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The relative contributions of ascorbic acid and phenolic antioxidants to the total antioxidant activity of orange and apple fruit juices and blackcurrant drink

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The total antioxidant activity (TAA) and antioxidant composition of orange juice, apple juice and blackcurrant drink have been studied. Phenolic antioxidants in these juices have been identified and values derived for their relative molar antioxidant activities or Trolox Equivalent Antioxidant Activity (TEAC). The bulk of the TAA of apple juice could be accounted for by chlorogenic acid and the phloretins, while that of orange juice was accounted for by hesperidin and narirutin. In contrast, the anthocyanins in blackcurrant drink contributed only a fraction of its non-vitamin C antioxidant activity and it is suggested that there is a significant unidentified antioxidant present in this beverage. After equalisation of the vitamin C content, juices were subjected to mild oxidation; the decline in ascorbate was of the order of apple > orange > blackcurrant drink. The results suggest that the phenolic antioxidants protect vitamin C against oxidative decomposition, with those in blackcurrant having the greatest vitamin C-sparing activity. © 1997 Elsevier Science Ltd

INTRODUCTION

Recent studies have highlighted the importance of the antioxidant constituents of fruits and vegetables in the maintenance of health and protection from coronary heart disease (Hertog *et al.*, 1993) and certain cancers. Although the majority of the evidence emphasises the roles of vitamin E, vitamin C and β-carotene, the presence of the phenolic antioxidants may also play a significant contributory role.

The polyphenols are effective hydrogen donors, particularly flavonols such as quercetin (Rice-Evans *et al.*, 1995), flavanols such as catechin gallate esters in green and black teas (Salah *et al.*, 1995), anthocyanins in wines (Frankel *et al.*, 1993) and phenylpropanoids (Castellucio *et al.*, 1995) including chlorogenic acid in apple juice (Miller *et al.*, 1995). Their antioxidant potential is dependent on the number and arrangement of the hydroxyl groups and the extent of structural conjugation, as well as the presence of electron-donating and electron-withdrawing substituents in the ring structure (Bors *et al.*, 1990; Rice-Evans *et al.*, 1995).

However, little is known about the bioavailability of the polyphenolic flavonoids in human subjects. The handling of these compounds by the GI tract and subsequent absorption and metabolism is in need of extensive study: some dietary polyphenols, such as quercetin (Gugler *et al.*, 1975; Hollman *et al.*, 1993) and catechin (Hackett & Griffiths, 1985), appear to be absorbed in the human but the extent is unclear. Although the study of urinary excretion of orally administered flavonoids has been employed by a number of investigators as a measure of absorption, flavonoid excretion has also been demonstrated through the bile (Ueno *et al.*, 1983). Furthermore, flavonoids (including those derived from citrus fruits) may undergo degradation in the intestine to low molecular weight compounds (such as the hydroxybenzoates and hydroxycinnamates) which are readily absorbed and subsequently excreted in the urine (Booth *et al.*, 1957).

The purpose of this study was to investigate the comparative antioxidant potentials of blackcurrant, orange and apple juice and the relationship with the antioxidant composition of these beverages, using the actual type of each beverage that is most widely consumed in the UK. The stability of the antioxidant activities of the beverages was assessed under mild oxidative conditions. The results suggest that the phenolic antioxidants of

fruit juices protect the vitamin C content from oxidative degradation, with the most active ascorbate-sparing antioxidants being found in blackcurrant juice.

MATERIALS AND METHODS

Chemicals

ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid,® Hoffman-La Roche) were obtained from Aldrich Chemical Co., Gillingham, Dorset SP8 4JL, England. Myoglobin (from horse heart), ascorbic acid, monophenolic acids, orthophenylenediamine and ferrozine were purchased from Sigma Chemical, Fancy Road, Poole, Dorset BH17 7BR, England. Anthocyanins and flavonoids were obtained from Extrasynthese, Z.I. Lyon-Nord, B.P. 62, 69726 Genay Cedex, France. All other chemicals were obtained from BDH (Merck Ltd, Merck House, Poole, Dorset BH15 1TD, England) and were of the highest quality available.

