Evaluation of the Total Antioxidant Activity as a Marker of the Deterioration of Apple Juice on Storage

Nicholas J. Miller,* Anthony T. Diplock, and Catherine A. Rice-Evans

Free Radical Research Group, Division of Biochemistry and Molecular Biology, UMDS - Guy's Hospital, University of London, London SE1 9RT, England

The total antioxidant activity (TAA) has been evaluated as a marker of the deterioration of apple juice during storage. Unfortified juice, juice fortified with vitamin C, and an apple "drink" (6% juice), to which vitamin C (300 ppm) is added during manufacture, were evaluated. Vitamin C activity represented a minor fraction of the TAA (*ca.* 1%) of "longlife" apple juice, with chlorogenic acid and phloretin glycosides as the major identifiable antioxidants (*ca.* 32% and 11% of the TAA, respectively). Antioxidant activity ascribable to these substances was stable under the storage conditions examined, whereas ascorbic acid added into the juice was unstable; in fortified whole apple juice the TAA value correlated significantly with the decline in the ascorbic acid concentration, while in the apple drink there was a direct relationship between TAA and ascorbate.

Keywords: *Apple; antioxidant; ascorbic acid; phenols; chlorogenic acid; phloridzin*

INTRODUCTION

The major antioxidant constituents of apple juice are the fruit polyphenols and ascorbic acid. In apples there are six classes of polyphenol (Figure 1) (Lea and Timberlake, 1974; Lea, 1978; Spanos *et al.*, 1990; Thompson *et al.*, 1972; Oleszek *et al.*, 1988; Whiting and Coggins, 1975a,b). The anthocyanins and flavonol glycosides are mainly found in the skin and might, however, be present in the juice. The phenolic acids are chlorogenic acid and *p*-coumaroylquinic acid, which belong to the cinnamate family. The dihydrochalcones are phloretin glucoside (phloridzin) and xyloglucoside. The main catechin is (−)-epicatechin, and the procyanidins are the 4-β-8-linked epicatechin series with some mixed (+)-catechin/(−)-epicatechin. Of the other antioxidants that occur naturally in foods, there is no significant glutathione in apples (Jones *et al.*, 1992) and none in apple juice. On the other hand, fresh apples may well contain up to 100 ppm of vitamin C, but during processing into juice this is rapidly lost (Lea, 1992). The addition of vitamin C prevents polyphenol oxidation, the major source of browning of apple juice during processing.

The biological role of the flavonoids and polyphenols remains to be elucidated, but there is growing evidence that an increase in dietary levels of these substances may be of long-term benefit to human health. The daily human intake of these polyphenols in the average American diet has been estimated to be 1 g or more (Kuhnau, 1976), which is significant since flavonoids have been shown to influence a wide range of biological functions. For example, *in vitro*, flavonoids scavenge free radicals, *e.g.* superoxide ions (Robak *et al.*, 1988) and singlet oxygen and lipid peroxyl radicals (Sorata *et al.*, 1982). Polyphenols, including flavonoids, inhibit 5-lipoxygenase and cyclo-oxygenase (Laughton *et al.*, 1991) and lipid peroxidation (Kappus *et al.*, 1979) and have diverse effects on immune and inflammatory cell function (Decharneux *et al.*, 1992). Flavonoids also have antihemolytic activities (Naim *et al.*, 1976), inhibit oxidation of low-density lipoprotein (LDL) by macrophages (De Whalley *et al.*, 1990), and prevent cytotoxicity of oxidized LDL on lymphoid cell lines (Negre-Salvayre *et al.*, 1992). In addition, flavonoids have been reported to affect capillary permeability, cellular secretory processes, cell membrane receptors, and carriers (Negre-Salvayre *et al.*, 1992). Mutagenic, antiviral, antibactericidal, and antifungal properties of flavonoids have also been demonstrated (Sichel *et al.*, 1991). A recent study by Hertog *et al.* (1993) showed that flavonoids in regularly consumed foods such as tea, onions, and red wine may reduce the risk of coronary heart disease in elderly men.

The objectives of this study were to evaluate (i) the relationship between the antioxidant content of apple juice and its "total antioxidant activity" (TAA), (ii) the stability of the antioxidants in apple juice on storage, and (iii) the application of the TAA as a marker of the deterioration of specific antioxidants on storage. The importance of the TAA method in this respect is that it can be used to detect the presence of unmeasured antioxidants (or the existence of a synergistic relationship between known antioxidants) (Miller *et al.*, 1993; Rice-Evans and Miller, 1994).

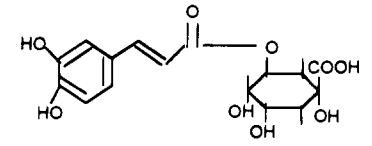
MATERIALS AND METHODS

Measurement of Total Antioxidant Activity (TAA). The method for measuring total antioxidant activity (TAA) estimates the relative ability of the antioxidant substance to scavenge the radical cation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) in the aqueous phase, as compared to standard amounts of the synthetic antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), the water-soluble vitamin E analogue (Miller *et al.*, 1993). This is a measure of antioxidant activity, as opposed to antioxidant concentration (which might include a proportion of biologically inactive antioxidant). It also permits the measurement of the antioxidant activity of mixtures of substances and, hence, can distinguish between additive and synergistic effects if the molar concentration of the contributing antioxidants is known. The assay is based on the interaction between antioxidants and the $ABTS^{\bullet+}$ radical cation, which has a characteristic long wave absorption spectrum showing maxima at 645, 734, and 815 nm. $ABTS^{\bullet+}$ can be generated by the interaction of ABTS

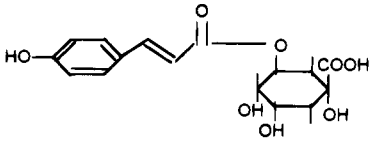
* Author to whom correspondence should be addressed (telephone 071-955-5000, ext 3104; fax 071-955-4983).

