The effect of method, standard and sample components on the total antioxidant capacity of commercial waters assessed by optical conventional assays

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Abstract

The total antioxidant capacity (TAC) of 28 flavoured water samples was assessed by ferric reducing anti- oxidant potential (FRAP), oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC) and total reactive antioxidant potential (TRAP) methods. It was observed that flavoured waters had higher antioxidant activity than the corresponding natural ones. The observed differences were attributed to flavours, juice and vitamins. Generally, higher TAC contents were obtained on lemon waters and lower values on guava and raspberry flavoured waters. Lower and higher TACs were obtained by TRAP and ORAC method, respectively. Statistical analysis suggested that vitamins and flavours increased the antioxidant content of the commercial waters.

Keywords

Total antioxidant capacity, FRAP, TEAC, ORAC, TRAP, Commercial waters

1. Introduction

Most living organisms have developed complex endogenous and exogenous antioxidant systems to counteract and prevent the deleterious effects of reactive oxygen species (ROS) (Cao & Prior, 1998). ROS (such as, hydrogen peroxide, H_2O_2; hydroxyl radical, HO , and superoxide radical, O_2^{-}) induce damage to the cells by reacting with biomolecules (proteins, lipids, among others) and cause serious lesions on DNA (Halliwell, 2009). Therefore, antioxidants appear to be very important in the prevention of many diseases, like as, atherosclerosis, diabetes mellitus, neurodegenerative disorders and certain types of cancer (Benherlal & Arumughan, 2008; Chen, Wang, Qi, & Xie, 2007).

Endogenous antioxidant systems include enzymes, such as, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione-*S*-transferase and catalase (Augustin, Wiswedel, Noack, Reinheckel, & Reichelt, 1997; Cao, Alessio, & Cutler, 1993). An additional protection can be provided by exogenous antioxidant compounds, such as, vitamins (A, E, C, b-carotene), phenolic compounds, minerals (selenium, zinc) or proteins (transferrin, ceruloplasmin, albumin) (Ferreira, Baptista, Vilas-Boas, & Barros, 2007). Increasing intake of dietary antioxidants may help to maintain an adequate antioxidant status and, therefore, the normal physiological functions of a living system. Some functional foods, vegetables, fruits and whole-grain cereals are good sources of exogenous antioxidants (Almajano, Carbó, Jiménez, & Gordon, 2008; Frankel, 2007; Huang, Wang, Eaves, Shikany, & Pace, 2009; Lee et al., 2009). Recently, to answer to consumer's preferences and considering that water is the most consumed drink all over the world, fla- voured waters were developed and commercialised. Flavoured waters are produced from mineral and spring waters. In the first semester of 2009, 6.23 million litters of this kind of water were consumed by Portuguese population (ANIRSF, 2009). This kind of water consist in the addition of flavours, juices and sugar or sweet- eners that provide water singular tastes and smells appreciated by consumers. Considering that flavours/aromas are fruit extracts, they should contain natural antioxidants, transferring them into the bottled water. So, drinking this type of water can increase the daily intake of natural exogenous antioxidants and may contribute to the protective system against ROS. However, there are no reports concerning the antioxidant capacity properties of these waters, although their macro and micromineral compositions are known. So, the presented research work is meant to find advantages/disadvantages of the consumption of these beverages with regard to its total antioxidant capacity (TAC).

Several methods have been reported to assess TAC in biological and food samples, because of the difficulty in measuring each antioxidant component separately and the interactions among different antioxidant components in the samples. Ferric reducing antioxidant potential (FRAP) (Benzie & Strain, 1999; Benzie & Szeto, 1999; Griffin & Bhagooli, 2004; Prior, Wu, & Schaich, 2005;

Pulido, Bravo, & Saura-Calixto, 2000; Sánchez-Alonso, Jiménez-Escrig, Saura-Calixto, & Borderías, 2007), trolox equivalent antioxidant capacity (TEAC) (Castro, Rogero, Junqueira, & Carrapeiro, 2006; Pellegrini et al., 2003), oxygen radical absorption capacity (ORAC) (Cao et al., 1993; Ou, Hampsch-Woodill, & Prior, 2001; Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002; Prior et al., 2005) and total reactive antioxidant potential (TRAP) (Ghiselli, Serafinni, Natella, & Scaccini, 2000; Prior et al., 2005; Sánchez-Moreno, 2002) are examples of methods used to determine TAC. Mechanistically, these methods are based either on single electron transfer or hydrogen atom transfer reactions between an oxidant and a free radical (Sánchez-Moreno, 2002). Change of absorbance of either antioxidant or oxidant is measured by an ultraviolet–visible spectrophotometer for FRAP, TEAC and TRAP methods and spectrofluorimetry for ORAC method. Absorbance value is used for the quantification of the reducing capability of the antioxidant (Ou et al., 2002).