Measurement of total antioxidant activity (TAA), total vitamin C and ascorbic acid

TAA was measured using the ferrylmyoglobin/ABTS spectrophotometric assay (Miller *et al.*, 1993) generating the chromogenic $ABTS^{\bullet+}$ radical cation from the interaction between ABTS (150 μM), metmyoglobin (2.5 μM) and hydrogen peroxide (375 μM) as previously described (Miller *et al.*, 1995). Trolox was used as an antioxidant standard; absorbance readings were taken at 734 nm. The Trolox Equivalent Antioxidant Capacity (TEAC) of pure compounds, an estimate of the molar activity of substances as antioxidants in comparison to Trolox, was measured as before.

Total vitamin C was measured by estimating dehydroascorbic acid (DHA) fluorescence with orthophenylenediamine after conversion of all the vitamin C to DHA by means of iodine (Deutsch & Weeks, 1965). Ascorbic acid was measured using the ferrozine assay (Butts & Mulvihill, 1975).

Determinations of TAA, total vitamin C and ascorbic acid were carried out in triplicate at three different dilutions, from which the mean value was taken, on at least three separate days ($n = 3$ or more), using a fresh sample of beverage on each day. The results were then expressed as the mean $\pm$ 1 s.d. (i.e. a minimum of 27 separate determinations spread over several days supporting each figure quoted).

Measurement of polyphenols

HPLC analyses of phenols, flavonoids and anthocyanins were carried out by Dr A. G. H. Lea, Reading Scientific Services, The Lord Zuckerman Research Centre, Whiteknights, P.O. Box 234, Reading RG6 2LA, England. Separate beverage samples were analysed in duplicate on three different days. Blackcurrant drink (Ribena®) samples for anthocyanins and free gallic acid were analysed by reverse phase high-performance liquid chromatography (HPLC) in an aqueous acetonitrile gradient at pH 1.5, monitoring by diode array detector at 280 and 520 nm. For assay of bound gallic acid, samples were hydrolysed by refluxing in 2M $HClO_4$ for 30 min before re-analysis. For assay of soluble polyphenols in orange juice, the juice was filtered and analysed by reverse phase (HPLC), using a gradient of between 0.5% acetic acid in water to 100% acetonitrile. For assay of phenolic acids and phloretin