0021-8561/95/1443-1794$09.00/0

1. Phenolic acids

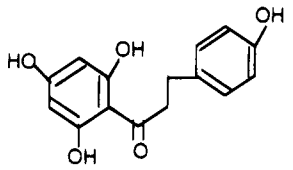


Chlorogenic acid (5-caffeoyl quinic acid)

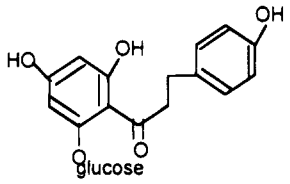


p-coumaroyl quinic acid

2. Dihydrochalcones

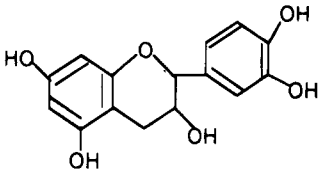


phloretin



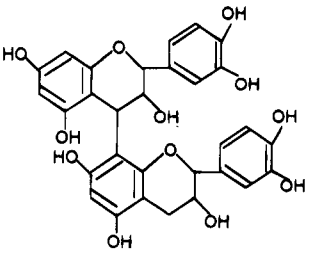
phloridzin (phloretin glycoside)

3. Catechins



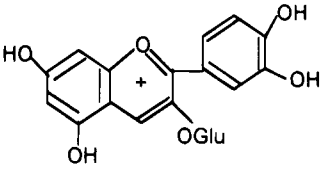
(-) epicatechin

4. Procyanidins



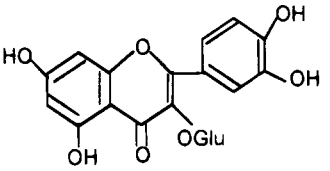
(the 4β-8-linked epicatechin series)

5. Anthocyanins (skin only, not in juice)



cyanidin - 3 - glucoside

6. Flavonol glycosides (skin only, not in juice)



quercetin - 3 - glucoside

Figure 1. Six classes of polyphenol found in apples.

(150 μM), H_2O_2 (375 μM), and metmyoglobin (2.5 μM). Antioxidant compounds suppress the formation of $ABTS^{\bullet+}$ to an extent and on a time scale dependent on the antioxidant capacity of the substance under investigation. With automated timing and reagent additions, this precise and stable assay has application to the measurement of the antioxidant activity of pure solutions and of mixtures of substances in a wide range of pharmacological and physiological situations.

Phosphate-buffered saline (5 mM) pH 7.4 (PBS), was used as a buffer. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Hoffman-La Roche) (2.5 mM) was prepared in PBS for use as an antioxidant standard. Working solutions of hydrogen peroxide were prepared from stock Aristar H_2O_2 (BDH) after an initial dilution in PBS to a concentration of 500 mM. ABTS (5 mM) was prepared in PBS. Mixed ABTS/myoglobin reagent was prepared so that on dilution by the analyzer the reagents were at the desired concentration (2.5 μM metmyoglobin, 150 μM ABTS, 375 μM H_2O_2, and 0.84% sample fraction). Instrument settings for the Cobas Bio centrifugal analyzer are shown in Table 1. Using these reagent concentrations, the end of the lag phase (*i.e.* the point at which the absorbance at 734 nm started to increase) for the 2.0 mM standard was at 170 s, which was taken as the measuring time for ΔA_{734nm} for all standards and analyticals. Recovery of freshly added ascorbate (as antioxidant activity) from both PBS and apple juice was checked and was found to be 99 ± 5% for PBS and 95 ± 9% for apple juice ($n = 3$ in both cases, at four levels of supplementation—150, 125, 75, and 30 ppm). Plots of the absorbance change in the assay for total antioxidant activity using samples of apple juice and apple juice supplemented with four levels of ascorbic acid are shown in Figure 2.

Table 1. Protocol for the Measurement of Total Antioxidant Activity (TAA) of Apple Juice with the Cobas Bio Centrifugal Analyzer (Miller *et al.*, 1993)

transfer	3.0 μL of sample	apple juice or Trolox standard
	30 μL of water	flushes transfer probe
	300 μL of reagent	(ABTS + metmyoglobin)
mix		
read	initial absorbance	
transfer	25 μL H_2O_2	final concn 375 μM
mix	H_2O_2 starts reaction	
incubate	at 30 °C	absorbance readings every 10 s
read	all cuvettes	170 s
plot	ΔA_{734nm} vs Trolox concn	logit/log 4 curve fit

Trolox Equivalent Antioxidant Capacity (TEAC) of Apple Juice Constituents. Data were obtained on the Trolox equivalent antioxidant capacity (TEAC) of substances present (or potentially present) in apple juice. The protocol was as follows. A 10 mM solution of the pure substance was prepared in ethanol or in PBS. This solution was analyzed for TAA according to the protocol described above. If it proved to have antioxidant activity, serial dilutions of the solution were analyzed until an estimate was obtained of the Trolox equivalent antioxidant capacity (the millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation).

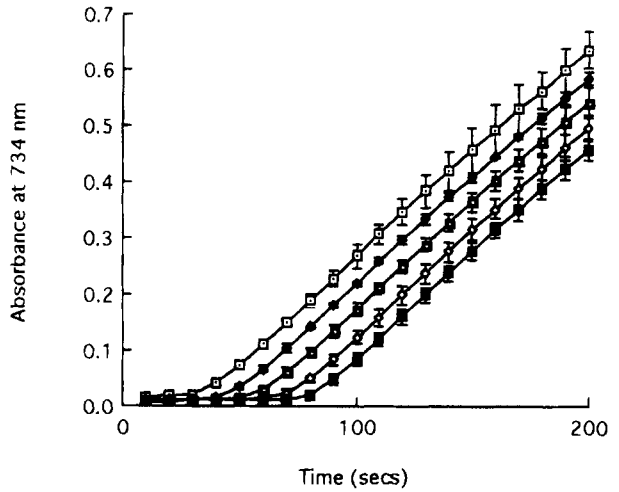


Figure 2. Plots of the absorbance change in the assay for total antioxidant activity using samples of apple juice and apple juice fortified with ascorbic acid. Conditions: 2.5 μM metmyoglobin, 150 μM ABTS, 375 μM H_2O_2; measuring time, 170 s from the addition of H_2O_2; temperature, 30 °C. "Near 100%" recovery of added ascorbic acid was measured in the fortified samples. $n = 3$; mean ± 1 SD shown.