These analytical methods include several advantages: technically they are simple with good repeatability and reproducibility. These techniques are also adaptable for assay on both hydrophilic and lipophilic antioxidants. However, no single assay really provides the TAC of a particular sample. The comparison of data from different studies is also difficult. In this context, a primary factor to consider when selecting a method is the mechanism of reaction and its relationship to what might occur in the envisioned application. It is also advantageous to select methods that are commonly accepted, validated and standardised, with a large body of comparable data available in the literature. Thus, to elucidate a full profile of TAC of complex samples such as flavoured waters it is essential to use various analytical methods (Prior et al., 2005).

Furthermore, TAC values also depend on the antioxidant standard used as reference. Ascorbic acid, gallic acid and trolox are widely used for this purpose. Ascorbic acid or vitamin C is a powerful antioxidant because it can donate a hydrogen atom and form a relatively stable ascorbyl free radical. As a scavenger of reactive oxygen and nitrogen oxide species, ascorbic acid has shown to be effective against the superoxide radical ion, hydrogen peroxide, the hydroxyl radical and singlet oxygen (Weber, Bendich, & Schalch, 1996). Trolox is a synthetic, hydrosoluble phenolic derivative of vitamin E and gallic acid is found both free and as part of tannins.

In the present study, the previously indicated methods were used to evaluate the TAC of all marketed waters found: 28 mineral and spring water samples, with flavours. Ascorbic acid, gallic acid and trolox were used as standards.

2. Materials and methods

2.1. Standard and reagents

Analytical grade chemicals were employed. Gallic acid, potassium persulphate monohydrate 2,4,6-tripyridyl-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), were obtained from Fluka. Ascorbic acid, sodium acetate trihydrate, glacial acetic acid, $FeCl_3 6H_2O$, dipotassium hydrogenphosphate anhydrous, phosphoric acid, hydrochloric acid were purchased from Merck. Fluorescein was obtained from Riedel-de Haën and 2,2′-Azobis(2-methyl-propanimidamide) (AAPH) was purchased from Aldrich.

2.2. Sampling procedure

Water samples were collected from several local supermarkets and stored aside from light at 4 °C. All waters found by that time were included in this study: 28 flavoured water samples (mineral and spring). Each brand included still or sparkling waters with different flavours and aromas. The natural water of each brand was always used as negative control. The gas was eliminated from the samples by sonication. Fig. 1 groups the sample distribution according to the labelled information, regarding the presence of vitamins, sweeteners and preservatives (no quantity was mentioned).

2.3. FRAP assay

FRAP assay measures the ability of antioxidants to reduce the ferric 2,4,6-tripyridyl-s-triazine complex $[Fe(III)\text{-}(TPTZ)_2]^{3+}$ to the intensely blue coloured ferrous complex $[Fe(II)\text{-}(TPTZ)_2]^{2+}$, in acidic medium, with an absorption maximum at 593 nm (Benzie & Strain, 1999; Benzie & Szeto, 1999; Griffin & Bhagooli, 2004; Prior et al., 2005; Pulido et al., 2000; Sánchez-Alonso et al., 2007). FRAP reaction detects compounds with redox potentials <0.7 V (redox potential of Fe^{3+}-TPTZ). So it cannot detect compounds that act by radical quenching (H transfer), particularly thiols and proteins (Ou et al., 2002). Absorbance was measured on a Shimadzu 160-A spectrophotometer. The experiment was conducted at 37 °C under pH 3.6 (to ensure Fe solubility). Blank trials were carried out in parallel. The working FRAP reagents were produced by mixing 300 mM acetate buffer, 10 mM TPTZ solution and 20 mM $FeCl_3 6H_2O$ in a 10:1:1 ratio before use and heated to 37 °C. The 300 mM acetate buffer was prepared with 3.1 g of acetic acid brought to 1 L with distilled water. TPTZ solution was prepared with 10 mM TPTZ in 40 mM HCl. These solutions were daily prepared. A mixture of 1500 μL working FRAP solution, 1300 μL acetate buffer and 200 μL water sample were added to a spectrophotometric cell. Absorbance readings were taken at 30 s and then every 20 min until minimal signal stabilisation. Calibration curves were traced for each time and analytical results were calculated for the corresponding measuring time. Final results were expressed in μM of ascorbic acid.

2.4. TEAC assay

This method is based on the ability of antioxidant molecules to quench the long-lived radical cation $ABTS^{+}$, a blue-green chromophore with typical maximum absorption at 734 nm, compared with to that of trolox, a water-soluble vitamin E analog. The addition of antioxidants to the preformed radical cation reduces it to ABTS, determining a decolourisation (Pellegrini et al., 2003). A stable stock solution of $ABTS^{+}$ was produced by reacting a 7 mM aqueous solution of ABTS with 2.45 mM potassium persulphate diluted in phosphate buffer (pH 7) and allowing the mixture to stand in the dark, at room temperature, for 12–16 h before use. At the beginning of each day, an $ABTS^{+}$ working solution (B) was obtained by dilution in phosphate buffer of the stock solution to an absorbance of 0.70 ± 0.02 a.u. Two thousand microlitres of this solution was added to 1000 μL of sample solution. The absorbance of this final solution was read after 15 min, at 734 nm. TAC was expressed in μM of trolox, ascorbic acid and gallic acid.

