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Antioxidant power quantification of decoction and cold infusions of *Hibiscus sabdariffa* flowers

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Abstract

In this paper the overall antioxidant power, expressed as Briggs–Rauscher antioxidant index, of decoction or cold infusions of dried *Hibiscus sabdariffa* flowers was determined at 25 and 37 °C, to compare the scavenger ability of the beverages at either room or physiological temperature. Total polyphenol contents and the absorbance of anthocyanin pigments were also determined, and the trend with the overall antioxidant capability is considered. Combined photometric and pH-metric titrations were acquired to obtain information on the colour-total acidity relationship of the product. The results show that the decoction preparation protocol provides *karkadè* with the highest nutritional value and that the polyphenol content can account for the antioxidant capability of *H. sabdariffa*-based beverages. Moreover, a quantitative relationship between acid–base and redox chemistry was found. The *H. sabdariffa*-based drinks can be considered as protective beverages and a regular consumption of *karkadè* might be proposed to ensure protection against free radicals.

Keywords: Anthocyanidins; Antioxidant power; Free radicals; *Hibiscus sabdariffa*; *Karkadè*; Redox chemistry

1. Introduction

The oxidative cellular stress, mainly caused by free radicals, is an ascertained cause of the degenerative pathologies and many strategies dedicated to its prevention are at present under study, particularly those related to food and nutritional supplements. Nutrition has been recognised as fundamental to prevent oxidative stress and a quantitative indication of the redox ability of food and beverages to scavenge physiological radicals is of primary importance for the quality of life.

Phenolic substances are the most common phytochemicals in fruits and vegetables (Rhodes, 1996) and polyphenols are known as potent antioxidants and natural antagonists of plant pathogens. Polyphenols show scavenger ability towards physiological free radicals. Most of them are classified in two principal groups: phenol carboxylic acids and flavonoids. The last are the most significant (Bitsch, 1996) and derive from 2-phenyl-benzopyran. The main subgroups are the colourless catechins and proanthocyanidins, the red to blue-coloured anthocyanidins and the light-yellow flavonols and flavones (Herrmann, 1994). Food products, such as fruit juices and vegetables, or dietary supplements, such as vitamins, are frequently employed for their preventive action because of the immediate effect on the redox status of the body fluids. The antioxidant activity of vegetable phenolic substances may also exceed that of the antioxidant vitamins C and E. Concentrates of red grapes, cherries and black currants show the highest efficiency of all concentrates currently analysed (Rice-Evans, Miller, & Pagana, 1997).

The *Hibiscus sabdariffa* flowers (family: Malvaceae) provide a soft drink – usually named *karkadè* or red tea – highly appreciated all over the world for the particular sensation of freshness conveyed. *H. sabdariffa*-based products are used in popular medicine to obtain an

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anti-hypertensive effect (Onyenekwe, Ajani, Ameh, & Gamaniel, 1999) as well as to prevent cardiovascular and hepatic diseases (Ali, Mousa, & El-Mougy, 2003). The *H. sabdariffa* petals are potentially a good source of antioxidant agents as anthocyanins (Ali et al., 2003) and ascorbic acid (Reaubourg & Monceaux, 1940). Studies (Chang-Che et al., 2004; Suboh, Bilto, & Aburjai, 2004) show how *karkadè* may be effective against low-density lipoprotein oxidation and hyperlipidemia.

In this paper, experiments were carried out in order to evaluate the effect of infusion time, temperature and ethanol on the extraction efficiency, and the influence of temperature on the nutritional status of the drink. The overall antioxidant power of either decoction or cold infusions of *H. sabdariffa* powdered flowers were determined at 25 and 37 °C, to compare the scavenger ability of the beverages at either room or physiological temperature. The experimental procedure, based on the kinetic response obtained by an oscillating reaction, is fully described by Prenesti, Toso, and Berto (2005). Total polyphenol contents (Folin–Ciocalteu reaction) and the absorbance of anthocyanin pigments (520 nm) were also determined, and the trend with the overall antioxidant capability examined.

Finally, a screening of the acid–base characteristics of the beverages is also proposed. It is well known that the acidity may contribute to the bacteriostatic effect and can support metal ion absorption into the organism, particularly acting against low-soluble salts formation. Furthermore, the sensation of freshness perceptible when drinking cold *karkadè* is very probably related to the acidity. Combined visible spectrophotometric spectra and pH-metric titrations were then also acquired to obtain information on the colour – total acidity relationship of the product, according to our previous findings on red wine (Prenesti et al., 2005).