At a minimum, three different dilutions of each stock solution were analyzed in triplicate on three separate days ($n = 3$, *i.e.* 27 determinations *in toto*). The TEAC was calculated for each dilution and the mean value of all the results calculated.

Due to its nonpolar nature, quercetin dissolved in ethanol did not prove to be fully miscible with the reaction mixture. This was reflected in poor agreement among triplicates obtained when the TEAC was derived. Quercetin was therefore dissolved in 70% aqueous DMSO at a concentration of 5 mM and also diluted in DMSO to working initial concentrations for assay (0.2, 0.3, and 0.4 mM). Dilutions of Trolox in 70% DMSO were then run as samples (in addition to Trolox standards in PBS), and the figure derived for quercetin was corrected for the value obtained for Trolox in DMSO; this corrected figure is quoted under Results as the TEAC for quercetin.

Measurement of Vitamin C. Total vitamin C (ascorbic and dehydroascorbic acids) was measured according to the method of Deutsch and Weeks (1965) (*o*-phenylenediamine fluorescence). The dose–response curve obtained by this method, plotting ascorbate (2–16 μM) vs relative fluorescence, was linear and passed through the origin. The relationship between the mass of ascorbic acid freshly added to apple juice and the measured vitamin C concentration ($n = 4$, at three levels over the range of the dose–response curve) showed that analytical recovery was satisfactory (97 ± 4%) (mean ± 1 SD).

Ascorbic acid was measured according to the ferrous chromogenic method of Butts and Mulvihill (1975). This method depends on the reduction of Fe^{III} to Fe^{II} ions by ascorbate, which is simultaneously converted to dehydroascorbate. The formed Fe^{II} ions are subsequently reacted with a chelator (Ferrozine), and the resulting complex is quantitated spectrophotometrically with the Cobas Bio analyzer, using an incubation time of 10 s. Other slower acting reducing substances may also generate Fe^{II} from Fe^{III}, but not to a significant extent during the 10 s period under the reaction conditions used. The iron/Ferrozine method quantitates only reduced ascorbic acid and not dehydroascorbic acid (DHA). In the protocol used an absorbance reading for a reagent blank was subtracted from standard and sample readings. Standards were analyzed in triplicate, and the dose–response curve (ascorbate concentration *vs* absorbance change) was taken as linear between the mean standard value and the reagent blank. The validity of this assumption was checked by analysis of apple juice to which known amounts of ascorbate had been added. Recovery of freshly added ascorbate from apple juice was found to be 98 ± 3% ($n = 3$, at four levels of supplementation—30, 75, 125, and 150 ppm).

Table 2. Antioxidant Substances and Activity of Apple Juice

component	type I apple juice mg/L (ppm)	type I apple juice μmol/L	type II apple juice mg/L (ppm)	type II apple juice μmol/L
A. Major Antioxidant Substances				
chlorogenic acid	4	12	91	257
p-coumaroylquinic acid	1	3	23	68
phloridzin	1	2	8	17
phloretin xyloglucoside	1	2	14	30
epicatechin	ND[a]		0.5	2
procyanidins	ND		ND	
ascorbic acid	300	1700	2	11
B. Total Antioxidant Activity (TAA)				
TAA		1807		1000

[a] ND, not detected.

Measurement of Polyphenols. HPLC analyses of the detailed phenolic composition of apple juice were carried out by Dr. A. G. H. Lea, Reading Scientific Services, The Lord Zuckerman Research Centre, Whiteknights, Reading, U.K. (Lea, 1982).

Apple Juice Samples. The TAA and vitamin C and polyphenol contents of two types of apple juice (type I, an apple "drink" containing 6% juice fortified with 300 mg/L of vitamin C; type II, long life apple juice, which had no specified vitamin C content) were investigated. The protocol used was as follows: six cartons of apple juice (same batch) were opened; 75 ppm vitamin C was added to two cartons, 150 ppm vitamin C was added to another two cartons, and two cartons were left unfortified. Assay of the TAA, total vitamin C (OPD fluoresence), and ascorbic acid (Ferrozine method) was performed on all six cartons (day 1). Three cartons (0, 75, and 150 ppm vitamin C) were stored at room temperature, and three cartons were stored under identical conditions but at 4 °C. On days 2, 3, 4, 5, and 10 the TAA, total vitamin C (OPD fluoresence), and ascorbic acid (Ferrozine method) of all six cartons were monitored. The experiment was repeated twice ($n = 3$), each time with six cartons of apple juice from the same batch.

RESULTS

Analysis of Antioxidant Content of Apple Juice. The results of the analysis of the phenolic and ascorbic acid composition of the apple juice, together with the TAA value for each juice (Table 2), show that vitamin C activity was the major contributor to the TAA in the type I drink, whereas in the unsupplemented type II juice it represented only a very minor fraction of the TAA, with phenolic compounds as the major identifiable antioxidants. The antioxidant with the highest concentration in whole apple juice (type II) was the hydroxycinnamate chlorogenic acid—present at 257 μmol/L. *p*-Coumaroylquinic acid, which is structurally related to chlorogenic acid—differing only in the lack of the 3-OH group—was present at 68 μmol/L. The flavonol glycosides, phloretin glucoside (phloridzin) and phloretin xyloglucoside, were the main flavonoid polyphenols present. A trace amount of (−)-epicatechin was detected. Other flavan-3-ols (such as catechin and the gallocatechins), which may have been present in the freshly expressed apple juice, were not detectable, presumably due to loss in the manufacturing process. The same applies to the procyanidins and flavonols (such as quercetin), which were not detectable in this type of apple juice.