2.5. ORAC assay

ORAC measures the antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer. In this assay, the peroxyl radical reacts with AAPH (radical) to form a non-fluorescent product, which can be quantified by spectrofluorimetry. TAC is determined by the decreased rate and amount of fluorescence decay over time (Cao et al., 1993; Ou et al., 2002; Prior et al., 2005). Sample (300 μL) and fluorescein (1000 μL, 14×10^{-3} μM) were added

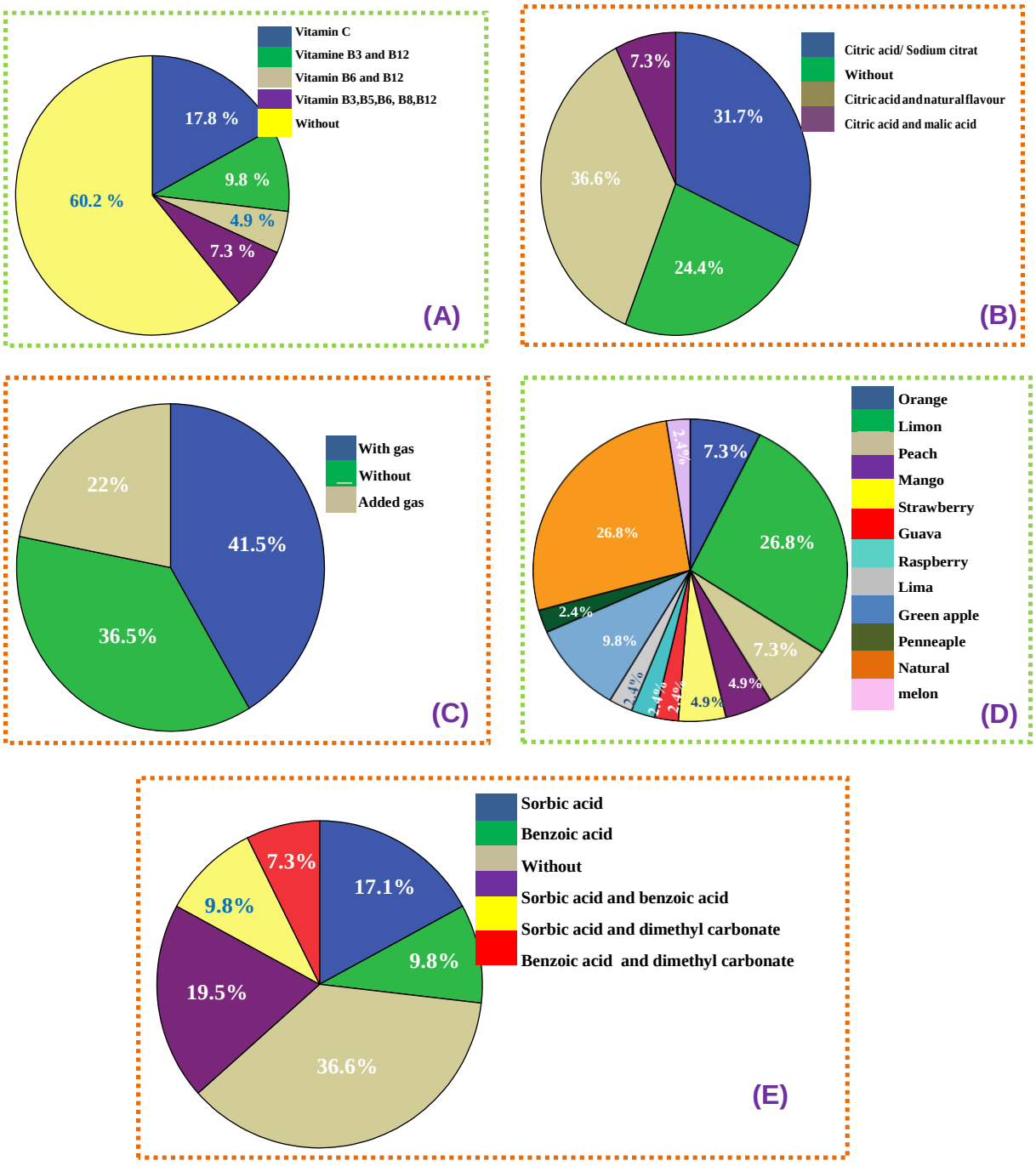

Fig. 1. Descriptive statistics of water labels, considering several factors: (A) vitamins; (B) preservatives; (C) gas; (D) flavour; and (E) acidity regulators.

to a spectrophotometric cell, and the mixture was incubated at 37 °C for 20 min, followed by addition of 1700 μL of radical solution (4.8 mM). Fluorescence was monitored in an AvaSpec 2048 fluorimeter with xenon lamp pulsed at 485 and 528 nm (excitation and emission wavelengths, respectively) until the final fluorescence was equal to 0.5% of the initial fluorescence. TAC was expressed in μM of trolox, ascorbic acid and gallic acid. A linear correlation was found between antioxidant concentration and the area under curve (AUC) that was calculated as:

$$AUC = 1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots f_{34}/f_0 + f_{35}/f_0 \quad (1)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time i.

