

Changes in plasma antioxidant status following consumption of diets high or low in fruit and vegetables or following dietary supplementation with an antioxidant mixture

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The aim of the present study was to examine the effect of consumption of a high-fruit and vegetable diet, or a spray-dried extract of selected fruits and vegetables of high antioxidant content, on indices of antioxidant status of individuals consuming a background diet with minimal antioxidant intake. Plasma antioxidant concentrations were determined in twenty-five men following a 2-week depletion period during which they consumed self-selected low-antioxidant diets (less than three servings of fruit and vegetables with no tea, coffee, red wine or fruit juice). Following this period the volunteers consumed either a self-selected diet containing five to seven servings of fruit and vegetables/d, or 30 g of a spray-dried supplement designed to provide the equivalent antioxidant activity of five to seven servings of fruit and vegetables for 2 weeks in a crossover trial. Following consumption of a high-antioxidant diet for 2 weeks, plasma concentrations of ascorbic acid, α - and β -carotene and lutein+zeaxanthin were all significantly increased ($P < 0.05$) over the depletion period. However, concentrations of lycopene, retinol and tocopherol were not affected. Consumption of the supplement also raised the concentrations of these same antioxidants in plasma. Despite the increases in the concentrations of measured antioxidant nutrients, the 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid-equivalent antioxidant capacity of plasma, as estimated by inhibition of metmyoglobin activity, was not significantly affected by any of the dietary treatments.

Antioxidants: Plasma: Dietary supplement

Over recent years considerable epidemiological evidence has been gathered to suggest an association between consumption of fruits and vegetables and a reduced risk of certain chronic diseases such as cardiovascular disease (Rimm *et al.* 1996), stroke (Gillman *et al.* 1995), hypertension (Ascherio *et al.* 1992), cataracts (Leske *et al.* 1998), macular degeneration (Seddon *et al.* 1994), cancers (Steinmetz & Potter, 1996) and DNA damage (Ames, 1998). In a number of cases the benefits of fruit and vegetable consumption may be a result of the consumption of dietary fibre.

However, fruits and vegetables are also a rich source of other food factors, including vitamins, minerals and non-nutritive phytochemicals which may exhibit biological activity in a number of ways. These phytochemicals may act as antioxidants, induce enzymes, act as pro- or antioestrogens, or modulate bacterial populations in the intestine (Lampe, 1999). The antioxidant activity of fruits, vegetables and beverages is often assumed to be of greatest

importance in combating a number of degenerative diseases, as free radical-related damage has been implicated in causing many of these conditions (Ames, 1998). However, the recommended daily consumption of five or more servings of fruit and vegetables (Baghurst *et al.* 1992) to accomplish this objective is rarely met in many Western diets. In an effort to provide a viable means of increasing the intake of nutrient and non-nutrient antioxidants we used data collected earlier (Record *et al.* 1998) to formulate a spray-dried plant-based supplement with high antioxidant activity. The antioxidant activity was equivalent to that contained in five to seven servings of fruit and vegetables, and was derived from a diverse range of antioxidants.

The efficacy of this supplement was compared in twenty-five volunteer males with a self-chosen dietary regimen when consumption of fruit and vegetables was encouraged *v.* a control diet based on low fruit, vegetable and polyphenol intake. The variables measured were plasma antioxidant capacity, plasma antioxidant vitamins and

Abbreviation: Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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Table 1. Composition of the spray-dried antioxidant supplement*

Ingredient	Measured antioxidant activity (mmol/g)	Level in supplement (g/kg)	Antioxidant contribution (mmol/d)
Green tea polyphenol	7200	10.6	2290
Turmeric	36	34.1	37
Capsicum	69	306.6	635
Carrot	6.4	400.6	77
Maize	4.0	202.1	24.2
Silverbeet (<i>Beta vulgaris</i>)	42	46.0	58
Total			3121

* Volunteers were asked to consume 30 g supplement/d in 2 × 15 g portions for 2 weeks.

carotenoids as well as albumin and uric acid, the other main non-nutrient antioxidants in plasma.