The polyphenol content of type I juice was compatible with its being a *ca.* 5% dilution of a juice such as the type II, but with 300 ppm of vitamin C added. The chlorogenic acid content of the type I juice was 12 μmol/

Table 3. Trolox Equivalent Antioxidant Capacity (TEAC) Values of Apple Juice Constituents, Including Phenolic Acids and Other Antioxidants Reportedly Present in Apple Juice[a]

substance	TEAC	n	SD
ascorbic acid	0.99	5	0.04
dehydroascorbic acid	0.00	3	
benzoic acid	0.00	3	
chlorogenic acid	1.24	6	0.02
caffeic acid	1.26	3	0.01
ferulic acid	1.90	9	0.02
protocatechuic acid	1.19	7	0.04
cinnamic acid	0.00	4	
quercetin	4.72	6	0.10
rutin (quercetin 3-rutinoside)	2.42	7	0.12
phloridzin	2.38	6	0.21
epicatechin	2.50	6	0.02
catechin	2.40	9	0.05
cyanidin	4.42	5	0.12
citric acid	0.00	3	
malic acid	0.00	3	
tartaric acid	0.00	3	
lactic acid	0.00	3	
aspartic acid	0.00	3	
asparagine	0.00	3	
γ-aminobutyric acid	0.00	3	
alanine	0.00	3	
serine	0.00	3	
proline	0.00	3	
fructose	0.00	3	
glucose	0.00	3	
sucrose	0.00	3	
sorbitol	0.00	3	

[a] Analyses were carried out on solutions of pure antioxidants. [b] TEAC, millimolar concentration of a Trolox solution having the antioxidant capacity equivalent to 1.0 mM solution of the substance under investigation.

L, which is 4.7% of the chlorogenic acid content of the type II juice. Only trace amounts of the phloretin glucosides were detected. The higher TAA value of the type I juice (1807 μmol/L, as opposed to 1000 μmol/L) is attributable to its enhanced vitamin C content.

To calculate the relative contribution of these concentrations of antioxidant substances to the TAA of the juices, the relative antioxidant activity of each compound was measured. The TEAC results for apple juice constitutents are shown in Table 3. By this method quercetin (TEAC = 4.72) proved to be the most powerful antioxidant potentially present in the apple juices (derived from the skins). Epicatechin (TEAC = 2.50) and phloridzin (TEAC = 2.38) were approximately half as active as antioxidants as quercetin (but still much more active than vitamin C). Chlorogenic acid (TEAC = 1.24) was also found to be a more active antioxidant than ascorbic acid (TEAC $\cong$ 1.0); because of its relatively high concentration in the whole apple juice, chlorogenic acid was the most significant antioxidant present in the type II juice (see below and Table 4). Dehydroascorbic acid was found to have no antioxidant activity, as was the case with benzoic acid and in total 17 of the 29 substances tested known to be present in apple juice (see Table 3).

Relative Contribution of Antioxidant Substances to the TAA of Apple Juice. The relative contribution of the different antioxidants to the TAA of apple juice can be derived by multiplying the TEAC of each substance by its molar concentration in the juice and expressing this figure as a percentage of the TAA. The results of these calculations are shown in Table 4.

Chlorogenic acid and p-coumaroylquinic acid together constituted 38.7% of the TAA of type II juice. Chlorogenic acid was thus the major single antioxidant in whole apple juice. Phloridzin and phloretin xyloglucoside constituted 11.7% of the TAA, while ascorbic acid contributed 1.0% to the TAA of this unsupplemented juice. Ascorbic acid constituted 94.1% of the TAA of the apple drink (type I), which was diluted apple juice that had been supplemented with vitamin C. Since dehydroascorbic acid is not an antioxidant (TEAC = 0), the small amount present in both juices made no contribution to the TAA.

Table 4. Relative Contribution of Antioxidant Substances to the TAA of Apple Juice (Based on TEAC × Concentration for Each Substance as a Percentage of the TAA)

	% contribution to the TAA	
substance	type I juice	type II juice
ascorbic acid	94.1	1.0
chlorogenic acid	1.0	31.9
p-coumaroylquinic acid[a]	0.2	6.8
phloretin glucosides[b]	0.5	11.2
epicatechin		0.5
remaining activity (unmeasured substances or synergistic interactions	4.2	48.6

[a] Assuming a TEAC of 1.0. [b] Phloridzin + phloretin xyloglucoside.

Unaccounted for were 4.2% of the observed TAA of type I juice and 48.6% of the observed TAA of the type II juice. This residual activity in the latter may have been due to either unmeasured substances in the juice or synergistic interactions between the measured components.

Fortification of Apple Juice with Vitamin C. Long life apple juice (designated type II) was fortified with ascorbic acid and tested during storage for 10 days at 4 °C and at room temperature. The results are depicted in Figures 3–5. The aim was to compare the recovered added antioxidant activity with the mass of added ascorbic acid, as a function of time and conditions of storage, so providing an estimate of the stability of the antioxidant content of apple juice under defined conditions as well as the antioxidant reactive material per unit of juice.

Figure 3A shows the measured change in the TAA of fortified and unfortified apple juice at room temperature over a 10 day period, while Figure 3B indicates the change at 4 °C. Figure 4 shows the changes in total ascorbate (OPD fluorescence) at room temperature and at 4 °C over a 10 day period.

Figure 5 depicts the changes in ascorbic acid (iron/Ferrozine method) at room temperature and at 4 °C over a 10 day period. Since the OPD fluoresence method measures total ascorbate and the iron/Ferrozine method measures ascorbic acid only, the difference between the two values for individual samples is equal to the dehydroascorbic acid content. Figure 5C plots the rise and fall in dehydroascorbic acid content (calculated as total ascorbate − ascorbic acid) in fortified and unfortified apple juice over 10 days at room temperature, while Figure 5D shows the rise and fall in dehydroascorbic acid content in fortified and unfortified apple juice over 10 days at 4 °C. Dehydroascorbic acid does not have antioxidant activity (Table 3) and therefore does not contribute to the total antioxidant activity. A decline in dehydroascorbic acid (see Figure 5C) represents irreversible hydrolysis of its ring structure with the formation of diketogulonic acid.