TRAP assay

TRAP assay monitors the ability of antioxidant compounds to interfere with the reaction between peroxyl radicals and a target probe (Araki et al., 1999; Prior et al. 2005; Ghiselli et al., 2000). AAPH was used as the peroxyl-radical generator to start the reaction. The final reaction mixture for the assay contained 2 mM AAPH and 75 μM ABTS in 50 mM acetate buffer (pH 4.3). The reaction mixture was incubated at 45 °C for 60 min and then brought to room temperature. A total of 600 μL of sample was added to 2600 μL of the reaction mixture in each cuvette. For the blank, 50 mM of acetate buffer (pH 4.3) was used in place of the sample. The decrease of absorbance at 734 nm elicited by the sample was

measured after 15 min at 25 °C. TAC was expressed in μM of trolox, ascorbic acid and gallic acid.

2.7. Assay validation

Calibration standards were daily prepared (all samples were determined in triplicate). The proposed methods were validated by linear range, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy. LOD and LOQ were defined, respectively, as 3 and 10 times the standard deviation of 10 blank signals divided by the slope of the calibration plot (Miller & Miller, 2000).

2.8. Data analysis

All results were expressed as mean ± standard deviation. Data were tested using one-way ANOVA to determine the main effect of method, standard, flavours, vitamins and preservatives added to the waters, on the TAC of flavoured waters. Differences at $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Descriptive statistics

As can be seen in Fig. 1C 41.5% of water samples were still and 58.5% were sparkling or without gas. Labelled information also indicated the presence of several compounds, some of them with biological activity and others added for technological purposes. Among these, flavours, juice fruits, vitamins, acidifying agents, sweeteners and preservatives are present. However it was not indicated the amount of ingredient added to the water (see Table 2).

Lemon was the predominant flavour (Fig. 1D), present in all water brands (A to J). Twelve different flavours were used for the production of flavoured waters: Lemon (10 samples), mango, strawberry, raspberry and lime (2 samples each), pineapple, apple and orange (3 samples each), peach (4 samples), guava, melon and green apple (1 sample each).

About 50% of the samples had fruit juices or concentrates. According to the literature (Orak, 2009; Xu et al., 2008) fruits are an important source of antioxidants such as vitamin C, carotenoids and phenolic compounds. Only flavoured waters from brands A, D and G did not refer the addition of this kind of ingredient.

The majority of samples did not have vitamins (Fig. 1A). Only eleven samples had vitamins of B complex (7 samples) and C (4 samples). Vitamins B complex are important for DNA synthesis and in the cancer prevention (Zhang et al., 2008). It is also important to refer that vitamin C is an antioxidant with the capacity of protection against oxidative stress, and is a cofactor in several vital enzymatic reactions. According to the labelled information, the added amounts are very different amongst the several brands and some of them only refer its presence (Birch, Brasch, McCaddon, & Williams, 2009; Weber et al., 1996).

Other bioactive compounds namely white and green teas, ginseng, ginkgo biloba and L-carnitine are present in some flavoured waters from different brands. Tea contains numerous components with antioxidant activity, such as polyphenols (catechins, epicatechin, epigallocatechin) and vitamins (Cabrera, Artacho, & Giménez, 2006; Neyestani, Gharavi, & Kalayi, 2009). Ginseng is an herbal medicine with antioxidant and anti-inflammatory activities (Radad, Gille, Liu, & Rausch, 2006). Ginkgo biloba is rich in phenolics and flavonoids (Liu et al., 2007). L-Carnitine is an amino acid with antioxidant activity, neuroprotective and neurotrophic actions, positive actions on mitochondrial metabolism and stabilisation of intracellular membranes (De Grandis, 2007).

Phenolic compounds are correlated with antioxidant activity and seem to have an important role in stabilising lipid oxidation. The total phenolic contents (TPC) of this kind of waters was evaluated (data not showed). It was observed that all flavoured waters presented phenolic compounds in its composition. Like it was expected, natural waters did not have TPC. The highest TPC levels were from citrus fruits flavours (tangerine, lime and lemon) and from waters with bioactive compounds, like, tea, ginseng and gingko biloba.

In what concerns to preservatives and the information contained in the label, each sample can contain one (Sorbic acid and benzoic acid) or two preservatives (sorbic acid and benzoic acid; sorbic acid and dimethyl dicarbonate; benzoic acid and dimethyl dicarbonate) simultaneously (Fig. 1E).