Materials and methods

Preparation of the antioxidant supplement

Antioxidant capacities of a range of fresh fruits and vegetables were determined on water-soluble portions of a number of fruits and vegetables, herbs and spices (Record *et al.* 1998). From these preliminary investigations it was possible to calculate a total antioxidant capacity range derived from the ingestion of five to seven normal servings of a variety of common fruit and vegetable foods. Based on this, a number of samples were evaluated for their suitability to be spray-dried following extraction. Edible portions of capsicum, carrot, maize and silverbeet (*Beta vulgaris*) were trimmed from fresh produce, except maize, which was from frozen maize kernels (McCain's, Doncaster, Victoria, Australia). The edible portions were milled in a commercial mill to pass through a 12.7 mm screen and pressed through a 0.5 mm screen to remove larger fragments. Maltodextrin (Starch Australasia, Sydney, NSW, Australia) was added to the maize, capsicum and carrot (5.5, 9.2 and 5.7 % (w/v) final concentrations respectively) to aid drying. The extracts were spray-dried in a commercial spray drier with an air inlet temperature range of 195–200°C and an air outlet temperature range of 95–100°C at a feed rate of approximately 220 ml/min. All spray-dried materials were prepared by Food Science Australia (Sydney, NSW, Australia). In addition, green tea polyphenols (50 % (w/w); Wing Lee, Hong Kong) and turmeric powder (Masterfoods, Wyong, NSW, Australia) were included in the final supplement. The formulation and measured antioxidant activity of each ingredient as well as that of the final supplement, as measured by the method of Miller *et al.* (1993), is presented in Table 1. The final

concentration of maltodextrin was calculated to be 6.2 % (w/w).

The supplement was analysed for the contents of several vitamins and carotenoids and these were compared with the Australian recommended dietary intake, where established (National Health and Medical Research Council, 1991). As can be seen from Table 2, the contribution of the supplement to the vitamin C recommended dietary intake (40 mg/d) was small. In terms of retinol equivalents (recommended intake of 500–750 retinol equivalents/d) the supplement supplied approximately 524 retinol equivalents ($0.167 \times (\beta\text{-carotene} + 0.5 \times \alpha\text{-carotene})$), as described by Holland *et al.* (1995), which was close to meeting the recommended daily requirements. The lutein content was also substantial; however, the supplement was found to contain only negligible amounts of lycopene, retinol and vitamin E.

Study participants and protocol

Following a newspaper advertisement and an initial screening to exclude individuals with clinical conditions or dietary habits that could compromise or be compromised by participation in the study, twenty-five males (mean age 48.3 (range 25–60) years; mean BMI 24.8 (20.1–30.3) kg/m²; all non-smokers) were recruited. The sample size was based on power calculations from previous studies (Miller *et al.* 1993) and were predicted to detect a 10 % change with a power of 0.95. Exclusion criteria included the use of dietary supplements (vitamins, carotenoids and minerals), adherence to vegetarian or macrobiotic diets, regular consumption of medications (e.g. salicylates) known to be antioxidants, or consumption of excessive amounts of alcohol (more than three standard glasses of alcohol per d). Volunteers attended an information night where background and study details were explained, and participants then received detailed instructions in smaller groups.

Subjects were instructed to consume diets low in antioxidants (i.e. no more than one piece of fruit and two servings of vegetables per d, and to avoid tea, coffee, red wine, fruit juices, antioxidant tablets and aspirin) for a 2-week washout period before the trial. Based on previous data (Record *et al.* 1998), the total antioxidant capacity of the diets due to fruit and vegetable consumption should have been approximately 1 mmol 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox) equivalents/d. Suggestions were made as to various menus and dishes that

Table 2. Measured vitamin and carotenoid composition of the spray-dried antioxidant supplement and the amount provided daily in 30 g taken as 2 × 15 g portions

	µg/g supplement	µg supplied/d
α-Carotene	34.5	1033
β-Carotene	87.6	2628
Lutein	18.1	543
Vitamin C	2.1	63

could be prepared. This period of 2 weeks was chosen because significant decreases in plasma ascorbic acid concentrations have been detected within this time with provision of a diet low in ascorbic acid (Samman *et al.* 1997). After an overnight fast, blood was withdrawn (with heparin as anticoagulant). Thereafter, half the subjects were given sachets (30 g) of the supplement and asked to consume this as 2×15 g portions suspended in water on a daily basis; this amounted to 3.0 mmol Trolox equivalents/d.