These results demonstrated the relative stability of the antioxidant activity of unfortified whole apple juice

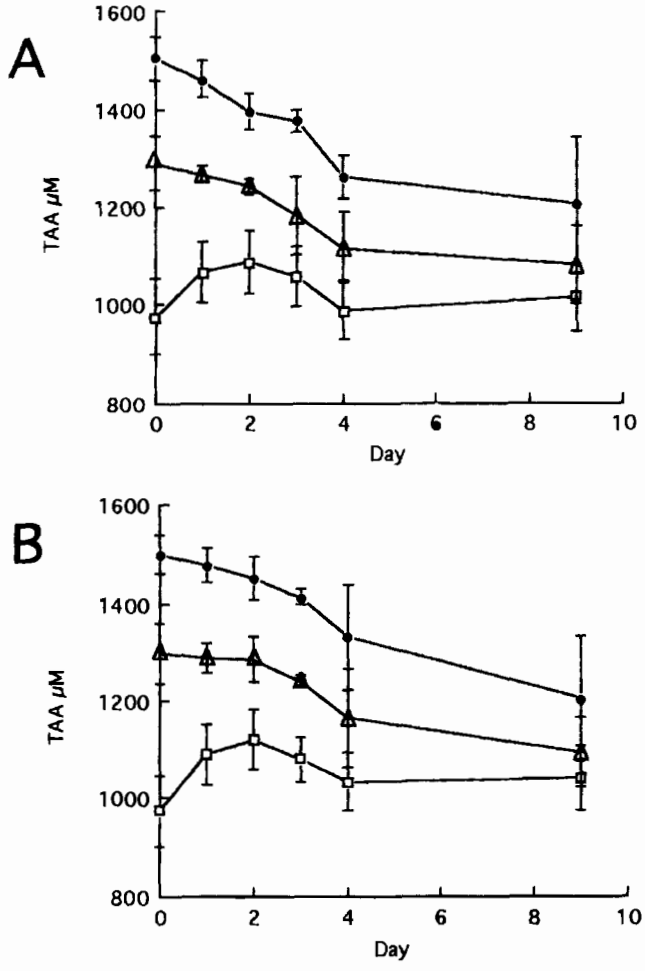


Figure 3. Effect of storage at (A) room temperature and (B) 4 °C for 10 days on the total antioxidant activity (TAA) of apple juice (type II) (□) without additional vitamin C fortification, (△) fortified with 75 ppm vitamin C, and (●) fortified with 150 ppm vitamin C.

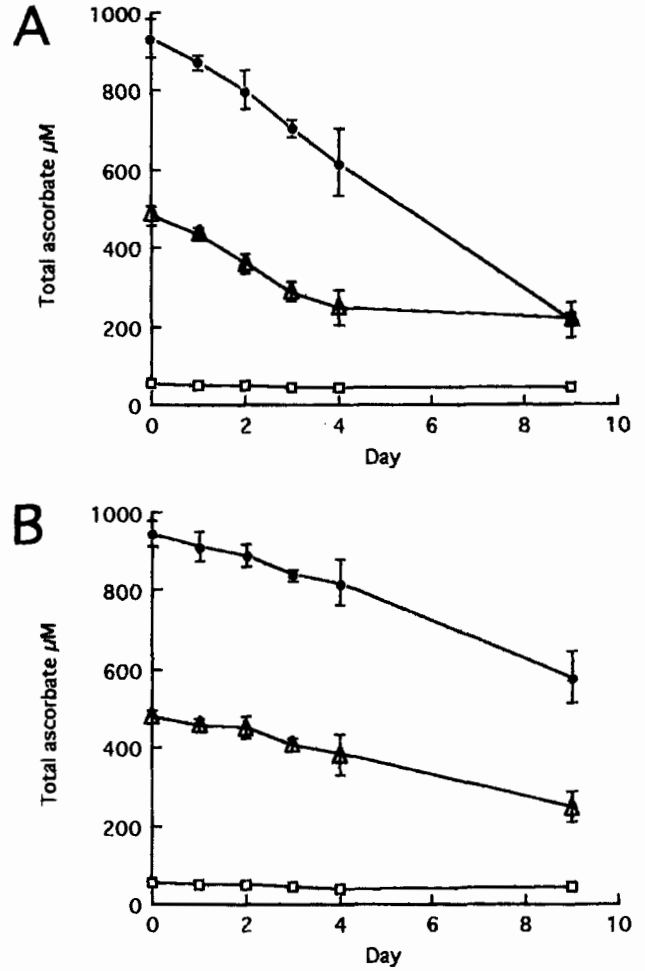


Figure 4. Effect of storage at (A) room temperature and (B) 4 °C for 10 days on the vitamin C content of apple juice (type II) (□) without additional vitamin C fortification, (△) fortified with 75 ppm vitamin C, and (●) fortified with 150 ppm vitamin C. $n = 4$; mean ± 1 SD shown.

both at room temperature and at 4 °C over a 10 day period (Figure 3). This is consistent with our observation that pure solutions of chlorogenic acid are stable for at least 10 days (data not shown). The antioxidant activity of ascorbate-fortified whole apple juice declined progressively over 10 days, somewhat more rapidly at room temperature than at 4 °C. The total ascorbate content of unfortified whole apple juice was low (55 ± 8 μmol/L or 9 ppm), of which only 18% was ascorbic acid (10 ± 2 μmol/L or 1.6 ppm). This explains why the total ascorbate of unfortified apple juice did not show a significant decline during 10 days of storage and underscores the fact that this type of apple juice is a poor dietary source of vitamin C. The ascorbic acid content of fortified apple juice showed a progressive decline over the 10 day storage period at both temperatures, while the dehydroascorbic acid content showed a concomitant rise.

The same determinations were carried out on type I juice (without additional vitamin C supplementation), and a comparison of the changes over a 10 day period in the ascorbate, dehydroascorbate, and TAA of unfortified type I and type II juices is shown in Table 5. The decline in the TAA and ascorbic acid content of type I at room temperature was virtually 100% at room temperature over 10 days.