Acidifying regulators are food additives added to change or maintain pH; in this work the most used was the blend of citric acid and sodium citrate (31.7%) (Fig. 1B). Citric acid is also an antioxidant, that can inhibit the decomposition of hydroperoxides by preventing their complexation with catalytic metal ions (Frankel, 2007).

Table 1
Main analytical features of the TAC assays.

Parameter	FRAP[d]	TEAC			ORAC			TRAP		
	Ascorbic acid	Ascorbic acid	Gallic acid	Trolox	Ascorbic acid	Gallic acid	Trolox	Ascorbic acid	Gallic acid	Trolox
Range concentration (μM)	1.00–24.7	1.00–19.5	0.50–4.0	1.00–19.0	10.00–50.0	10.00–56.0	10.00–50.0	1.00–11.5	0.8–5.6	0.9–9.4
Slope ($n = 3$)	0.075	−0.036	−0.169	−0.040	0.017	0.048	0.060	−0.028	−0.067	−0.027
Intercept ($n = 3$)	0.012	0.678	0.626	0.671	0.012	0.157	−0.022	0.492	0.361	0.447
Correlation coefficient ($n = 3$)	0.996	0.991	0.994	0.993	0.990	0.999	0.992	0.988	0.989	0.993
LOD (μM)	0.023	0.049	0.010	0.044	0.104	0.037	0.029	0.063	0.026	0.065
LOQ (μM)	0.078	0.163	0.035	0.147	0.345	0.122	0.098	0.210	0.088	0.217
Precision/accuracy										
Added	10.00	3.75	2.50	3.75	20.00	20.00	20.00	1.25	2.50	1.25
Found	10.51	3.50	2.53	3.47	20.20	19.88	20.04	1.36	2.50	1.38
RSD[a]	6.0	1.5	14.0	0.5	1.0	0.0	6.5	12.0	9.0	16.0
RE[b]	+5.10	−6.67	+11.20	−7.47	+1.00	−0.60	+0.20	+8.80	+0.00	+10.40
REC[c]	105.1	93.2	101.2	92.5	101.0	99.4	100.2	108.6	100.0	110.0

[a] RSD: relative standard deviation.
[b] RE: relative error.
[c] REC: recovery.
[d] For 180 min.

3.2. Method validation

The linear relationship between absorbance (FRAP, TEAC, TRAP methods) or net area (ORAC method) and antioxidant concentration was evaluated for trolox, ascorbic acid and gallic acid. The corresponding analytical results are presented in Table 1.

Linearity ranges were from 1.0 to 24.7, 0.5 to 19.5, 10.0 to 56.0 and 0.8 to 11.5 μM for FRAP, TEAC, ORAC and TRAP methods, respectively, depending on the standard. In general ascorbic acid and trolox provided wider linear ranges, while gallic acid showed linear behaviour along a narrower range of lower concentration.

The calculated LOD values ranged from 0.010 to 0.104 μM. LOQ values lied within 0.035 and 0.345 μM. Lowest LOD and LOQ values were found using gallic acid as standard antioxidant in TEAC method.

Precision and accuracy values are also shown in Table 1. Relative standard deviation (RSD) values ranged from 0.0% to 16.0%, and confirmed the high precision of the method. Recovery (REC) and relative error (RE) values assessed the accuracy of the results; RE were always <11% and recovery trials ranged from 92% to 110%.

3.3. TAC evaluation

FRAP assay is a simple and inexpensive procedure that measures the total antioxidant level in samples. However, any compound with redox potential lower than that of the redox pair Fe(III)/Fe(II), can theoretically reduce Fe(III) to Fe(II), contributing to the antioxidant level of the sample (Prior et al., 2005).

TAC values obtained by FRAP method were based on different times of reaction, ranging between 0 and 250 min (Fig. 2). Ascorbic acid was always used as reference standard. Calibration curves were plotted for different times of reaction. TAC values were increased with the reaction time until 160 min; signal stabilisation was reached after 180 min for all the water samples. Some authors indicated that FRAP redox reactions proceeded so rapidly that all reactions were complete within 4 and 6 min, but in fact this is not always true. FRAP results can vary tremendously, depending on the time scale of analysis. Pulido observed that dietary polyphenols react more slowly and require longer reaction times (P30 min) for total quantification, and depending on the analysis time, the order of their reactivity was changed (Pulido et al., 2000). In this work the reduction of Fe(III) took at least 180 min.

TAC values obtained using FRAP method were between 3.18 and 19.42 μM for still water and 0.11–219 μM for sparkling water, considering 180 min for reaction time (Fig. 2). Still waters presented lower TACs than sparkling waters. The highest values were obtained in sparkling waters of brand G that were added of vitamin C. As expected, natural waters had the lowest TAC values sometimes below the LOD of the method. Lemon flavour produced the highest TAC values for all brands (exception for brand B). This

Table 2
Label information in flavoured water.

