+06	−6.0	0.998
		60	2.84E+06	−3.4	0.998
		240	2.55E+06	3.4	0.998
Ascorbic acid	Concentration (mM)	0.25	1.76E+06	7.8	0.976
		0.30[a]	4.60E+06	−9.5	0.996
		0.35	4.31E+06	−12.2	0.987
	pH	5.4	3.04E+06	−1.1	0.997
		Water	4.60E+06	−9.5	0.969
		7.4	5.12E+06	−13.5	0.995
	Time (s)	15	2.03E+06	−3.6	0.982
		60	2.46E+06	−9.5	0.982
		240	2.76E+06	4.8	0.985
Caffeic acid	Concentration (mM)	0.20	4.99E+06	−3.2	0.987
		0.30[a]	4.27E+06	1.8	0.988
		0.45	4.21E+06	6.5	0.974
	pH	5.4	4.64E+06	8.3	0.995
		Water	4.27E+06	1.8	0.988
		7.4	5.77E+06	9.2	0.957
	Time (s)	15	4.12E+06	8.6	0.987
		60	4.24E+06	6.2	0.990
		240	4.94E+06	−0.3	0.985
Gallic acid	Concentration (mM)	0.06	1.85E+07	−5.5	0.948
		0.10[a]	1.16E+07	0.8	0.990
		0.15	9.88E+06	9.1	0.971
	pH	5.4	1.32E+07	12.7	0.999
		Water	1.40E+07	8.8	0.990
		7.4	1.27E+07	−8.1	0.991
	Time (s)	15	1.16E+07	0.8	0.991
		60	1.71E+07	3.6	0.999
		240	1.50E+07	10.1	0.973
Catechin hydrate	Concentration (mM)	0.08	9.37E+06	3.9	0.988
		0.10[a]	9.83E+06	5.8	0.996
		0.15	8.25E+06	9.7	0.971
	pH	5.4	1.58E+07	20.0	0.989
		Water	9.45E+06	7.8	0.995
		7.4	1.48E+07	8.8	0.990
	Time (s)	15	9.83E+06	5.8	0.988
		60	8.65E+06	11.7	0.994
		240	1.58E+07	20.1	0.997
α-Tocopherol	Concentration (mM)	0.10	5.50E+06	−2.2	0.948
		0.15	6.64E+06	−1.7	0.997
		0.20[a]	6.15E+06	−2.6	0.976
	pH	5.4	7.37E+06	6.5	0.971
		Water	6.44E+06	15.2	0.989
		7.4	9.40E+06	−1.7	0.999
	Time (s)	15	6.44E+06	15.2	0.989
		60	8.44E+06	2.4	0.990
		240	1.09E+07	3.9	0.968

[a] Selected concentrations used for further studies.

Table 4
Comparison between the results obtained by the developed SIA method and by reference procedure in analysis of food samples of different origins

Carrier	Sample	Concentration (mM of ascorbic acid) by SIA	Concentration (mM of ascorbic acid) by the batch procedure
Water	Beer 1	0.54 ± 0.04	0.62 ± 0.09
	Beer 2	0.72 ± 0.05	0.77 ± 0.12
	Pasteurised milk 1	0.65 ± 0.02	0.85 ± 0.05
	Pumpkin	0.36 ± 0.04	0.42 ± 0.03
	Tea based refreshing drink	0.85 ± 0.06	0.84 ± 0.08
pH 7.4	Tea	0.37 ± 0.09	0.39 ± 0.02
	Yoghurt, fortified	10.63 ± 1.9	10.68 ± 3.1
	Tomato juice 1	2.19 ± 0.5	2.81 ± 0.2
	Pasteurised milk 2	13.18 ± 0.02	12.3 ± 0.2
	Pumpkin 1	1.18 ± 0.04	1.33 ± 0.1
	Green tea 1	14.46 ± 1.1	12.23 ± 0.6
	Mandarine	5.00 ± 0.03	4.33 ± 0.3
	Apple juice 1	1.15 ± 0.03	1.6 ± 0.2

the reaction. In the case of ascorbic acid, caffeic acid, catechin hydrate and α-tocopherol the slope increased with time. As expected [28], for trolox no significant change was found between 15 and 240 s. Gallic acid showed no systematic tendency, although linearity was clearly better for the 60 s stop period.

3.3. Analysis of food and beverage samples

To evaluate the applicability of the developed methodology, samples were analyzed by the developed method and by the comparison batch procedure [10].

Statistical treatment of results (Table 4) was made by establishing a relation type of $C_{SIA} = C_0 + SC_{batch}$, being $C_0 = -0.021$ (±0.573), $S = 0.923$ (±0.796) and $R = 0.905$ for water as carrier and $C_0 = -0.422$ (±0.951), $S = 1.13$ (±0.13) and $R = 0.994$ for pH 7.4 carrier. Between brackets, the confidence limits for a 95% significance level [27] are presented. These figures indicate that there is no significant difference between the results obtained by the developed methodology and by the ones obtained by the batch procedure.

Complex samples or sample solutions of diverse origin were also aspirated in variable volume and linear calibration curves (% of decolouration as a function of injected volume) were obtained between 20 and 80% decolouration, indicating that for these samples the antioxidant capacity was not affected by the dilution neither by the intrinsic absorbance of the sample matrix. Under these conditions, the slope of the "sample calibration curve" can be compared with the slope of the standard (ascorbic acid) calibration curve, and the ratio should reflect the TAC of the sample solution.

