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Antioxidant capacity of some caramel-containing soft drinks

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ABSTRACT

Antioxidant properties of foods and beverages have been widely studied; however, few data have been reported on the antioxidant capacity of soft drinks. Apart from fruit juice-based drinks, some of the most common soft drinks contain as a colouring agent one of the four caramel colours allowed in foods (E150 d). Caramels contain melanoidin compounds, which have been reported to contribute to the antioxidant powers of some foodstuff. This study aimed to ascertain the contribution to the antioxidant activity of some caramel-containing soft drinks, such as cola drinks, and chinotto, an original Italian soft drink. Some commercial caramel colours were analysed for main parameters, i.e. HMF (5-(hydroxymethyl)-2-furfural), residual glucose and fructose content, total reducing compounds by the Folin–Ciocalteau reagent, and the antioxidant activity by the FRAP and DPPH® methods. Similar analyses were performed on various soft drinks coloured with E150 d. The results showed that even if soft drinks have a lower antioxidant activity than other beverages such as tea, coffee or chocolate, they may contribute to the antioxidant pool assumed with the diet, since the antioxidant activity ranged from 0.2 for cola-like soft drinks to 1.0 mmoles Trolox equivalent/l for chinotto drinks.

1. Introduction

Recent studies have widely demonstrated that a diet rich in antioxidants can protect from a variety of degenerative pathologies (Block, Patterson, & Subar, 1992; Kinsella, Frankel, German, & Kanner, 1993; Renaud & de Lorgeril, 1992). In particular, polyphenols have anti-inflammatory (Subbaramaiah et al., 1998), anti-carcinogenic (Kuroda & Hara, 1999), anti-atherogenic and cardioprotective (Dell'Agli, Buscialà, & Bosisio, 2004) effects, and also play a protective role towards brain degenerative processes (Conte, Pellegrini, & Tagliazucchi, 2003). Polyphenols are present in drinks derived from fruits such as wine and fruit juices, from malted seeds such as beer, from roasted seeds such as coffee and cocoa or from leaves such as tea. For example, in wines the amount of phenolic compounds (expressed as GAE, measured by the Folin–Ciocalteau reagent) can range from less than 500 mg/l in white wines to some g/l in red wines (Lee, Kim, Lee, & Lee, 2003). A lower level of phenolics, derived from cereal grains and added hops, was observed in dark and lager beers (Lugasi & Hóvári, 2003). A cup of espresso coffee (30 ml) contains 130–160 mg GAE [unpublished data]. Much higher levels of total phenolics (611 mg of GAE) are present in cocoa beverages (200 ml per serving); whilst the GAE content of tea varies from about 120 GAE for black tea to 165 for green tea (200 ml per serving) (Lee et al., 2003). Recently, the antioxidant power of some ready-to-drink polyphenol-rich beverages available in the United States has been reported (Seeram et al., 2008). With regard to cola-like drinks, recipes are generally covered; only two papers (Atawodi et al., 2007; Rababah, Hettiarachchy, & Horax, 2004) report a partial characterisation of African cola, and the reported phenolic content is very poor. Therefore, phenolics cannot be assumed to contribute to the antioxidant power of these drinks.

Most of the above-mentioned beverages suffer from some drawbacks as the alcoholic content in wine and beer or the presence of stimulant compounds, such as caffeine in coffee and tea and theobromine in chocolate drinks. Therefore, due to medical (children and pregnant women), practical (i.e. driving a car), or ethical–religious reasons, many consumers turn their attention to different beverages, such as the so-called "soft drinks". Concerning these kinds of beverages, most of them contain a rather high content of sugars, that contribute to energy intake and this fact must be taken into account too.

The aim of this study was to evaluate the antioxidant capacity of soft drinks coloured with caramel, such as the cola-type carbonated beverages, which are largely consumed around the world and do not have the main drawbacks of most of the above-mentioned beverages. Owing to their manufacture procedures, some caramels contain melanoidins that were reported to act as antioxidant (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002; Wang et al., 2007).