Water	Ascorbic acid (labelled)	Flavour	Preservatives	Acidifying regulators	Sweeteners
A1	–	Lemon	Potassium sorbate	Citric acid	Acesulfame-K
A2	–	Mango	Sodium benzoate	Sodium citrate	
A3	–	Strawberry			
	–				
B1	–	Pineapple/orange	Potassium sorbate	Citric acid	Acesulfame K
B2	–	Lemon			Aspartame
	–				
C1	–	Lemon/magnesium	Potassium sorbate Dimethyl dicarbonate	Citric acid	
C2	–	Apple/white tea	Potassium sorbate		
C3	–	Pineapple/fibre	Potassium sorbate Dimethyl dicarbonate		
	–				
D1	–	Apple	Sodium benzoate	Citric acid	Sucralose
D2	–	Orange/peach	Dimethyl dicarbonate		Acesulfame-K
D3	–	Lemon			
	–				
E1	–	Lemon	Potassium sorbate	Citric acid	Acesulfame K
E2	–	Orange/raspberry	Sodium benzoate	Sodium citrate	Aspartame
E3	–	Peach/pineapple			
E4	–	Guava/lime			
	–				
F1	–	Lemon/green tea		Citric acid	
F2	–	Raspberry/ginseng			
F3	–	Peach/white tea			
F4	–	Mango/gingko beloba			
F5	–	Melon/mint			
	–				
G1	12 mg/250 mL	Lemon	Potassium sorbate	Citric acid	Sucralose
G2		Lime			Acesulfame-K
G3	12 mg/250 mL	Apple	Potassium sorbate	Citric acid	
G4	12 mg/250 mL	Peach	Potassium sorbate	Citric acid	
	–				
H1	30 mg/250 mL	Lemon	Sodium benzoate Potassium sorbate	Citric acid	Aspartame
	–				
I1	–	Lemon	Sodium benzoate	Citric acid	Aspartame
I2	–	Green apple			Sucralose
I3	–	Strawberry			Aspartame
	–				
J1	–	Lemon	Potassium sorbate	Citric Acid Sodium citrate	Aspartame Acesulfame-k

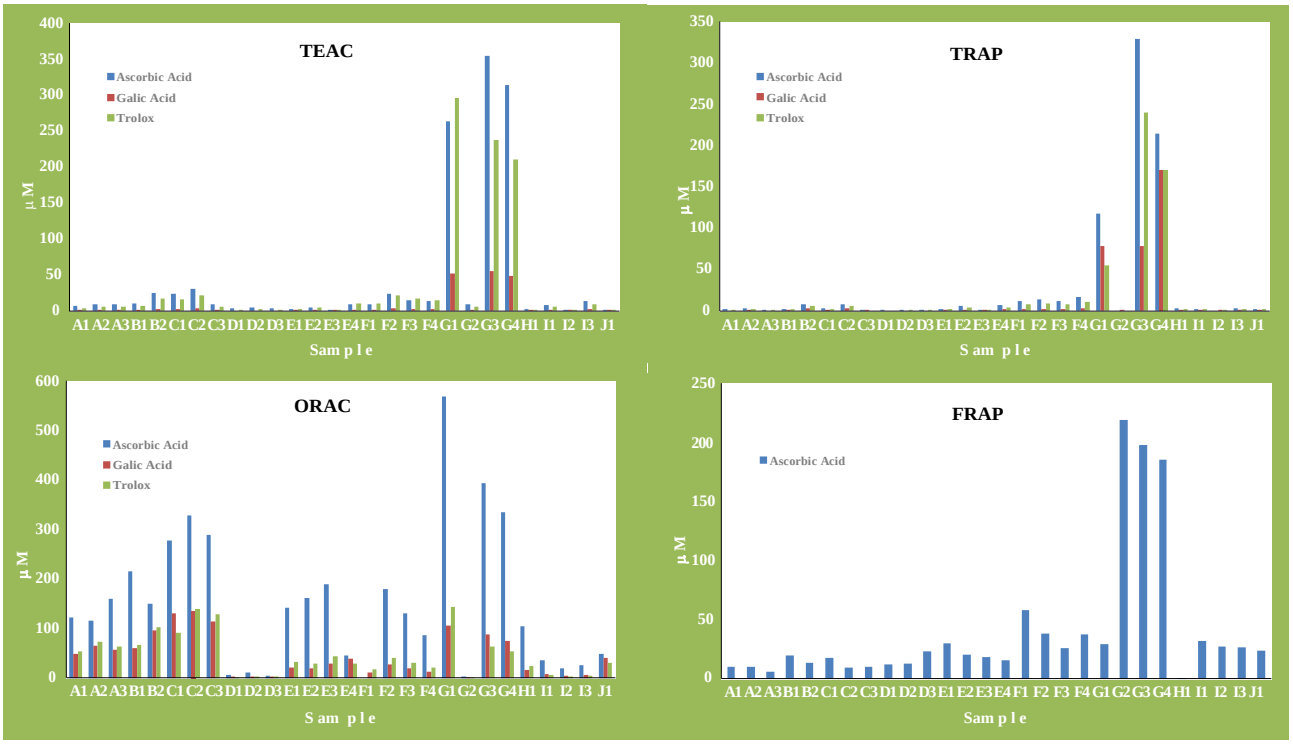

Fig. 2. TAC by TEAC, ORAC and TRAP methods expressed in 1M of ascorbic acid or gallic acid or trolox.