The remaining volunteers were instructed to consume a diet high in antioxidants (i.e. five to seven servings of fruit and vegetables per d, together with fruit juices), which would be expected to provide approximately 1.7–2.4 mmol Trolox equivalents/d. As with the low-antioxidant phase, suggestions were given as to the composition of meals and menus that would yield a high proportion of fruits and vegetables.

After a further 2 weeks, all subjects returned for repeat blood sampling. At this time those subjects in the group consuming the supplement were asked to consume 15 g of the supplement in water and a further blood sample was taken 1 h later. Volunteers consuming the high- or low-antioxidant diet were given a light breakfast of water and toast spread with margarine as a low-antioxidant comparison with the supplement, and a second blood sample was taken 1 h later. For the final 2 weeks before blood sampling, subjects reversed the regimen, with those consuming a high-antioxidant diet consuming the supplement and vice versa. The postprandial sampling time of 1 h was chosen because several other studies (for examples, see Maxwell *et al.* 1994; Day & Stansbie, 1995; Cao *et al.* 1998; Benzie *et al.* 1999) detected increased plasma antioxidant activity after similar periods. Compliance with the dietary regimen was assessed by interview with a dietitian, using a modified food intake questionnaire as a prompt at each visit. Consumption of the product was monitored by a brief questionnaire and only two of the subjects reported missing the supplement for more than 1 d.

Informed consent was obtained from each volunteer, and the CSIRO Health Sciences and Nutrition Human Ethics Committee approved all procedures.

Laboratory analyses

Immediately after collection blood samples were placed on ice in the dark, and plasma was separated from the whole blood within 1 h and snap-frozen in liquid N₂ before storage at -80°C . Antioxidant activity in plasma was determined by the method of Miller *et al.* (1993). Deproteinisation of the plasma was carried out by ultrafiltration (Centrisort 1, 20000d; Sartorius, Melbourne, Victoria, Australia). Metmyoglobin and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (diammonium salt) and H₂O₂ were obtained from Sigma Chemicals (Sydney, NSW, Australia). Trolox was purchased from Aldrich Chemicals, Sydney, NSW, Australia. Ascorbic and uric acids were measured by HPLC following stabilisation of the plasma with metaphosphoric acid immediately after isolation (Nagy & Degrell, 1989). Analysis of the plasma samples for antioxidants was carried out with slight modifications to the procedures described by Yang & Lee (1987) and Khachik *et al.* (1992). A Shimadzu LC 10

HPLC (Shimadzu, Kyoto, Japan) fitted with a refrigerated autosampler and an SPD-M10Avp photodiode array detector with a class LC10 chromatography workstation was used for analysis of the prepared samples. Isocratic separations of the fat-soluble vitamins and carotenoids were carried out on a Rainin (Walnut CK, CA, USA) (4.6 mm i.d. \times 250 mm length) C₁₈ (5 μ spherical particles) reverse-phase column. The mobile phase was a mixture of (% v/v): acetonitrile 55, methanol 22, hexane 11.5, dichloromethane 11.5; the flow rate was 1.0 ml/min. Ammonium acetate (0.01 %, w/v) was added to the mobile phase for stabilisation of the carotenoids. *Trans*- α - and β -carotene, lycopene, lutein, retinol, α -tocopherol and α -tocopheryl acetate were all obtained from Sigma Chemicals. Solvents, hexane, methanol, acetonitrile, ethanol and dichloromethane were all of analytical HPLC grade. A standard reference material (National Institute of Standards and Technology product 968b) was analysed with each batch of samples. The reference material with certified concentrations of retinol, α -tocopherol, lutein and β -carotene was used to monitor the daily variation in analyses. The CV for the analyses ranged from 2.45 % for retinol to 2.9 % for α -tocopherol. Statistics were evaluated by paired Student's *t* test with $P = 0.05$ as the criterion for significance.