DISCUSSION

The suppression of $ABTS^{\bullet+}$ formation is a reproducible and practical way to estimate antioxidant activity. By comparison with Trolox we have demonstrated a 20-fold range of molar antioxidant activities (Trolox equivalent antioxidant capacity or TEAC values) among plant-derived flavonoids (Rice-Evans *et al.*, 1995). As is shown in this work, TAA measurement can also be used to analyze mixtures of antioxidants in food matrixes.

These results indicate that vitamin C activity was the major contributor to the TAA of the type I drink and that in the type II juice vitamin C represented only a very minor fraction of the total antioxidant activity, with cinnamic acids as the major identifiable antioxidants. The TAA and ascorbic acid concentrations of all samples showed a highly significant correlation (Figure 6, $P < 0.001$, paired t-test). The TAA value at the y-intercept in Figure 6 (where ascorbate = 0) is approximately equal to the original TAA of the unsupplemented juice. A substantial percentage of the TAA in whole apple juice (type II) was unaccounted for: the correlation between the TAA and the ascorbic acid concentrations in these samples suggests that this fraction represents either the presence of unmeasured antioxidants or an augmentation of activity due to a synergistic interaction between polyphenol components.

Glucose, fructose, sucrose, and sorbitol are the main carbohydrates found in apple juice. Major organic acids

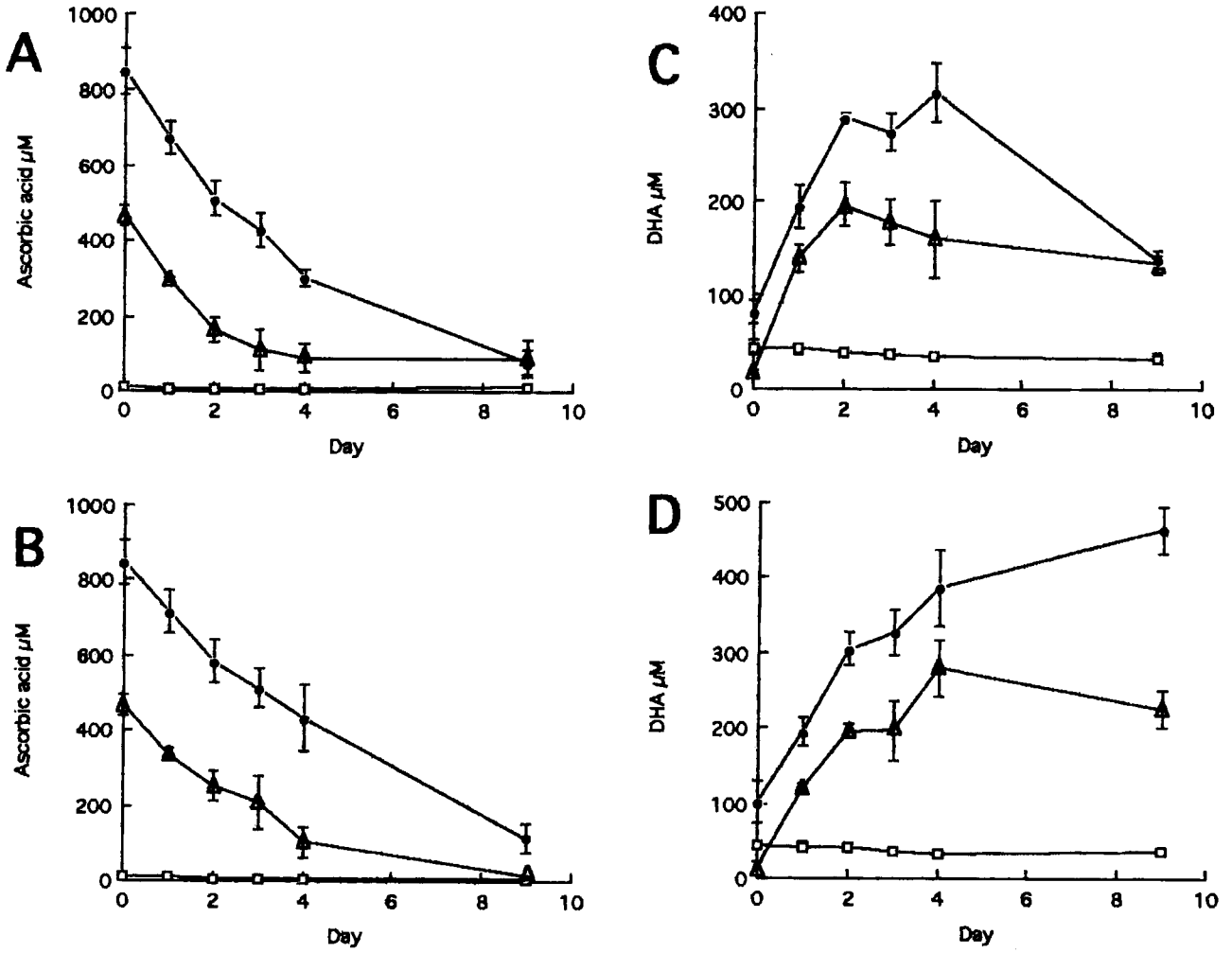


Figure 5. Effect of storage at (A and C) room temperature and (B and D) 4 °C for 10 days on the ascorbic acid content of apple juice (type II) (□) without additional vitamin C fortification, (△) fortified with 75 ppm vitamin C, and (●) fortified with 150 ppm vitamin C.