2. Materials and methods

2.1. Chemicals

Folin–Ciocalteu reagent, ethanol (>99.8% v/v), hydrogen peroxide (30% w/w), malonic acid (>99%) and gallic acid (>98%) were from Fluka (Buchs, SG, Schweiz). Salts KCl and KIO_3, from VWR International (Milano, Italy), and $MnSO_4 \cdot H_2O$ was from Carlo Erba (Milano, Italy). Standard NaOH, KOH and HCl solutions were prepared by diluting Fluka concentrate products and standardised against potassium hydrogenphthalate (Fluka, puriss.) or sodium carbonate (Fluka, puriss.), respectively. All solutions were prepared using grade A glassware and deionised plus twice-distilled water.

2.2. Sample preparation

The dried *H. sabdariffa* calices, coming from Egypt, were gently powdered with a homogeniser (model Blendor, from Waring, Torrington CT, USA). The amount of powder used for each aqueous extraction corresponds to the typical mass of herbs found in the commercial bags for home-made tisanes. We prepared a decoction, boiling 2.000 g of petals at 100 °C for 3 min with about 100 ml of twice-distilled water, and a series of cold infusions characterised by various infusion times. Different samples were prepared, leaving 2.000 g of petals in 100 ml of cold twice-distilled water for 5, 30, 180, 540 or 930 min, without stirring. Besides, in order to verify the effect of alcohol on the extraction efficiency, a 30 min cold infusion was prepared by the addition of 12% v/v of ethanol to the extractive solution. The amount of ethanol was chosen to simulate the average percentage of alcohol in table wine. Extraction conditions are provided in Table 1. Each extract was rapidly filtered through a Buchner funnel, and filled according to the calibrated volume flask (100 ml). The solutions were stored at 4 °C and analysed within three days after preparation.

2.3. Overall antioxidant power measurement

The method of measurement used in this paper, previously developed by our group on red wine (Prenesti et al., 2005), starting with studies by Cervellati, Honer, Furrow, Neddens, and Costa (2001, 2000), allows quantification of the overall antioxidant power of a fluid. The Briggs–Rauscher oscillating reaction (henceforth: BR) (Briggs & Rauscher, 1973) generates hydroperoxyl radicals and supplies a synthetic environment able to simulate natural pro-oxidant conditions. A reference scale, based on the response of gallic acid, was considered to achieve a quantitative response and the "Briggs–Rauscher Antioxidant Index", BRAI, was defined (Prenesti et al., 2005) to express, quantitatively, the overall antioxidant power. Gallic acid was chosen as a standard phenolic substance also to simplify the comparison with the total polyphenol content, expressed as units of gallic acid equivalents.

As in the previous work, the BRAI was determined at 25 and 37 °C.

In order to obtain suitable inhibition time values, the volumes of undiluted *karkadè* solution added to the BR mixture, freshly prepared, were from 0.060 to 0.150 ml, for the measurement at 25 °C, and from 0.160 to 0.300 ml, for the measurement at 37 °C.

The inhibition times were measured potentiometrically with a platinum electrode (see "*Potentiometric apparatus*") as well as by direct chronometric detections; as previously found (Prenesti et al., 2005), chronometric and potentiometric devices exhibited equivalent accuracy in measuring the inhibition time. A chronometer (Oregon Scientific, Agrate Brianza, MI, Italy, mod. SL888T) was then used. The temperature control was achieved by means of a circulation of water around the reaction vessel, from a thermo cryostat (model DI-G Haake). Each reactant was maintained at a controlled temperature before and during the experiment. Each measurement of the inhibition time was repeated at least three times.

Table 1
Extraction conditions, absorbance values at 520 nm (dilution 1/10 v/v), total polyphenols content (GAE) and Briggs–Rauscher antioxidant index (BRAI) for each *karkadè* solution under investigation

Sample name	Extraction time (min)	A_{520}	GAE[c]		BRAI[d]			
					25 °C		37 °C	
1	5	0.422	25.3[e]	12.7[f]	197[e]	99[f]	461[e]	231[f]
2	30	0.458	28.5	14.2	265	132	571	285
3	180	0.478	31.0	15.5	298	149	522	261
4	540	0.477	32.4	16.2	–	–	–	–
5	930	0.458	29.2	14.6	–	–	–	–
6	30[a]	0.446	27.9	13.9	–	–	–	–
7	3[b]	0.612	39.1	19.6	320	160	665	332

Uncertainty in BRAI at 25 °C ranges between 12.7% and 18.1% while, at 37 °C, it ranges between 9.5% and 17.1%
[a] 12% (v/v) Ethanol in the extraction solution.
[b] Extraction temperature: 100 °C (decoction).
[c] Total polyphenols content expressed as gallic acid equivalent.
[d] Overall antioxidant power expressed as Briggs–Rauscher antioxidant index.
[e] $mg_{gallic\ acid}/100\ ml_{karkadè}$.
[f] $mg_{gallic\ acid}/g_{petals}$.