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2. Materials and methods

2.1. Materials

2.1.1. Samples

Four commercial caramel colours (E150 a, b, c and d) were kindly provided by SICNA S.r.l. (Cassina Nuova di Bollate, Milan, Italy). Eleven samples of caramel coloured soft drinks, three classic colas (A, B and C), three colas light (D, E and F), two colas without caffeine (G and H), one cola with lemon juice (I) and two soft drinks flavoured with chinotto (*Citrus myrtifolia*) (Italian law: DPR 19/5/1958, art. 5), were also analysed (chinottos A and B).

2.1.2. Chemicals

HPLC-grade methanol, glucose, fructose, sucrose, Folin–Ciocalteau reagent, Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tripyridyl-striazine), $FeCl_3 \cdot 6H_2O$, DPPH* (1,1-diphenyl-2-picrylhydrazyl radical) and HMF (5-(hydroxymethyl)-2-furfural) were purchased from Sigma–Aldrich Italia (Milano – Italy). All solutions were prepared using deionised water.

2.2. Methods

2.2.1. Dry weight for caramel colours

The analysis was carried out according to the AOAC 925,45 D method (AOAC, 1995, chap. 44).

2.2.2. Colour intensity EBC units

This parameter was determined according to the EBC procedure no. 6.8: colour of caramel (European Brewery Convention, Anaytica-EBC, 2004). The absorbance of all samples was read at 430 nm, after a suitable dilution, in 10 mm pathlength cells, using a Beckman DU 650 spectrophotometer.

2.2.3. Spectrophotometric parameters

The absorbance of caramel colours (0.1% w/v, dry matter) and undiluted soft drinks using deionised water as reference was determined in a 10 mm pathlength cell at 610, 560 and 280 nm; the ratio $R_{280/560}$ values were also calculated this being an important parameter to distinguish between caramel colours II and IV (Licht et al., 1992).

2.2.4. HPLC determination of HMF

This analysis was performed according to the method of Porretta and Sandei (1991). The HPLC system consisted of a Merck Hitachi L-7100 pump, a Merck Hitachi UV detector L-7400 (set at 280 nm) and Merck Hitachi integrator D-7500. A Spherisorb ODS2 column was used, equipped with a C18 pre-column. The flow rate was 1.2 ml/min and injection volume was 20 μl. Isocratic elution was carried out with 10% methanol in water. A calibration curve was obtained with different amounts of HMF standard. All samples were filtered through Waters HA 0.45 μm filters before injection. Results are expressed as g/kg dry matter (d.m.) for caramels and as mg/l for the analysed soft drinks.

2.2.5. HPLC determination of simple sugars

Glucose and fructose contents were determined essentially as described by Calull, Marsé, and Borrull (1992). The HPLC system used consisted of a Waters 600 E Multisolvent Delivery System, a RI detector (1037 A, Hewlett Packard), an Aminex HPX-87 H column (Bio–Rad, Richmond, USA) equipped with a Microguard Cation H^+ pre-column (Bio–Rad). Samples were eluted with 0.01 N sulphuric acid at a flow rate of 0.7 ml/min at 60 °C. Before injection (20 μl), samples were filtered through a 0.45 μm membrane (Waters HA). The amounts of glucose and fructose were calculated from the respective calibration curves and were expressed as g of sugar per litre of sample.

2.2.6. Total reducing substances content

These were determined using the Folin–Ciocalteau reagent (Singleton & Rossi, 1965). Briefly, 1.0 ml of appropriately diluted sample was mixed with a small amount of water and 1.0 ml of the Folin–Ciocalteau reagent; after ca. 4 min, 4 ml of 10% w/v sodium carbonate solution was added; the flask was then filled to the mark with distilled water. The mixture was incubated in the dark at room temperature for 90 min before reading the A_{750} nm using distilled water as the reference. Results were expressed as mg/l of gallic acid equivalents (GAE), a standard curve being prepared using pure gallic acid.

2.2.7. Ferric reducing antioxidant power (FRAP)

The procedure of Benzie and Strain (1996) was followed with some modifications. Briefly, 10 ml of freshly prepared reagent containing 10 mM TPTZ in 40 mM HCl, 20 mM $FeCl_3 \cdot 6H_2O$ and 300 mM acetate buffer, pH 3.6, in the ratio of 1:1:10 (v:v:v) were mixed with 330 μl of appropriately diluted sample. The mixture was allowed to stand for 60 min at room temperature before absorption was measured at 593 nm. Aqueous solutions of $FeSO_4 \cdot 6H_2O$ in the concentration range of 0.125–1.0 mM were used for calibration of the FRAP assay, and Trolox was used as a standard (in the range from 0.1 to 0.5 mM). FRAP values were expressed as mmol Trolox equivalents (TE) per kg dry matter for caramel colours and as mmol TE/l of sample for soft drinks.