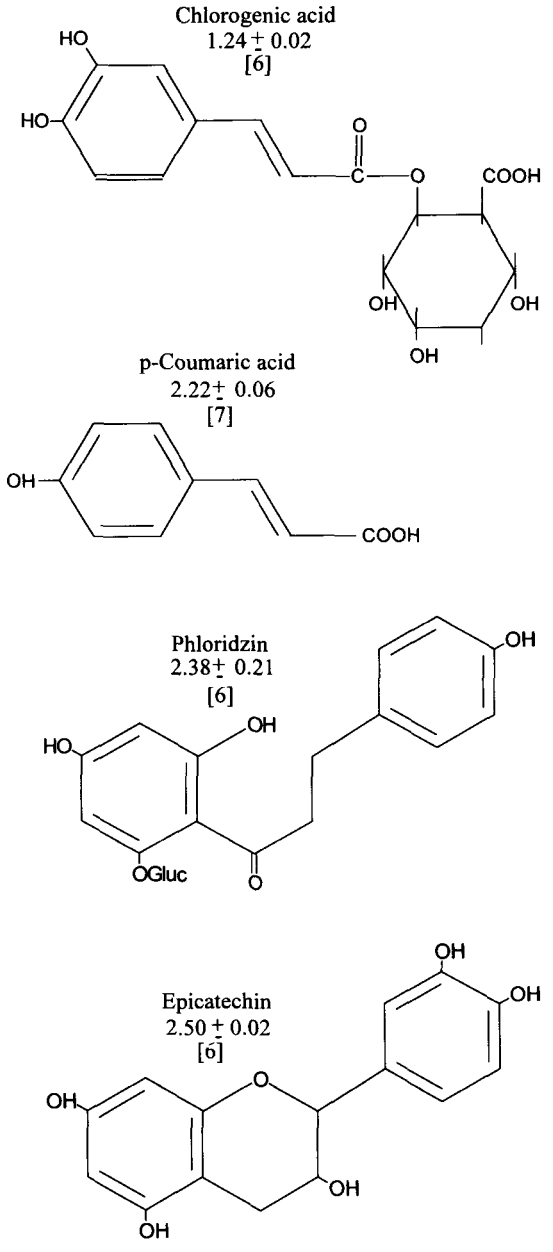

Fig. 1. Phenolic antioxidants found in longlife apple juice. (TEAC values shown in parentheses). Chlorogenic acid, p-coumaric acid, phloridzin, epicatechin.

derivatives in apple juice, the sample was analysed by reverse-phase HPLC with diode array detection in a water-acetonitrile gradient acidified to pH 2.5 (Lea, 1982, 1988).

Beverages

Longlife apple juice (J. Sainsbury plc, Stamford Street, London SE1 9LL), pH 3.3 and citric acid 90 mmol litre^{-1}, longlife orange juice (Sainsbury's), pH 1.9 and citric acid 145 mmol litre^{-1}, and ready-to-drink Ribena (®SmthKline Beecham Consumer Healthcare, Brentford, TW8 9BD, UK), pH 2.8 and citric acid 44 mM, were investigated. Ribena in this form is supplied as a 16.7% dilution of the concentrate: appropriate volumes of blackcurrant extract (Ribena — no added sugar) were also added to aliquots of Ribena drink to raise the concentration to 25%.

Oxidation of beverage samples

Beverage samples were subjected to mild oxidation after adjustment of the vitamin C concentration to 3.5 mM to facilitate comparisons with Ribena blackcurrant drink. The conditions chosen to promote oxidation were maintenance at 37°C for 24 h away from direct light and with a minimum of aeration. It was established that, under these conditions, 3.5 mM vitamin C (Redoxon, Roche Products Ltd, Welwyn Garden City, England) dissolved in bottled mineral water (Sainsbury's Caledonian Spring) was fully degraded. Small aliquots were removed at zero time and after 6 and 24 h and analysed immediately for TAA, total vitamin C and ascorbic acid.

RESULTS

Figures 1–3 show the structures and TEAC values of selected phenylpropanoids, anthocyanins and flavonoids present (or potentially present) in apple juice, orange juice and blackcurrant drink. The antioxidant potentials of the fruit beverages longlife apple juice, longlife orange juice and blackcurrant drink (Ribena) are shown in Tables 1–3. Blackcurrant drink has the highest TAA (5070 ± 190 μmol litre^{-1}) and apple juice the lowest (840 ± 50 μmol litre^{-1}). The antioxidant constituents of the fruit beverages are also listed in the tables together with their vitamin C levels. The antioxidant activity of the individual phenolics is shown and their contribution towards the antioxidant activity calculated.

The identifiable soluble phenolic components of orange juice were hesperidin and narirutin, but the major antioxidant was ascorbic acid (2270 ± 210 μmol litre^{-1} or 87% of the measured TAA). The values obtained for apple juice are consistent with our previous findings (Miller *et al.*, 1995) that the antioxidant activity of apple juice can be accounted for essentially by