2.4. Potentiometric apparatus

pH-metric measurements were carried out at $T = 25 \pm 0.1$ °C with a Metrohm 713 potentiometer equipped with a combined glass electrode (Metrohm, Herisau, Switzerland, mod. 6.0204.100). The titrant was dispensed with a 765 Dosimat burette from Metrohm (minimum volume deliverable equal to 0.001 cm^3). The alkalimetric titrations were carried out in a stream of purified nitrogen gently bubbled in the titration cell to avoid O_2 and CO_2 contamination. The temperature control was achieved as described in the previous section. Each titration was repeated at least twice. The alkalimetric titrations were carried out on *karkadè* solutions diluted 1/5 v/v with water, and utilising standardised 0.2 M KOH as titrant. A combined platinum electrode (Metrohm mod. 6.0402.100 (LE)) was used to monitor, potentiometrically, the kinetic trend of the oscillating reaction together with a Metrohm automatic computer-assisted potentiometric apparatus (Basic Titrino 794).

2.5. Spectrophotometric apparatus

The visible spectrophotometric determinations were carried out with a Jasco V-550 UV/VIS double beam spectrophotometer (optical path length 1.000 cm). As for coupled photometric/volumetric measurements of *karkadè*, the examined solution was transferred from the potentiometric to the optical cell using a peristaltic pump, in order to record visible spectra, or absorbance values at a fixed wavelength, as a function of the pH value of the solution, thus strongly reducing equilibrium inconveniences.

3. Results

3.1. Spectrophotometric determinations of total polyphenols

The total polyphenol content was determined by means of the Folin–Ciocalteu method (Ough & Amerine, 1988). Gallic acid was chosen as standard molecule for the calibration. The working curve was constructed at 752 nm, according to the position of the experimental maximum of absorbance. The reaction was carried out on *karkadè* solutions diluted 1/100 v/v. The total polyphenol content was expressed using the gallic acid equivalents (GAE) value: mg of gallic acid *per* 100 ml of *karkadè* solution, or mg of gallic acid *per* gramme of dry petals of *H. sabdariffa*. The results are shown in Table 1.

3.2. Overall antioxidant power at 25 and 37 °C

The overall antioxidant power of *karkadè* was expressed by means of the Briggs–Rauscher antioxidant index, BRAI. In this case, the BRAI value can be referred to a fixed volume of beverage, obtained under a specific preparation control, or to dry weight of *H. sabdariffa* petals.

The gallic acid calibration details are collected in our previous work (Prenesti et al., 2005); Table 2 shows the parameters of the straight-line equations of gallic acid useful for current application. A series of kinetic

Table 2
Parameters of the straight-line equations utilised for the calculation of the Briggs–Rauscher antioxidant index (BRAI) of some *karkadè* samples, at 25 and 37 °C

	Analyses temperature (°C)	Slope ($\pm s$)	Intercept	R
Gallic acid[a]	25	667 ± 55	−1.3	0.9901
	37	77 ± 6	30.7	0.9945
Sample 1	25	1315 ± 60	−10.7	0.9979
	37	355 ± 34	−31.3	0.9909
Sample 2	25	1765 ± 176	−62.0	0.9902
	37	439 ± 36	−36.3	0.9932
Sample 3	25	1991 ± 101	−55.5	0.9974
	37	401 ± 25	−22.0	0.9961
Sample 7	25	2134 ± 105	−27.6	0.9976
	37	511 ± 10	−34.3	0.9996

[a] From previous work (Prenesti et al., 2005).

measurements was randomly repeated to verify the repeatability of the calibration.

The sample volume added to BR-mixture and the corresponding inhibition time value are proportional dimensions and, under our experimental conditions, an excellent linear trend was found for each *karkadè* solution; results are in Fig. 1. The straight-line parameters of each sample are collected in Table 2. Kinetic analyses were carried out on selected samples, taking into account the polyphenol contents and the infusion time.