Table 5 presents the parameters of the "sample calibration curves" and the results obtained for antioxidant capacity of some food and beverages analysed by this slope comparison method. These values were calculated in comparison to ascorbic acid calibration curve: $Y = 1.03$ (±0.09) × $V + 6.24$ (±12.4) and $R^2 = 0.996$ (±0.003), where Y is the percentage of decolouration and V is the aspirated volume. The values between brackets are the relative deviation of the parameters corresponding to determinations at four independent days.

Table 5
Results obtained in the determination of total antioxidant activity, by the slope comparison method

Sample	Slope	R^2	Concentration (mM)[a]
Green tea 2	0.968	0.958	7.1 ± 0.6
Tea based refreshing drink 2	0.925	0.991	0.39 ± 0.03
Apple juice 2	0.642	0.904	0.28 ± 0.02
Pumpkin 2	0.679	0.986	0.20 ± 0.02
Mango juice	0.526	0.974	3.8 ± 0.3
Tomato juice 2	1.471	0.987	4.3 ± 0.4
Beer 3	1.439	0.997	0.42 ± 0.03
Beer 4	1.444	0.994	0.42 ± 0.03

[a] Expressed as ascorbic acid content of the undiluted sample.

In conclusion, the presented approach for the determination of the TAC was successfully applied to pure substances and commercial food samples of diverse origin. The developed system showed good stability and repeatability. The incorporation of the mixing chamber into the manifold made possible to use one single standard to perform the calibration procedure and simplified the manipulation without the necessity of increasing the analytical cycle. The decrease of sample and standard manipulation outside the manifold also avoided the excessive exposure of the sensitive compounds to ambient air. The configuration of the system allows accommodating the other spectrophotometric reactions for assessing the total antioxidant capacity.

Acknowledgements

To IFADAP through project Agro 273. Ildikó Tóth thanks FCT and FSE (III Quadro Comunitário de Apoio), for the financial support through grant SFRH/BPD/563/2001. M.J.R. Lima also thanks to Fundação Calouste Gulbenkian.

References

[1] S.A. Stanner, J. Hughes, C.N.M. Kelly, J. Buttriss, Public Health Nutr. 7 (2004) 407.
[2] B. Halliwell, Annu. Rev. Nutr. 50 (1996) 16.
[3] C. Sanchez-Moreno, Food Sci. Technol. Int. 8 (2002) 121.
[4] M. Antolovich, P.D. Prenzler, E. Patsalides, S. McDonald, K. Robards, Analyst 127 (2002) 183.
[5] N. Pellegrini, M. Serafini, B. Colombi, D. Del Rio, S. Salvatore, M. Bianchi, F. Brighenti, J. Nutr. 133 (2003) 2812.
[6] C. Sanchez-Moreno, J.A. Larrauri, F.J. Saura-Calixto, J. Sci. Food Agric. 76 (1998) 270.
[7] M.I. Gil, F.A. Tomás-Barberán, B. Hess-Pierce, D.M. Holcroft, A.A. Kader, J. Agric. Food Chem. 48 (2000) 4581.
[8] I.F.F. Benzie, J.J. Strain, Anal. Biochem. 239 (1996) 70.
[9] A. Ghishelli, M. Nardini, A. Baldi, C. Scaccini, J. Agric. Food Chem. 46 (1998) 361.
[10] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Free Rad. Biol. Med. 26 (1999) 1231.

[11] M.B. Arnao, Trends Food Sci. Technol. 11 (2000) 419.
[12] J. Chen, L. Gorton, B. Åkesson, Anal. Chim. Acta 474 (2002) 137.
[13] S. Buratti, N. Pellegrini, O.V. Brenna, S. Mannino, J. Agric. Food Chem. 49 (2001) 5136.
[14] M. Sariahmetoglu, R.A. Wheatley, Y. Çakýcý, A. Townshend, Pharmacol. Res. 48 (2003) 361.
[15] J.W. Costin, N.W. Barnett, S.W. Lewis, D.J. McGillivery, Anal. Chim. Acta 499 (2003) 47.
[16] B. Tang, L. Zhang, Y. Geng, Talanta 65 (2005) 769.
[17] H. Ukeda, Y. Adachi, M. Sawamura, Talanta 58 (2002) 1279.
[18] I.I. Koleva, H.A.G. Niederlander, T.A. van Beek, Anal. Chem. 73 (2001) 3373.
[19] N. Pellegrini, D. del Rio, B. Colombi, M. Bianchi, F. Brighenti, J. Agric. Food Chem. 51 (2003) 260.
[20] H. Ukeda, Bunseki Kagaku 53 (2004) 221.
[21] S. Bompadre, L. Leone, A. Politi, M. Battino, Free Rad. Res. 38 (2004) 831.
[22] J. Ruzicka, G.D. Marshall, Anal. Chim. Acta 237 (1990) 329.
[23] K. Nakamura, Y. Ohba, N. Kishikawa, N. Kuroda, Buseki Kagaku 53 (2004) 925.
[24] P.C.A.G. Pinto, L.M.F.S. Saraiva, S. Reis, J.L.F.C. Lima, Anal. Chim. Acta 531 (2005) 25.
[25] M. Polásek, P. Skála, L. Opletal, L. Jahodá, Anal. Biochem. 379 (2004) 754.
[26] I.P.A. Morais, M.R.S. Souto, A.O.S.S. Rangel, J. Flow Inj. Anal. 20 (2003) 187.
[27] J.C. Miller, J.N. Miller, Statistics for Analytical Chemistry, third ed., Ellis Horwood, New York, USA, 1993, pp. 120–124.
[28] D. Villano, M.S. Fernández-Pachón, A.M. Troncoso, M.C. García-Parrilla, Talanta 64 (2004) 501.
[29] E.P. Labrinea, C.A. Georgiou, Anal. Chim. Acta 526 (2004) 63.