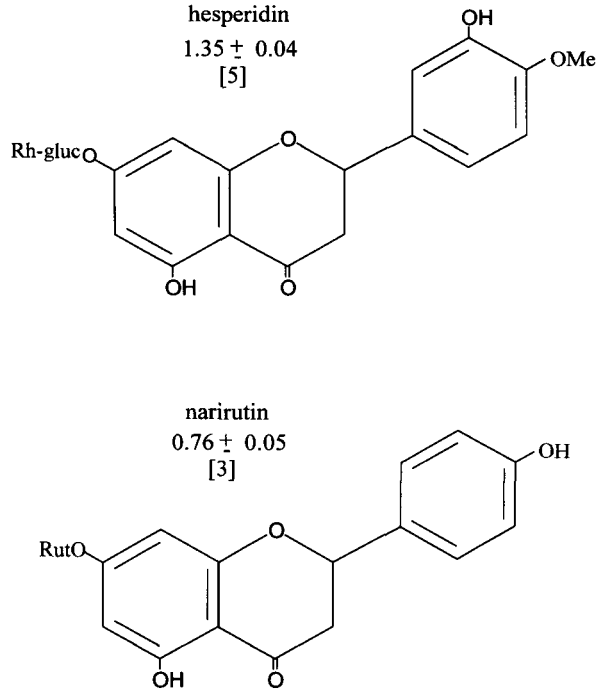

Fig. 2. Phenolic antioxidants found in longlife orange juice. (TEAC values shown in parentheses). Hesperidin, narirutin.

chlorogenic acid and a range of constituents that are present at lower concentrations.

The predominant identifiable antioxidant constituent of blackcurrant drink (Ribena) is ascorbic acid (3726 ± 185 μmol litre^{-1} or 73% of the measured TAA) with smaller contributions from the anthocyanins. The large discrepancy (1209 μmol litre^{-1}) between the calculated (3861 μmol litre^{-1}) and measured TAA (5070 μmol^{-1}) suggests the presence of a major unidentified antioxidant.

The TAA was evaluated as a marker of the deterioration of apple juice, orange juice and Ribena drink under oxidative conditions. Samples were subjected to oxidising conditions (37°C for 24 h with a limited amount of aeration), during which time TAA measurements were made in tandem with total vitamin C and ascorbic acid determinations. Juice samples were also fortified with vitamin C to a similar concentration to Ribena drink (3.5 mmol litre^{-1} vitamin C) and subjected to the same procedure. As well as packet Ribena (16.7%), fortified Ribena (25%) was evaluated. The oxidation of vitamin C (Redoxon) dissolved in commercial bottled drinking water was studied and the conditions adjusted so that vitamin C in mineral water was completely degraded over a 24 h period. Vitamin C in apple juice lost 70% of its ascorbic acid activity over 24 h under the same conditions, while vitamin C in orange juice lost 58% of its activity. In contrast, packet Ribena lost 47% of its ascorbate activity and fortified Ribena only lost 9% of its activity under these experimental conditions. The results suggest that the

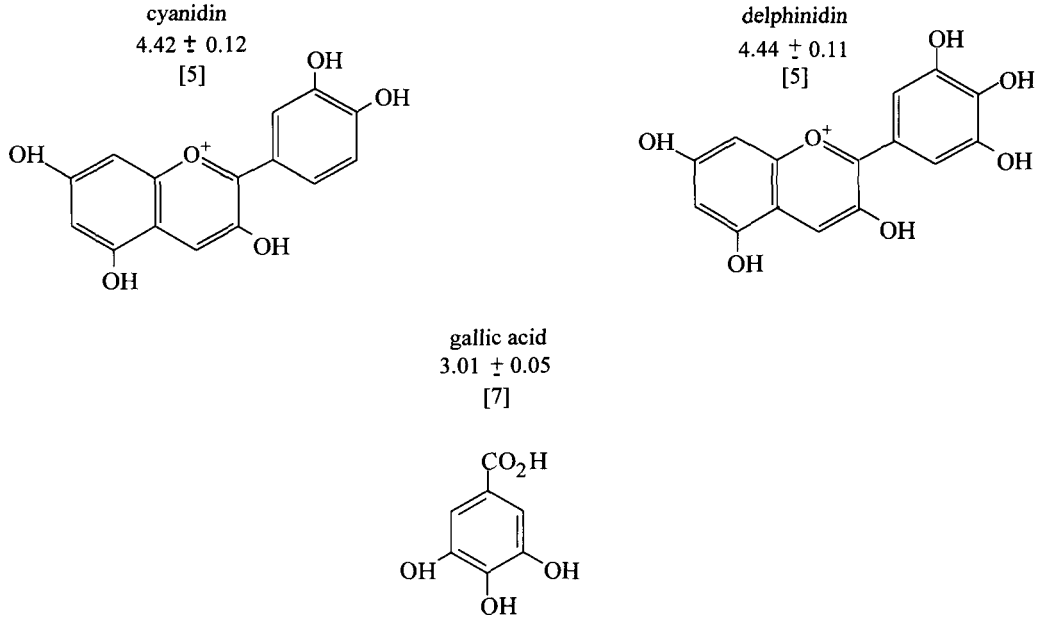

Fig. 3. Phenolic antioxidants found in Ribena blackcurrant drink. (TEAC values shown in parentheses). Delphinidin, cyanidin, gallic acid.

polyphenol constituents of these beverages can retard the oxidative decomposition of vitamin C, with those contained in Ribena having the greatest vitamin C-sparing activity (Fig. 4).