suggested that the addition of flavour and other ingredients to water increased their antioxidant content. According to labelled information, the following compounds were able to increase the TAC in the sample: ascorbic acid, citric acid, flavour, fruit juice and bioactive compounds. However, false positive results may occur by the concomitant production of Fe(II) by means of reducing compounds co-existing in the sample. In addition, compounds that absorb at the wavelength of the determination may interfere, causing overestimation of the FRAP value.

TAC values obtained using TEAC methods for all water samples are presented in Fig. 2. Thermodynamically, any compound that has a redox potential lower than that of $ABTS^{+}$ may react with the radical (Sánchez-Moreno, 2002). As the results obtained are related to each standard antioxidant and each antioxidant showed a specific kinetic behaviour, the results provided by this assay depend on the time of analysis. Since the experiments are carried out always for the same time, it is expectable that each sample has different TAC, according to the antioxidant used as standard. These compounds may be ascorbic acid, citric acid, flavours, fruit juice and bioactive compounds.

Generally, the addition of flavours increased TAC values of water samples, when compared to the natural ones. TAC values were higher when ascorbic acid was used. TAC values ranged from 0.05 to 354, 0.03 to 55 and 0.25 to 296 1M using ascorbic acid, gallic acid and trolox standards, respectively. Natural waters had the lowest TAC values and some of these were below LOD. TAC values obtained in flavoured waters were similar with exception of those of brand G. As observed on FRAP method, TEAC values were higher in brand G for the three antioxidant standards, this could have been a result of the addition of vitamin C.

Flavoured water samples with white tea, ginseng or gingko beloba as ingredients (from brands C and F) have TAC values higher than other samples. Indeed there are some reports in literature indicating that tea has high antioxidant levels due to catechin components (Coyle, Philips, Morrisroe, Chancellor, & Yoshimura, 2008). In general terms, the observed differences may outcome from the sample background and from the kinetics of the standard reaction. It is clear that flavoured waters have more TAC than non-flavoured ones, although the observed differences may not be attributed to a single compound or flavour. In addition, it is important to refer that this method has been criticised as the $ABTS^{+}$ radical is not representative of biomolecules and not even found in any biological system. However, this spectrophotometric assay is technically simple, which accounts for its application for screening and routine determinations.

The ORAC assay is one of the most common methods for assessing $ROO^{\bullet}$ scavenging capacity. Because the protective effect of antioxidants is evaluated from the AUC, this method can be applied for antioxidants that exhibit distinct lag phases and also to those samples that have no lag phases accounting for kinetic differences within samples and standard. It takes into account the initial reaction rate and the total extent of inhibition, which includes the action of slow-reacting or secondary antioxidant products formed.

Fig. 2 displays the TAC contents in waters using ORAC method. Generally, TAC values were higher when ascorbic acid was used as calibration standard. The highest TAC value, like in the previously referred methods, was obtained in waters from brand G (for every antioxidant standards).

TAC values of still flavoured waters using ORAC method ranged from 0.55 to 328, 0.52 to 136, 0.17 to 138 1M using ascorbic acid, gallic acid and trolox, respectively. Brand D had the lowest TAC results: the corresponding sample had no vitamins, preservatives, acidifying regulators and sweeteners.

ORAC results in sparkling flavoured waters expressed in ascorbic acid, gallic acid and trolox ranged from 2.0 to 569, 0.5 to 105 and 0.2 to 144 1M, respectively. As previously reported to other methods, the natural water samples did not display significant antioxidant capacity, and the addition of some ingredients to the water increased their TAC values. In addition, the highest TACs were obtained for samples with vitamin C. Other samples with high antioxidant capacity values contained vitamins of the B complex, which could have contributed for a TAC increase.

TRAP method is the simplest one in terms of laboratorial experiment. TRAP results are presented in Fig. 2, and ranged from 0.05 to 8.37 1M in still waters and 0.13 to 329 1M in sparkling waters.

Generally, still waters presented the lowest TRAP values when compared with the sparkling ones. When gallic acid was used for calibration, brand D displayed antioxidant capacities below the LOD of the method. In sparkling flavoured water, the highest values were obtained with samples with vitamina C from brand G. Natural waters did not show antioxidant capacity.

3.4. Statistical evaluation

Several factors influence the TAC values of flavoured waters, such as, method and standard, ingredients added to the water (flavour, vitamins, and preservatives) and commercial brand of the water. Their influence in the TAC level will be appreciated individually, facing the inherent limitations of a small sampling number (as commercially available). It is important to mention that tests made on waters of controlled chemical composition would be essential to confirm the statistical data presented below.