The Briggs–Rauscher antioxidant index is calculated as the ratio of the slopes of the kinetic straight-line equations obtained for each sample (slope $= t_{\text{inhib}}./\text{ml}_{\text{karkadè}}$) and for the standard molecule (slope $= t_{\text{inhib}}./\text{mg}_{\text{gallic acid}}$). The ratio of slopes thus obtained, $\text{mg}_{\text{gallic acid}}/\text{ml}_{\text{karkadè}}$, was finally referred to 100 ml of beverage, or per gramme of dry petals of *H. sabdariffa*. The results are collected in Table 1. Moreover, based on standard deviation ($\pm s$) of the gallic acid and *karkadè* straight-line slopes, we estimated the following mean uncertainty values for BRAI: 15.0% at 25 °C and 14.1% at 37 °C. Values higher than those obtained for red wines (Prenesti et al., 2005) include little variability caused by the preparation step.

3.3. Colour-acidity relation and photometric estimation of the total acidity

The colour of beverages containing polyphenols, such as *karkadè*, tea, blueberry juice or wine depends on pH value. In this paragraph, as for wine (Prenesti et al., 2005), a relationship between colour and pH is investigated via coupled pH-metric and photometric measurements. We have also recorded the spectrum of *karkadè* solutions (dilution 1/10 v/v), in the range 250–750 nm, in order to acquire the absorbance values at 520 nm (see Table 1). This absorbance value is related to the polyphenols content since the peak at 520 nm is characteristic of anthocyanin pigments.

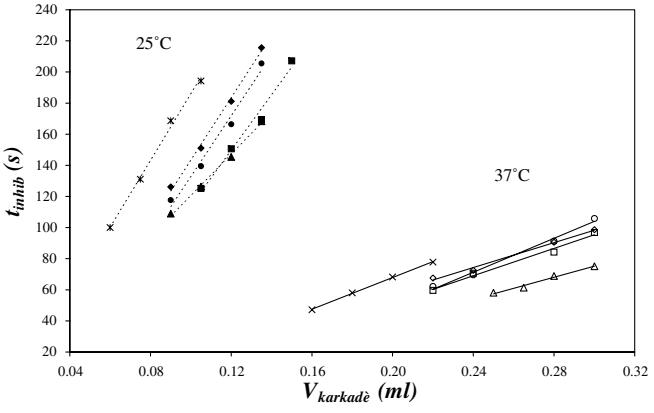

Fig. 1. Kinetic test (BR reaction) of each *karkadè* solution: plot t_{inhib}. (s) vs. $V_{\text{karkadè}}$ (ml) with linear fittings, at 25 °C (symbols: ∗, for sample 7; ▲, for sample 1; ■, for sample 2; ◆, for sample 3; ●, for sample 6) and 37 °C (symbols: ×, for sample 7; △, for sample 1; □, for sample 2; ◇, for sample 3; ○, for sample 6).

The UV–Vis absorption spectrum of *karkadè* solutions exhibits absorption maxima around 520, 330 and 280 nm. With increasing pH of beverage, the spectrum changes are as shown in Fig. 2. The colour of solution changes from red to blue-green and the most significant modifications in absorption features are found around the inflection point (pH ∼ 7.0) of the alkalimetric titration. Absorbance value at 520 nm decreases regularly before reaching the inflection point (see Fig. 3) while, in alkaline conditions, absorbance values at 600 and 365 nm increase with pH. The plot A_{520}/pH (see Fig. 3) shows the dependence of photometric behaviour of anthocyanidins pigments on pH conditions.

Total acidity of *karkadè* solutions was obtained both from potentiometric and from photometric measurements, as for wine (Prenesti et al., 2005). The acid/base titrations were achieved on two *karkadè* samples, one obtained by infusion (sample 2), and one by decoction (sample 7). The total acidity values obtained by the plots absorbance/titrant volume at 600 and 365 nm are in fair agreement with the results obtained potentiometrically. The pH and acidity data are shown in Table 3 and an example of absorbance/titrant volume diagram is shown in Fig. 4.