2.2.8. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH*) assay

The antioxidant activity was measured by the DPPH* assay (Sanchez-Moreno, Larrauri, & Saura-Calixto, 1998), which measures the free radical scavenging capacity**.** Briefly, 30 μl of diluted sample was added to 5 ml of methanolic DPPH* solution (30 mg/l). After 30 min at 40 °C ($A_{t=30}$), the absorbance at 515 nm was measured using methanol as the reference. At least four different dilutions were assayed for each sample, in order to obtain a percentage inhibition between 20% and 80% (in our case, dilutions were in the range 1–5 fold). The antioxidant activity was expressed as TE by using Trolox as a standard for calibration.

2.3. Statistical analysis

All samples were analysed at least in triplicate, and data were reported as mean value ± standard deviation (SD). One-way ANOVA and the multiple range test were carried out using Statgraphics plus 5.1 (Graphics Software Systems, Rockville, USA). Multivariate analysis was performed applying the PCA (Principal Components Analysis), in order to discriminate samples on the basis of their analytical characteristics. Afterwards analytical data were correlated with the antioxidant capacity using the PLS algorithm (Partial Least Squares). PCA and PLS processing were performed with The Unscramble v. 9.7 software (Camo, Inondhaim, Norway).

Table 1
Spectrophotometric parameters of the four caramel samples.

Sample	EBC units	A_{560}	A_{280}	$R_{280/560}$	A_{610}
E150 a	7.040 ± 28	0.072 ± 0.001	7.04 ± 0.21	97.78	0.032 ± 0.001
E150 b	18.300 ± 460	0.242 ± 0.001	10.58 ± 0.07	43.72	0.121 ± 0.000
E150 c	34.850 ± 141	0.496 ± 0.001	15.41 ± 0.08	31.07	0.256 ± 0.001
E150 d	40.425 ± 247	0.892 ± 0.004	12.05 ± 0.16	13.51	0.513 ± 0.001

The absorbance values were recorded on a 0.1% (w/v, dry matter) solutions. The EBC units are expressed according to the European Brewery Convention, Anaytica-EBC (2004), as A 430 * dilution factor * 25.

Table 2
HMF and reducing sugar contents of the four caramel samples (all data expressed as g/kg dry matter, except for dry matter content).

Sample	Dry matter (%)	HMF	Glucose	Fructose
E150 a	61.91 ± 0.08	12.477 ± 0.013	206.58 ± 2.53	95.75 ± 3.73
E150 b	64.34 ± 0.31	28.849 ± 0.054	308.07 ± 0.54	25.83 ± 0.48
E150 c	71.24 ± 0.18	nd	8.46 ± 0.05	53.99 ± 0.15
E150 d	54.12 ± 0.06	0.800 ± 0.012	47.05 ± 0.45	100.08 ± 1.91

nd = not detectable.

Table 3
Reducing substances and antioxidant activity of the four caramel colours.

Sample	GAE[a]	DPPH[b]	FRAP[b]
E150 a	29.92 ± 0.48	61.96 ± 0.75	79.35 ± 0.73
E150 b	41.22 ± 0.06	133.24 ± 5.87	169.63 ± 2.00
E150 c	112.30 ± 0.13	318.90 ± 1.81	349.68 ± 3.54
E150 d	78.75 ± 0.25	294.44 ± 1.87	382.22 ± 4.10

[a] Expressed as gallic acid equivalent (mg/kg, dry matter).
[b] Expressed as Trolox equivalents (mmoles/kg, dry matter).