Table 5. Changes in the Antioxidant Composition of Apple Juice on Storage: Comparison of Type I and Type II Apple Juices[a]

	room temp			4 °C		
apple juice and day	ascorbic acid, μM (± SD)	dehydroascorbic acid, μM (± SD)	TAA, μM (± SD)	ascorbic acid, μM (± SD)	dehydroascorbic acid, μM (± SD)	TAA, μM (± SD)
type I						
day 0	1700 (±65)	23 (±11.)	1807 (± 24.)	1700 (± 65)	23 (± 11)	1807 (± 24)
day 4	721 (± 10)	79 (± 3)	893 (± 56)	1684 (± 47)	25 (± 13)	1909 (± 77)
day 9	5 (± 1)	327 (± 48)	0	1304 (± 19)	231 (± 9)	1329 (± 23)
type II						
day 0	10 (± 2)	45 (± 7)	974 (± 62)	10 (± 2)	45 (± 5)	974 (± 62)
day 4	7 (± 2)	37 (± 3)	991 (± 61)	7 (± 2)	34 (± 3)	1024 (± 60)
day 9	7 (± 2)	34 (± 5)	1014 (± 69)	7 (± 1)	36 (± 3)	1042 (± 66)

[a] Mean values ± 1 SD shown (n = 3).

include malic acid (which is structurally closely related to the glucoside moiety of chlorogenic acid) and citric acid, while asparagine is the predominant amino acid (893 ppm or 6760 μmol/L). Aspartic acid, γ-aminobutyric acid, alanine, and serine are also relatively abundant components of apple juice (Lea, 1991). Total phenolic compounds, including chlorogenic acid, are present at *ca.* 500 ppm, or approximately 1400 μmol/L, in freshly expressed apple juice. Apart from the phenolic compounds, which are demonstrated here to possess widely varying levels of antioxidant activity against radicals in the aqueous phase (as judged by their scavenging of $ABTS^{\bullet+}$), none of this heterogeneous group of substances is an antioxidant. Chlorogenic acid has been identified as the principal antioxidant of apple juice, contributing 32% of the TAA. This substance is present in apple juice in high concentration and has a TEAC value greater than that of ascorbic acid. The presence of chlorogenic acid and other antioxidants in apple juice will enhance both the nutritional value of the juice and its storage capacity in an oxidative environment.

Kuhnau (1976) described the flavonoid phenolics found in apple juice. Many of these substances originate in the skin of the apple and are found to various extents in the freshly expressed juice. They include flavonols (quercetin galactoside, arabinoside, glucoside, and rhamnoside) and anthocyanins (cyanidin as the galactoside, arabinoside, arabinosylgalactoside, and glucoside) (Dick *et al.*, 1987) which are responsible for the red color in

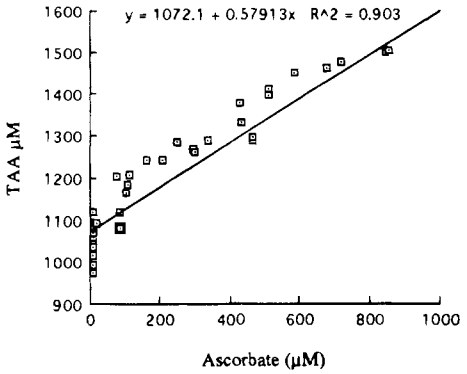


Figure 6. Correlation between TAA (μM) and ascorbate concentration (μM) in apple juice (all samples): unfortified/+ 75 ppm vitamin C/+ 150 ppm vitamin C/+ 300 ppm vitamin C—freshly opened/stored at room temperature/stored at 4 °C. $R^2 = 0.903$. $P < 0.001$ (*** paired t-test).

apple skin. As well as chlorogenic acid, the flesh of the apple contains catechin, epicatechin, and the gallocatechins and epigallocatechins and also a series of biflavans (procyanidins A_1, A_2, B_1, B_2, and B_4) (Lea and Timberlake, 1978). Anthocyanidins and other biflavans that may have been present in the freshly expressed juice are more readily oxidized and precipitated than chlorogenic acid and the cinnamics (Siegelman, 1955) and hence less likely to survive through a manufacturing process in sufficient quantities to be detected in the final juice product. Phloridzin in apples is derived from the core tissue and seeds (Durkee and Poapst, 1965): of the flavonols in the apple juice examined above, the most significant amounts were of phloridzin (phloretin glucoside and xyloglucoside).

Storage at room temperature rather than 4 °C is unlikely to affect the phenolic composition of apple juice over a 10 day period. Spanos *et al.* (1990) reported that a high temperature during initial processing (by diffusion extraction) produced up to a 5-fold increase in the recovery of phloretin glucosides as compared to that obtained in a conventionally pressed juice without temperature elevation. These authors also reported total loss of procyanidins, 60% loss of phloridzin and quercetin, and 36% loss of cinnamics over a 9 month storage period, together with (hydroxymethyl)furfural formation in a "browning" reaction.

The TAA of the diluted apple drink and its ascorbic acid activity (which is the major contributor to the TAA of type I juice) did not survive 10 days of storage at room temperature and were poorly maintained at 4 °C. In contrast, the TAA of whole apple juice (type II) was maintained well after 10 days of storage at both temperatures. Ascorbic acid levels fell more rapidly that total vitamin C levels (ascorbic acid + dehydroascorbic acid) both at room temperature and at 4 °C in vitamin C fortified juice. This demonstrates the importance of the presence of stable antioxidants (such as chlorogenic acid and the phloridzins) in apple juice for the maintenance of antioxidant quality after package opening. The presence of phloridzin in apple tissue has previously been associated with suppressing firmness loss in apples (Oleszek *et al.*, 1988) and with "brown core" discoloration during cold storage (Durkee and Poapst, 1965) and was identified in apple juice by Lea and Timberlake (1974).

Vitamin C activity, however, represents a small part of the antioxidant activity of processed apple juice, unless the juice is fortified with vitamin C on packaging. In this work it was found that such exogenous vitamin C was readily oxidized after package opening, particularly at room temperature, although it was relatively well maintained at 4 °C for 10 days. In those cases where the TAA had been augmented by fortification of the juice with vitamin C, the results showed that the decline in the TAA on storage correlated well with the decline in the ascorbic acid concentration (R^2 0.903, *** $P < 0.001$, paired t-test).

ACKNOWLEDGMENT

The cooperation of Professor R. Swaminathan, Department of Clinical Chemistry, Guy's Hospital, London, is gratefully acknowledged.