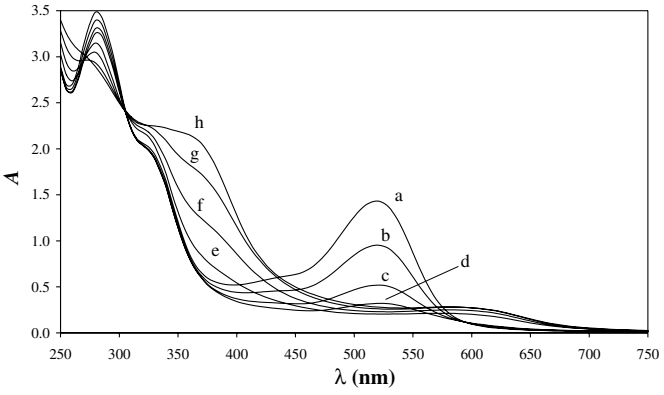

Fig. 2. UV–Vis absorption spectra of diluted sample 7 (1/5 v/v): (a) pH 2.76; (b) pH 3.17; (c) pH 3.68; (d) pH 4.14; (e) pH 7.12; (f) pH 7.79; (g) pH 8.20; (h) pH 8.46.

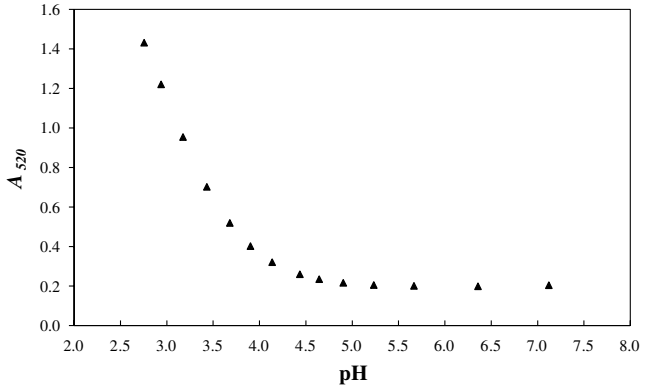

Fig. 3. Trend of absorbance values at 520 nm during alkalimetric titration of a *karkadè* solution (sample 7 diluted 1/5 v/v). Titrant: standardised 0.2 M KOH.

Table 3
Total acidity evaluation in *karkadè* solutions by pH-metric ($pH/V_{titrant}$) or photometric ($A/V_{titrant}$) detection

Sample	pH	Total acidity (mM)		
		pH-metric detection	Photometric detection	
			365 nm[a]	600 nm[a]
2	2.75	48.5	50.5	45.1
7	2.76	50.9	50.4	48.0

[a] Detection wavelength.

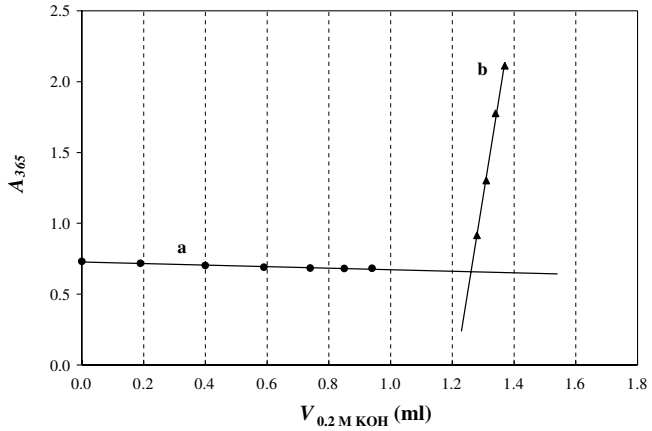

Fig. 4. Estimation of the total acidity. Plot A_{365} vs. $V_{titrant}$: photometric detection during the alkalimetric titration (titrant 0.2 M KOH) of sample 7 (diluted 1/5 v/v). Linear fittings: ●, $y = -0.0548x + 0.7274$ ($R = 0.9781$); ▲, $y = 13.54x - 16.414$ ($R = 0.9980$).

The absorbance values at 520 nm were not utilised for acidity quantification because, past the inflection point, the signal is spoiled owing to the overlapping of next peak tails.

4. Discussion

4.1. Polyphenols and overall antioxidant power

The results collected in Table 1 show that the temperature of extraction plays an important role in the polyphenols content of *karkadè* beverages. The decoction provides the highest polyphenols content and the highest antioxidant power. This means that hot water leads to more efficient extraction (though for only 3 min of contact) and does not damage the antioxidant ability of the phenolic molecules. Results obtained on cold infusions show that the extraction efficiency is related to the infusion time. Little difference of GAE is recorded between the infusions obtained in 30 or 180 min. Moreover, the contribution of ethanol to the extraction power is negligible. Nevertheless the alcohol increases the storage time: in fact, after three days from preparation, only water/ethanol infusion does not decay, showing a longer life time than the simple water infusions.