Figure 4 illustrates the rate of decline of the TAA, the ascorbic acid and the total vitamin C levels of these beverages under mildly oxidative conditions. In the case of vitamin C in mineral water, all the TAA (Fig. 4A) and ascorbic acid (Fig. 4B) and the majority of the total vitamin C (Fig. 4C) were lost over 24 h (leaving 15% of the original total vitamin C as dehydroascorbic acid). In contrast, when 3.5 mmol litre^{-1} of vitamin C was dissolved in longlife apple juice, 30% of the TAA and the ascorbic acid was preserved after 24 h. Longlife orange juice, with an initial vitamin C content adjusted to the same level, had 40% of the TAA and 42% of the ascorbic acid still present after 24 h. Ready-to-drink Ribena kept 80% of its TAA and 53% of its ascorbic acid after 24 h and, if the concentration of the beverage was increased from 16.7 to 25%, 90% of the TAA and ascorbic acid was maintained after 24 h. The difference between the percentage decline in TAA and ascorbic acid for all beverage samples and vitamin C in mineral water was statistically significant (Mann–Whitney U-test, $P < 0.001$).

DISCUSSION

Our previous studies and those of others have demonstrated structure-activity relationships of phenols and polyphenols in chemical systems (Bors *et al.*, 1990; Rice-Evans *et al.*, 1995). In this investigation, the antioxidant potential of blackcurrant juice drink has been determined in relation to its antioxidant content and compared to apple juice and orange juice. The contribution

Table 1. Antioxidant content and activity of longlife apple juice

Constituent	TEAC[a] [1 s.d.] $n \geq 6$	Concentration mean μmol litre^{-1} [1 s.d.] $n = 3$	Activity (conc. × TEAC) μM
Chlorogenic acid	1.24 [0.02]	274 [95]	340
p-Coumaroyl quinic acid	2.22[b] [0.06]	74 [24]	164
Phloridzin	2.38 [0.06]	23 [4]	55
Phloretin xyloglucoside	2.38[c] [0.06]	35 [10]	83
Epicatechin	2.50 [0.02]	< 14	35
Ascorbic acid	1.00 [0.04]	51 [13]	51
Calculated TAA			728
Measured TAA			840 [50]
TAA unaccounted for			112

[a]TEAC, millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation.
[b]Pure substance not available for testing: activity taken as that of p-coumaric acid.
[c]Pure substance not available for testing: activity taken as that of phloridzin.

Table 2. Antioxidant content and activity of longlife orange juice

Constituent	TEAC[a] [1 s.d.] $n \geqslant 3$	Concentration mean μmol litre^{-1} [1 s.d.] $n = 3$	Activity (conc. × TEAC) μM
Hesperidin[b]	1.08 [0.03]	141 [49]	152
Narirutin [b]	0.76 [0.05]	62 [16]	47
Ascorbic acid	1.00 [0.04]	2270 [210]	2270
Calculated TAA			2469
Measured TAA			2610 [50]
TAA unaccounted for			141

[a]TEAC, millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation.
[b]Soluble fraction only.

of vitamin C to the antioxidant potential has been determined, together with its stability under mildly oxidative conditions.