LITERATURE CITED

Butts, W. C.; Mulvihill, H. J. Centrifugal analyzer determination of ascorbate in serum or urine with Fe^{3+}/ferrozine. *Clin. Chem.* **1975**, *21*, 1493–1487.

Decharneux, T.; Dubois, F.; Beauloye, C.; Warriaux-de Coninck, S.; Wattiaux, R. Effect of various flavonoids on lysosomes subjected to an oxidative or an osmotic stress. *Biochem. Pharmacol.* **1992**, *44*, 1243–1248.

Deutsch, M. J.; Weeks, C. E. Microfluorimetric assay for vitamin C. *J. Assoc. Off. Anal. Chem.* **1965**, *48*, 1248–1256.

De Whalley, C. V.; Rankin, S. M.; Hoult, J. R. S.; Jessup, W.; Leake, D. S. Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochem. Pharmacol.* **1990**, *39*, 1743–1750.

Dick, A. J.; Redden, P. R.; DeMacro, A. C.; Lidster, P. D.; Grindley, T. B. Flavonoid glycosides of Spartan apple peel. *J. Agric. Food Chem.* **1987**, *35*, 529–531.

Durkee, A. B.; Poapst, P. A. Phenolic constituents in core tissues and ripe seeds of McIntosh apples. *J. Agric. Food Chem.* **1965**, *13*, 137–145.

Hertog, M. G. L.; Feskens, E.; Hollman, P. C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. *Lancet* **1993**, *342*, 1007–1011.

Jones, D. P.; Coates, R. J.; Flagg, E. W.; Eley, J. W.; Block, G.; Greenberg, R. S.; Gunter, E. W.; Jackson, B. Glutathione in foods listed in the National Cancer Institute's health habits and history food frequency questionnaire. *Nutr. Cancer* **1992**, *17*, 57–75.

Kappus, H.; Koster-Albrecht, D.; Remmer, H. 2-Hydroxyoestradiol and (+)-cyanidanol-3 prevent lipid peroxidation of isolated rat hepatocytes. *Arch. Toxicol.* **1979**, Suppl. 2, 321–326.

Kuhnau, J. The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet* **1976**, *24*, 117–191.

Laughton, M. J.; Evans, P. E.; Moroney, M. A.; Hoult, J. R. S.; Halliwell, B. Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. *Biochem. Pharmacol.* **1991**, *42*, 1673–1681.

Lea, A. G. H. The phenolics of ciders: oligomeric and polymeric procyanidins. *J. Sci. Food Agric.* **1978**, *29*, 471–477.

Lea, A. G. H. Reversed-phase high-performance liquid chromatography of procyanidins and other phenolics in fresh and oxidizing apple juices using a pH shift technique. *J. Chromatogr.* **1982**, *238*, 253–257.

Lea, A. G. H. Apple juice. In *Production of Non-carbonated Fruit Juices and Beverages*; Hicks, D., Ed.; Blackie: Glasgow, 1991; pp 182–225.

Lea, A. G. H. Flavor, color and stability in fruit products: the effect of polyphenols. In *Plant Polyphenols*; Hemingway, R. W., Laks, P. E., Eds.; Plenum Press: New York, 1992; pp 827–847.

Lea, A. G. H.; Timberlake, C. F. The phenolics of ciders. *J. Sci. Food Agric.* **1974**, *25*, 1537–1545.

Miller, N. J.; Rice-Evans, C. A.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84*, 407–412.

Naim, M.; Gestetner, B.; Bondi, A.; Birk, Y. Antioxidative and antihemolytic activities of soybean isoflavones. *J. Agric. Food Chem.* **1976**, *24*, 1174–1177.

Negre-Salvayre, A.; Salvayre, R. Quercetin prevents the cytotoxicity of oxidized LDL on lymphoid cell lines. *Free Radical Biol. Med.* **1992**, *12*, 101–106.

Oleszek, W.; Lee, C. Y.; Jaworski, A. W.; Price, K. R. Identification of some phenolic compounds in apples. *J. Agric. Food Chem.* **1988**, *36*, 430–436.

Rice-Evans, C. A.; Miller, N. J. Total antioxidant status in plasma and body fluids. *Methods Enzymol.* **1994**, *234*, 279–293.

Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* **1995**, *22*, 375–383.

Robak, J.; Gryglewski, R. J. Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.* **1988**, *37*, 837–841.

Sichel, G.; Corsaro, C.; Scalia, M.; Di Bilio, A. J.; Bonomo, R. P. In vitro scavenger activity of some flavonoids and melanins ogainst $O_2^{\cdot-}$. *Free Radical Biol. Med.* **1991**, *11*, 1–8.

Siegelman, H. W. Quercetin glycosides of Grimes Golden Apple skin. *J. Biol. Chem.* **1955**, *213*, 647–654.

Sorata, Y.; Takahama, U.; Kimura, M. Protective effect of quercetin and rutin on photosensitized lysis of human erythrocytes in the presence of hematoporphyrin. *Biochim. Biophys. Acta* **1982**, *799*, 313–317.

Spanos, G. A.; Wrolstad, R. E.; Heatherbell, D. A. Influence of processing and storage on the phenolic composition of apple juice. *J. Agric. Food Chem.* **1990**, *38*, 1572–1579.

Thompson, R. S.; Jacques, D.; Haslam, E.; Tanner, R. J. N. Plant proanthocyanidins. Part 1. *J. Chem. Soc., Perkin Trans. 1* **1972**, 1837–1841.

Whiting, G. C.; Coggins, R. A. 4-*p*-coumaroyl quinic acid in apple fruits. *Phytochemistry* **1975a**, *14*, 593–597.

Whiting, G. C.; Coggins, R. A. Estimation of the monomeric phenolics of ciders. *J. Sci. Food Agric.* **1975b**, *26*, 1833–1839.

Received for review November 22, 1994. Accepted April 18, 1995.[⊗] This work was funded by the Ministry of Agriculture, Fisheries and Food (U.K.).

JF9406572

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1995.