Considering the analytical method and analysing Fig. 2, the lower TAC values were obtained in TRAP method and the higher ones were provided by ORAC method. This behaviour was generally in agreement with Pulido and Prior (Prior et al., 2005; Pulido, Bravo, & Saura-Calixto, 2000). Considering each standard separately, the Levene's test for equality of variances indicated that TAC values of each method were statistically different ($P < 0.05$). The only exception was TRAP method with trolox as standard ($P > 0.05$). The observed lack of correlation within all methods is also in agreement with Ou et al. (2002); that analysed freeze-dried vegetable samples and the results indicated that the FRAP and ORAC values did not correlate well. The same statistic behaviour was obtained in this work. The observed differences may be attributed to the differences in reaction mechanisms, oxidant and target/probe species, and reaction conditions for all TAC assays.

Regarding standards, ascorbic acid standard produced generally higher values than the other standards and gallic acid the lowest ones. SPSS test analysis also indicated that TAC values from different antioxidant standards were found statistically different ($P < 0.05$).

Flavours are added to plain water in order to attribute specific taste and smell. Technologically, flavours are added as fruit extracts, and fruits are rich in antioxidants. Typically, vitamin C is present in almost all fruits such as citrus, strawberry, pineapple; carotenoids in citrus fruit; tocopherol (vitamin E) in raspberry, melon and peach; and flavonoids in red fruit and nearly in all fruits. In this work, flavoured waters presented higher TAC levels when compared with the corresponding natural ones due to the increase of antioxidants by means of fruit extracts addition. One-way ANOVA for 95% level of significance confirmed that the presence of flavour influenced the TAC of the analysed samples ($P < 0.05$). TAC values from natural and flavoured waters obtained by TRAP method were not statistically different ($P > 0.05$), suggesting that this method maybe unsuitable for the present study.

Generally, higher TAC contents were obtained in apple, peach and lemon flavoured waters and lower values in guava and raspberry flavours. This behaviour was similar for all methods and antioxidant standards. Statistical analysis of flavour factor against TAC methods indicated however no statistical difference between all flavours ($P > 0.05$). This observation was most probably a result of the strong variability amongst all methods for each flavour.

Eleven flavoured water samples had vitamins C and B complex. Vitamin C was present in 4 samples, and vitamins of the B complex in 7 flavoured water samples; different kinds of vitamins B were added to the commercial water: either a mixture of B_3 and B_{12} (4 samples); or a blend of B_3, B_5, B_6, B_8, B_9, B_{12} (3 samples).

Both vitamins C and B have antioxidant properties. Therefore, samples with these compounds should have higher TAC. Indeed, samples with vitamins C showed the highest TAC values, reaching 221, 314, 569, 329 1M in FRAP, TEAC, ORAC and TRAP assays, respectively. The maximum TAC values for vitamins B were 30, 31, 328 and 8 1M, respectively. There is a strong variability between these samples and some of them have low TAC. This wide range of TAC values points out no significant differences between samples with and without vitamins.

However, a straight comparison between samples where only vitamins are the difference in composition indicates otherwise. These results point out that vitamin produced a large increase on the antioxidant. This behaviour was obtained in all methods and all antioxidant standards. Statistical evaluation confirmed that the presence of vitamins influenced with 95% level of significance the TAC values of the flavoured waters ($P < 0.05$).

Preservatives are added to foodstuffs to preserve them. These compounds are not typically considered as antioxidant, for which it is not expected that their presence would change TAC values of samples. This assumption was confirmed statistically, either by grouping all samples or by comparing samples of similar background. Therefore, no statistical differences were observed in TAC values regarding the preservatives added to the flavoured waters ($P > 0.05$).

In the consumer's perspective, the brand is most probably the most important factor, leading to the selection of a specific product. Combining all results obtained, brand G seems to provide the highest antioxidant contents. Despite the corresponding waters show the higher TAC, this is most probably correlated to the vitamin C addition, and not to the brand itself. Confirming this, the only flavoured water from this brand that has no vitamins displays very low TAC. This sample is the responsible for three wide ranges of TAC values for this brand. In practical terms, this wide range of TAC values tends to eliminate the statistical differences between the commercial brands. Other brands have only single commercial flavoured water, providing no significance to their effect upon this study.

4. Conclusions

Flavours in waters had higher TAC than the natural ones from which they were obtained. The magnitude of this increase depended however on the method/standard used to estimate the TAC. Apple, pineapple and lemon waters provided in general the highest TAC values and guava and raspberry the lowest. There was however no statistical differences observed due to the strong variability between similar samples. The addition of vitamin C has been correlated to the highest increase in TAC. This has been confirmed statistically. The brand and the preservatives in the samples seem to display insignificant effects. In practical terms, it may be said that the additives in general are responsible for the TAC of flavoured waters, attributing vitamin C the main role for an increase in TAC. This information would be better confirmed if applied to a higher number of samples, but these are not commercially available.

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