While vitamin C activity represented a minimal fraction of the TAA of longlife apple juice, the hydroxycinnamate chlorogenic acid was shown to be its major identifiable antioxidant, as shown previously (Miller *et al.*, 1995). Of the flavonoid antioxidants, phloridzin was the most prominent compound present in apple juice. In both orange juice and Ribena, vitamin C (which is added during manufacture) was the major antioxidant. The identifiable soluble phenolic components of orange juice were hesperidin and narirutin, but the major antioxidant was ascorbic acid (2270 ± 210 μmol litre^{-1}, 87% of the measured TAA). Insoluble phenolics contributed significantly to the antioxidant composition of orange juice but, according to the results shown here, did not contribute greatly to the *in-vitro* antioxidant activity, given that the ascorbic acid activity combined with the soluble phenol activity accounted for the bulk of the measured TAA. Orange juice also contains carotenoids, mainly cryptoxanthins, lutein, anthoxanthin, violaxanthin (Lea, 1988) which will also not be mixed in with the water-soluble antioxidant components. The flavanones hesperidin and narirutin are abundant in orange juice (Rouseff *et al.*, 1987) in a partially dissolved/partially suspended/partially colloidal form. The results are consistent with the notion that only the dissolved fractions of these substances in orange juice will be measured as *in-vitro* antioxidants in this assay. Blackcurrant drink (Ribena) contains delphinidin and cyanidin in the glucoside and rutinoside form. The bulk of the measured activity found in the apple and orange beverages can be accounted for by the known antioxidant constituents, but little of the non-vitamin C TAA of Ribena can be accounted for: it is suggested that there are significant antioxidants present in Ribena which are not identified by these procedures, but whose activity can be detected in the TAA assay.

The stabilising effect of blackcurrant anthocyanins on ascorbic acid subjected to oxidative stress was reported by Hooper and Ayres (1950), who ascribed this effect to the inhibition of oxidising enzymes. Timberlake (1960) reported the relative stability of ascorbic acid in blackcurrant juice and concluded that its oxidation occurred via a non-enzymic mechanism. The stability of ascorbic acid in model systems containing phenols and polyphenols was investigated by Clegg and Morton (1968) who concluded that quercetin had the greatest protective effect, followed by dihydroquercetin > kaempferol > quercitrin > chlorogenic acid = p-coumaric acid. These workers also found that anthocyanins had an antioxidant effect only when ascorbate oxidation was promoted by copper and not when simple aerobic

Table 3. Antioxidant content and activity of Ribena blackcurrant drink

Constituent	TEAC[a] [1 s.d.] $n \geqslant 3$	Concentration mean μmol litre^{-1} [1 s.d.] $n = 3$	Activity (conc. × TEAC) μM
Delphinidin-3-glucoside	2.47[b] [0.03]	5.9 [4.0]	150
Delphinidin-3-rutinoside	3.25[c] [0.10]	19.9 [11.8]	65
Cyanidin-3-glucoside	2.47 [0.03]	2.4 [1.7]	6
Cyanidin-3-rutinoside	3.25 [0.10]	15.0 [7.8]	49
Gallic acid	3.01 [0.05]	Undetectable	
Ascorbic acid	1.00 [0.04]	3726 [185]	3726
Calculated TAA			3861
Measured TAA			5070 [190]
TAA unaccounted for			1209

[a]TEAC, millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation
[b]Pure substance not available for testing: since TEAC values for the anthocyanidins delphinidin (4.44 ± 0.11) and cyanidin (4.42 ± 0.12) were nearly identical, the activity was taken as that of cyanidin-3-glucoside
[c]Pure substance not available for testing: activity taken as that of cyanidin-3-rutinoside (see above).

oxidation was taking place. The same researchers, using a Cu^{2+}-stimulated oxidising system, concluded that the factor in blackcurrant juice stabilising ascorbic acid was not the anthocyanins (Harper *et al.*, 1969), due to the low activities recorded for these compounds relative to quercetin. In contrast, the protective action of ascorbic acid against the oxidation of anthocyanins has also been investigated (Macheix *et al.*, 1990).