3. Results and discussion

Table 1 shows analytical data of the four caramel colours analysed. They have different solid contents (ranging between 54.12% for E150 d and 71.24% for E150 c). The colour intensity and the colouring power are expressed as EBC units derived from absorbance at 430 nm. It can be noticed that the four caramel colours differ in their features, in particular, caramel colours E150 c and d were darker than the others, when analysed at all specific wavelengths (430, 560 and 610 nm). The differences amongst the samples are also highlighted by the A_{280}/A_{560} ratios ($R_{280/560}$). Some differences were found amongst the four caramel colours with regard to the HMF and sugar content (Table 2); in particular, whilst E150 a and b contained a fairly good amount of HMF, this compound was not detectable at all in E150 c and was present at a very low level in E150 d, which is the caramel colour used in soft drinks. E150 c also showed the lowest amount of simple sugars. In the four caramel colours analysed, the glucose/fructose ratio varied in the range 0.2–11.5.

Caramel colours (Table 3) were also found to contain variable amounts of reducing compounds according to the Folin–Ciocalteau method. This method is usually applied to measure total phenolic content in wine, fruits and vegetables, but every compound that can act as a reducing compound in the experimental conditions employed in that assay gives a response. This is the case, for example, of sulphite in wine that must be removed before applying the Folin–Ciocalteau method (Di Stefano & Guidoni, 1989), or of ascorbic acid that can be removed in a similar way (Georgé, Brat, Alter, & Amiot, 2005). It was not the aim of our work to investigate the presence of phenolic compounds (if any) in the analysed soft drinks. However, several studies suggested that melanoidins are responsible for the strong antioxidant properties showed by coffee beverages (Anese & Nicoli, 2003; Borrelli et al., 2002; Nicoli, Anese, & Lerici, 1997), so we use the Folin–Ciocalteau method to rapidly measure the presence of any reducing compound. These data also emphasised the difference between caramel colours c and d and types a and b. The amount of Folin–Ciocalteau reactive substances ranged from about 30 to 112 mg of GAE/kg of sample (dry matter). Antioxidant activity was assayed on caramel colours using two different methods: the DPPH* and the FRAP assay. As shown in Table 3, the highest antioxidant activity was found in caramel E150 c. Values obtained by the DPPH* assay were well correlated with those found with the FRAP assay (linear correlation coefficient 0.97).

The same analysis as for caramel colours, except for the solid content determination, was performed on the 11 beverages considered in this study. Table 4 shows the spectrophotometric properties of these drinks. Even though the values varied little amongst the samples, the beverages were statistically different amongst them ($p < 0.001$) as determined by one-way ANOVA. It can be noticed that colas without caffeine (G and H) had the lowest value for A_{280}, as for the two chinottos at 610 nm. Colour intensity was also determined at 430 nm according to the Anaytica-EBC procedure, usually used for beer and beverages obtained from malt (European Brewery Convention, Anaytica-EBC, 2004).

HMF, glucose and fructose concentrations of samples are also shown in Table 4. All beverages had a slight content of HMF (ranging from 0.80 to 2.00 mg/l). The chinotto drinks contained higher amounts of HMF, especially the chinotto A that presents HMF content 40 times higher than chinotto B. The glucose and fructose contents were about 100 g/l, except for the light cola samples [D, E and F], that presented very low amounts of these sugars, probably related to the sugar content of caramel colours added as colouring agent. In fact, the ratio between glucose and fructose in cola light D was found to be very similar to the one of E150 d (about 0.5).

Table 5 shows the total reducing compounds content (Folin–Ciocalteau method) and the antioxidant capacity, as determined by the DPPH* and FRAP assays. As it can be seen, the values obtained with the DPPH* and FRAP assays were rather similar, whilst

Table 4
Spectrophotometric parameters, HMF and sugar contents of the analysed soft drinks.