Results

Concentrations of antioxidants present in the plasma are presented in Table 3. Ascorbic acid concentrations in the plasma samples taken after consumption of the antioxidant-depleted diet for 2 weeks were significantly lower ($P < 0.0001$) than those of the other two dietary treatments. The concentrations of α -carotene, β -carotene and lutein were all significantly increased ($P < 0.005$ or less) over the baseline concentrations following consumption of either the high-antioxidant diet or the supplement. Concentrations of α -tocopherol, lycopene and retinol were largely unchanged during the course of the study, although there was a statistically, but probably non-physiologically, significant increase in fasting plasma retinol concentrations between the low-antioxidant dietary period and the high-antioxidant dietary period ($P = 0.03$). Plasma uric acid concentrations were unchanged during the study, but plasma albumin concentrations were significantly lower than those after either the high-antioxidant dietary period ($P < 0.09$) or consumption of the supplement ($P < 0.004$).

Plasma antioxidant activities from whole and deproteinised plasma samples collected after the overnight fast and 1 h after the breakfast are presented in Table 4. There were no significant differences between any of the groups, either before or after the light breakfast or 15 g supplement. Removal of protein, which is a significant contributor to the antioxidant capacity of plasma, had no effect on antioxidant activity (Table 4) in the postprandial samples. Owing to insufficient material, deproteinised fasting plasma samples could not be analysed.

Discussion

As one of the major aims of the study was to monitor changes against a background diet low in antioxidants,

Table 3. Antioxidants in plasma* from volunteers following 2-week period of consumption of low-antioxidant diets, diets high in antioxidants or the low-antioxidant diet together with 30 g dietary supplement†

	Low-antioxidant diet	High-antioxidant diet	Statistical significance of difference: <i>P</i> <	Supplement	Statistical significance of difference‡: <i>P</i> <	Pooled SE
Albumin (g/l)	41.8	44.0	0.04	44.7	0.0009	0.05
α-Tocopherol (μmol/l)	28.8	28.8	NS	28.1	NS	0.9
Ascorbic acid (μmol/l)	29.9	47.1	0.0001	46.2	0.0001	1.3
α-Carotene (μmol/l)	0.11	0.17	0.0001	0.20	0.0001	0.02
β-Carotene (μmol/l)	0.56	0.74	0.0001	0.80	0.0001	0.02
Lutein+zeaxanthin (μmol/l)	0.33	0.46	0.003	0.40	0.02	0.03
Lycopene (μmol/l)	0.56	0.56	NS	0.50	NS	0.04
Retinol (μmol/l)	2.09	2.16	NS	2.09	NS	0.05
Uric acid (μmol/l)	352	347	NS	359	NS	7.7

* Fasting blood samples were collected after an overnight fast.

† For details of diets and supplement, see Tables 1 and 2 and p. 460, and for details of procedures, see p. 460.

‡ Relative to low-antioxidant diet.

baseline samples (i.e. samples collected whilst the subjects consumed their usual diets) were not collected. Following the adoption of a largely self-selected antioxidant-poor diet for 2 weeks, transition to a diet rich in fruits and vegetables resulted in significant increases in plasma concentrations of vitamin C and some, but not all carotenoids. Samman *et al.* (1997) showed significant reductions in plasma vitamin C concentrations after 2 weeks on a low-vitamin C dietary regimen. The observation that plasma albumin concentrations were significantly lower after the depletion period than after either supplementation period is difficult to interpret.

Rock *et al.* (1992) monitored depletion of carotenoids in plasma of subjects consuming diets low in carotenoids for up to 2 months. In their study plasma carotenoids, in particular lycopene, declined most rapidly over the first 2 weeks of the depletion period, followed by a slower reduction over the remaining period. From their results, they suggested that carotenoids exist in two pools in the body; one with rapid turnover, and another (possibly in tissue stores) with greater resistance to depletion. In the

Table 4. Antioxidant (AO) activities (μmol 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid equivalents/l) of whole and deproteinised plasma samples of volunteers following 2 weeks of consumption of low-AO diets, diets high in antioxidants or the low-AO diet together with 30 g of the dietary supplement.*†

(Mean values with their standard errors for twenty-five volunteers per treatment group)

	Fasting		Postprandial	
	Mean	SE	Mean	SE
Whole plasma				
Low-AO diet	1.54	0.05	1.52	0.05
High-AO diet	1.51	0.05	1.50	0.06
Supplement	1.51	0.05	1.51	0.06
Deproteinised plasma				
Low-AO diet	ND		0.56	0.02
High-AO diet	ND		0.56	0.03
Supplement	ND		0.56	0.03

ND, not determined.