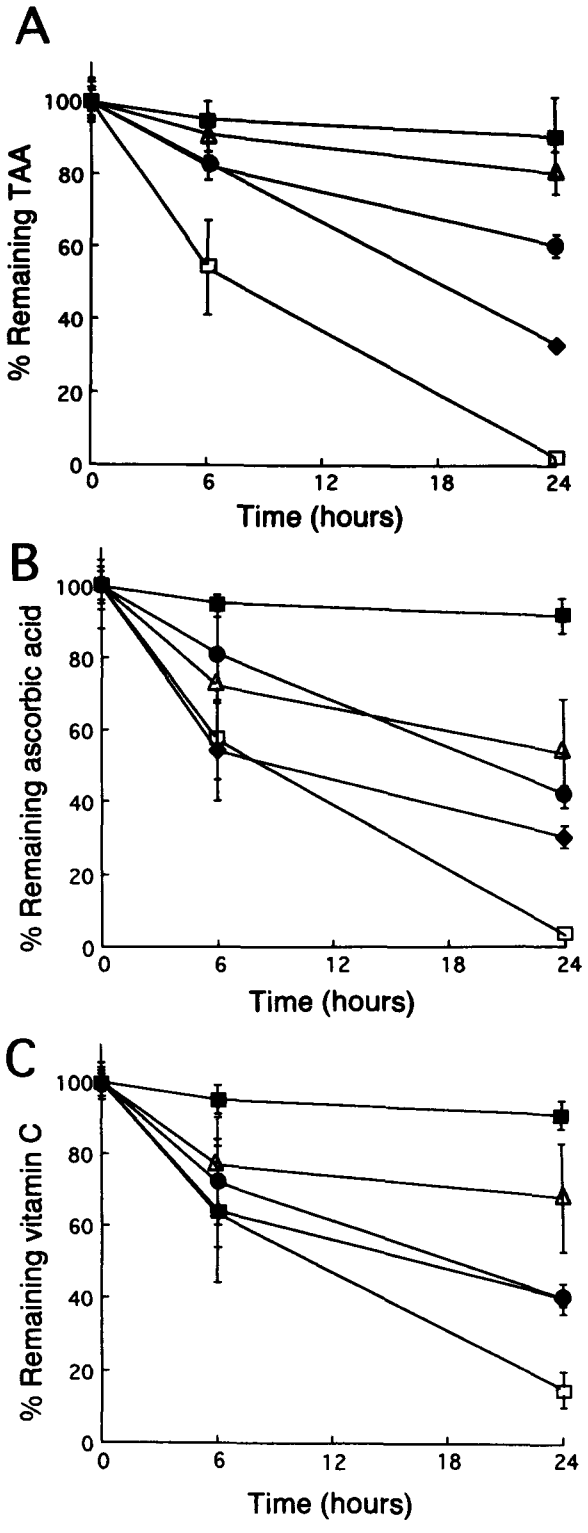

Fig. 4. The effect of mild oxidation (24 h) on ■, 25% Ribena; ▽, 'ready-to-drink' 16.7% Ribena; ●, long life orange juice with added vitamin C; ◆, long life apple juice with added vitamin C and □, Redoxon in mineral water; A, plotting % remaining TAA; B, ascorbic acid and C total vitamin C. $n = 3$ or more; mean ± 1 s.d. shown.

The results of this investigation show that the total antioxidant activities (TAA) of the beverages studied are in the order of blackcurrant > orange > apple. However, of these, the major source of potential dietary phenolics is clearly apple juice. Fortification of the beverages with vitamin C and studies of preservation under mild oxidative conditions demonstrate that the phenolics in fruit juices have an ascorbate-sparing effect in the order of blackcurrant > orange > apple; the minimal effectiveness of apple juice may be attributed to the fact that the phenylpropanoids are less efficient at conserving ascorbate than are the polyphenolic flavonoids. Increasing the 'in the glass' concentration of Ribena drink leads to more than 90% of the ascorbic acid content being preserved under the experimental conditions. This underlines the close relationship between the different antioxidants in the food matrix. The very low measured concentrations of anthocyanins in Ribena drink in comparison to the phenols and polyphenols in apple and orange juice raises the question as to whether this activity is due to anthocyanins, anthocyanin polymers, procyanidins or other as yet unrecognised phenols present in blackcurrant juice.

ACKNOWLEDGEMENT

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