Sample	EBC units	A_{280}	A_{560}	$R_{280/560}$	A_{610}	HMF[a]	Glucose[a]	Fructose[a]
Cola A	60.98 i	11.457 d	0.772 l	14.83	0.441 h	1.05 b	55.08 c	48.81 e
Cola B	56.44 g	13.035 f	0.711 i	18.34	0.406 g	1.36 c	48.32 b	32.71 b
Cola C	48.08 c	12.301 e	0.611 e	20.14	0.344 c	2.01 f	57.11 cd	49.06 ef
Cola D	52.13 e	12.857 f	0.660 g	19.22	0.373 e	1.36 c	0.01	0.290 a
Cola E	48.45 cd	8.759 b	0.540 b	16.01	0.300 a	0.84 a	0.00	nd
Cola F	45.34 b	13.880 g	0.578 d	24.01	0.332 b	1.63 d	0.00	nd
Cola G	53.25 f	8.190 ab	0.669 h	12.24	0.382 f	1.33 c	77.19 h	37.19 c
Cola H	48.94 d	8.130 a	0.669 h	12.15	0.350 cd	2.01 f	59.14 de	51.57 f
Cola I	49.05 d	13.320 fg	0.619 f	21.52	0.353 d	0.81 a	61.23 ef	37.42 c
Chinotto A	58.54 h	31.511 h	0.567 c	55.52	0.295 a	79.57 g	62.17 f	49.34 ef
Chinotto B	43.54 a	10.650 c	0.522 a	20.40	0.297 a	1.85 e	71.14 g	40.65 d
p	***	***	***		***	***	***	***

*** $p < 0.001$. Different letters in each column indicate significant differences at 95% confidence level as obtained by the LSD test. The EBC units are expressed according to the European Brewery Convention, Anaytica-EBC (2004), as A430 * dilution factor * 25.
nd = not detectable.
[a] HMF expressed as mg/l; glucose and fructose as g/l. The absorbance values were reported to undiluted samples.

Table 5
Reducing substances and antioxidant activity of analysed soft drinks.

Sample	GAE[a]	FRAP[b]	DPPH[b]
Cola A	98.47 d	0.328 c	0.288 h
Cola B	80.62 bc	0.259 a	0.232 g
Cola C	79.29 bc	0.260 a	0.184 b
Cola D	57.54 a	0.267 ab	0.186 bc
Cola E	59.31 a	0.290 b	0.170 a
Cola F	56.38 a	0.253 a	0.200 e
Cola G	72.10 b	0.270 ab	0.192 cd
Cola H	87.99 c	0.266 a	0.198 de
Cola I	72.19 b	0.319 c	0.211 f
Chinotto A	213.81 f	1.442 e	0.986 l
Chinotto B	136.03 e	0.792 d	0.543 i
p	***	***	***

*** $p < 0.001$. Different letters in each column indicate significant differences at 95% confidence level as obtained by the LSD test.
[a] Expressed as gallic acid equivalent (mg/l).
[b] Expressed as Trolox equivalents (mmol/l).

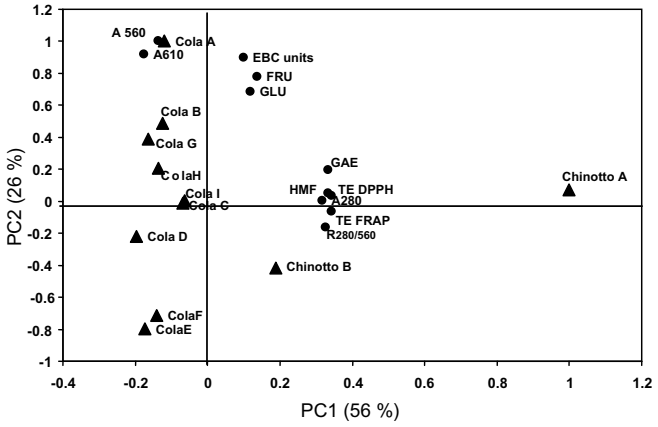


Fig. 1. Biplot obtained from the PCA analysis applied to the analytical parameters of soft drinks.

different values were obtained using the Folin–Ciocalteau test. This depends on the fact that caffeine gave positive response in the DPPH* and FRAP assays, whilst being unresponsive to the Folin–Ciocalteau assay. However, the antioxidant capacity and the total reducing compounds content pointed out the different behaviours of the two chinotto drinks. In fact, all beverages were characterised by low values, whilst chinotto A showed higher values: more than 200 mg/l GAE, 1.44 mM TE and 0.99 mM TE for FRAP and DPPH* assay, respectively. Moreover, chinotto B differed from other cola soft drinks as well, even though with values half of those found for chinotto A. From a statistical point of view, we noticed significant differences amongst cola soft drinks with the DPPH* assay, whilst the FRAP method showed a lower sensitivity.