* Fasting blood samples were collected after an overnight fast and the postprandial samples 1 h after consumption of toast and water (low- and high-AO diets) or 15 g of the supplement.

† For details of diets and supplement, see Tables 1 and 2 and p. 460, and for details of procedures, see p. 460.

present study the dietary supplement contained little lycopene, so it is surprising that subjects consuming the supplement directly after the low-antioxidant period showed no change in their plasma lycopene concentrations. Indeed, plasma lycopene concentrations remained constant throughout the study, even when the participants were consuming a self-selected high-antioxidant diet. In addition, ascorbic acid concentrations were not different when the subjects consumed their chosen high-antioxidant diet or the supplement, despite the fact that the ascorbate content of the supplement was minimal. It is possible that inclusion of extra antioxidants in the diet via the supplement allowed ascorbate to be spared. Other studies have reported increased plasma antioxidant activity in periods ranging from less than 1 h up to 4 h following consumption of tea or tea polyphenols (Serafini *et al.* 1996; Benzie *et al.* 1999; Nakagawa *et al.* 1999), red wine or grape extracts (Maxwell *et al.* 1994; Day & Stansbie, 1995; Whitehead *et al.* 1995; Cao *et al.* 1998; Durak *et al.* 1999). Cao *et al.* (1998) also reported increased plasma antioxidant activity following consumption of strawberries, spinach and vitamin C. The extract used in the present study contained antioxidants equivalent to approximately five to seven servings of fruit and vegetables per d. On the last day of the intervention with the supplement, antioxidant activities of plasma taken 1 h after consumption of the equivalent of 2.5–3.5 servings of fruit and vegetables was unchanged from the sample obtained after the overnight fast. Despite the measurable lower concentrations of antioxidants in the plasma after the depletion period, the antioxidant activity of the plasma samples did not increase after 2 weeks of dietary change or consumption of the extract. After removal of plasma albumin, which represents a major contributor to the total antioxidant capacity of plasma (Cao & Prior, 1998), the antioxidant capacity due to the lower-molecular-weight components did not change. Of the limited number of similar studies involving dietary manipulation over significant periods of time, Young *et al.* (1999) failed to find any increase in the antioxidant capacity of plasma measured either as the Fe³⁺-reducing ability of plasma (Benzie & Strain, 1996) or as Trolox-equivalent antioxidant capacity (Miller *et al.* 1993). Castenmiller *et al.* (1999) also failed to find any changes in antioxidant capacity in subjects supplemented with either

β -carotene or spinach-based products, even though significant elevations in plasma carotenoid concentrations were detected. Cao *et al.* (1998) have compared the Trolox-equivalent antioxidant capacity assay as described by Miller *et al.* (1993) and commercialised by Randox with two other methods, the Fe³⁺-reducing ability of plasma assay (described by Benzie & Strain, 1996) and the oxygen radical absorbance assay developed by Cao *et al.* (1995). In their hands the Trolox-equivalent antioxidant capacity assay was less sensitive than either of the other assays to changes in plasma antioxidant activity in the 4 h following consumption of antioxidant foods. Differences between the methodologies of the assays might account for the lack of effect seen in the present study, although it is possible that the antioxidant capacity of plasma is under tight homeostatic control and can be overridden only with acute consumption of large amounts of antioxidant-rich foods or beverages.

Conclusions

Consumption of a dietary supplement representing antioxidants from five to seven servings of fruit and vegetables has been shown to increase the major antioxidant nutrients in human plasma to concentrations similar to those obtained through inclusion of fruits and vegetables in the diet. Lycopene and ascorbic acid concentrations were maintained or increased, even though the amounts supplied by the supplement were minimal. Despite the significant changes in plasma antioxidant components, there were no detectable changes in plasma antioxidant activity detectable by the Trolox-equivalent antioxidant capacity assay.

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