During our study on red wine (Prenesti et al., 2005), we found that $BRAI_{37} > BRAI_{25}$, correctly indicating (overall antioxidant power)$_{37}$ < (overall antioxidant power)$_{25}$. The kinetic activity of the radicals formed in the test environment is higher at 37 °C and, as a consequence, we found that the antioxidant ability decreased. With the antioxidant activity lowered at 37 °C, more gallic acid, with respect to the amount required at 25 °C, must be used to stop the radical reactivity, under equal inhibition time conditions.

It is now interesting to evaluate the relationship between the different data relating to polyphenol molecules. The values of GAE are closely correlated with the absorbance values at 520 nm, as expected: the correlation coefficient R calculated between the two quantities is 0.971. In Fig. 4 a graphical representation of the connection between BRAI (25 °C) and GAE values is furnished. The two quantities are closely related, though the BRAI is an evaluation of activity while the GAE is an evaluation of concentration. Therefore, it is reasonable to suppose that the antioxidant power of the beverage is exclusively related to total polyphenols content (see Fig. 5). The presence of ascorbic acid in *karkadè* drinks can be considered negligible with regard to the antioxidant ability; on the other hand, literature reports indicate low concentration values, from 100 to 40 mg of ascorbic acid for 100 g of dried material, (Reaubourg & Monceaux, 1940).

4.2. Acidity and colour

The total acidity data (see Table 3) show that the extraction conditions do not significantly affect the acid content of *karkadè*, probably because acids are easily extractable (rather than polyphenols) in aqueous solution. The two samples analysed, the decoction (sample 7) and the 30 min cold infusion (sample 2), show comparable total acidity values and pH. The photometric trend of *karkadè* solution with varying pH confirms the data obtained with red wines (Prenesti et al., 2005). In the polyphenol containing beverages, acid–base and redox chemistry are related: the acidity plays a protective role in the phenolic molecules, thus preserving both colour and antioxidant power. Moreover, the quoted relationship may be considered in a quantitative fashion: comparing data obtained from pH-metric and photometric titrations (see details in Table 3), the

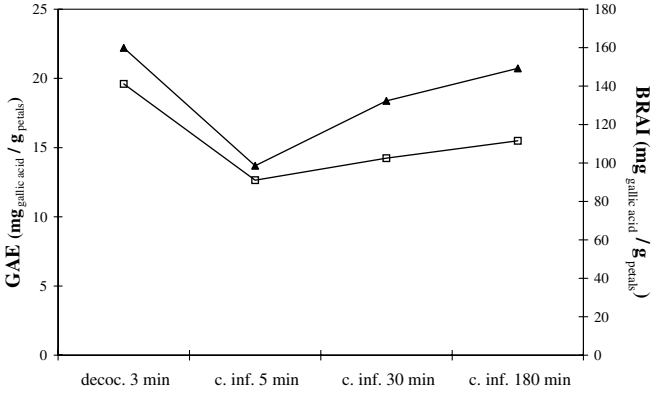

Fig. 5. GAE (symbol: □) and BRAI (symbol: ▲) values of four *karkadè* samples: the decoction (decoc.) and three cold infusions (c. inf.) with extraction times of 5, 30 and 180 min.

agreement is fairly satisfactory: the greatest discrepancy observed (sample 2, photometric detection at 600 nm) was 7%, assuming, as reference, the pH-metric equivalent volume. Accuracy of the comparison is close to what found for red wine (Prenesti et al., 2005).

5. Conclusions

This paper identifies a suitable recipe in order to obtain a *karkadè* with high nutritional status and shows that, on the basis of the correlation between BRAI and GAE values, the polyphenol content can account for the antioxidant capability of *H. sabdariffa*-based beverages. Comparing the BRAI values of *karkadè* solutions with those previously obtained for some Italian table red wines (Prenesti et al., 2005), it is clear that red wine provides higher antioxidant activity. However, we can describe *H. sabdariffa*-based drinks as protective beverages and a regular consumption of *karkadè* might be proposed as an alternative to red wine (Renaud & de Lorgeril, 1992) for those persons who cannot deal with ethanol and for children. Moreover, in summer, people are strongly dissuaded from consumption of ethanol-based beverages; alternative protective drinks are hence suitable to ensure the organism a food contribution against sunlight-induced free radicals. Cold infusion is, moreover, able to preserve vitamin C of *H. sabdariffa*, which is a useful partner of polyphenols in their angio-protective activity.

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