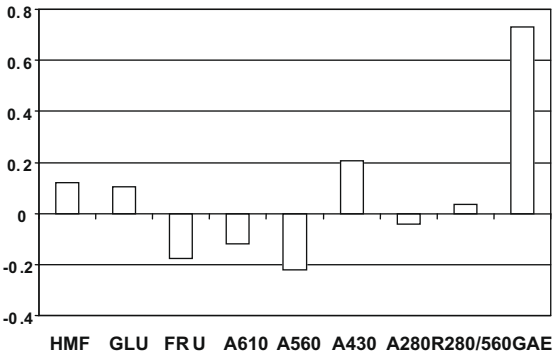
All analytical data were subjected to multivariate statistical analysis by using PCA and PLS. The biplot (Fig. 1) is the overlay of the scores of all samples with the loading of all variables on the plane defined by the two principal components, which explain 82% of the total variance. It can be noticed that along PC1 (56% of the total variance explained), the separation of cola samples from chinottos was obtained, whilst cola samples were separated along PC2 (26% of the total variance explained).

All variables related to the antioxidant capacity, total reducing compounds, HMF content, A_{280} nm and the $R_{280/560}$ discriminated chinotto drinks from all other soft drinks along PC1; chinotto A results were well separated from chinotto B along PC1, due to the higher values of the above-mentioned parameters. All colas are in the PC1 negative plane and are characterised by low values related to the antioxidant capacity, whereas they are discriminated one from the others along the PC2, mainly due to the differences in their absorbance values in the visible spectrum and in sugar content. Light cola soft drinks, which did not contain sugars, are obviously grouped in the opposite quadrant with respect to all the other colas. In a further elaboration, the chemical parameters listed in Fig. 2 were correlated to the antioxidant activity of soft drinks (determined by the FRAP assay) using the PLS algorithm.

Determination coefficients $R^2 = 0.989$ and 0.904 were obtained in calibration and cross-validation, respectively; these values indicate a good correlation between the response variable (antioxidant activity) and the predictive parameters. The regression coefficients obtained for the predictive parameters (Fig. 2) show that gallic acid equivalents and, to a lesser extent, A_{430} and A_{560} nm are the variables, which are significantly correlated to the antioxidant activity of soft drinks.

4. Conclusion

Wine and beer or other beverages such as coffee and tea, besides adding more or less antioxidants to the diet, contain some compounds, such as alcohol or stimulants that can have adverse health effects in people (as in pregnancy, suckling or in childhood) must be avoided for religious reasons or may be the cause of danger when driving cars. We considered cola-like soft drinks and two samples of chinotto, an Italian characteristic drink, all of them coloured with caramel. Cola drinks are supposed (recipes are confidential) to contain extracts from kola nuts and coca leaves, deprived of their dangerous and addicting compounds. A very poor phenolic content has been found in African cola (Atawodi et al.,



Predictive parameters	Regression coufficients
HMF	0,12
GLU	0,105
FRU	-0,178
A610	-0,118
A560	-0,219
A430	0,207
A280	-0,0415
R280/560	0,0353
GAE	0,729

Fig. 2. PLS regression coefficients of predictive parameters towards the dependent variable (antioxidant activity, FRAP).

2007; Rababah et al., 2004) and no other data are reported in the literature. Therefore, no other compounds, in particular phenolics, can be assumed to confer to the considered drinks their antioxidant power. Beverages with higher antioxidant activity, such as tea and coffee, contain also very high amounts of caffeine per serving. Caffeine is normally added by producers in cola soft drinks (Chou & Bell, 2007), but its content in cola and chinotto is lower than that in espresso coffee.

Our data show that some compounds present in the beverages analysed can contribute to the antioxidants pool that people can consume with the diet.

Among all beverages analysed, soft drinks flavoured with chinotto have the highest value of antioxidant capacity that ranges from two to five times the values found in cola samples. For this reason, it would be interesting to try to promote the consumption of this pleasant Italian beverage, that even if prepared with the addition of extracts obtained from the homonymous fruit (Italian law: DPR 19/5/1958, art. 5) cannot be grouped with all the other fruity beverages, because the juice content is well under the value requested by law (12%). However, chinotto and cola-like soft drinks have rather high sugar content (Table 4), so an excessive assumption must be avoided in order to control the calorific intake. Concerning the cola-like beverages the problem can be faced by drinking light colas; it might therefore be interesting to produce a "light" chinotto too.

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