Effect of acute ingestion of fresh and stored lettuce (*Lactuca sativa*) on plasma total antioxidant capacity and antioxidant levels in human subjects

Mauro Serafini*, Rossana Bugianesi, Monica Salucci, Elena Azzini, Anna Raguzzini and Giuseppe Maiani

Antioxidant Research Laboratory at the Unit of Human Nutrition, Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (INRAN), Rome, Italy

The present study investigated whether storage under modified-atmosphere packaging (MAP) affected the antioxidant properties of fresh lettuce (*Lactuca sativa*). Eleven healthy volunteers (six men, five women) consumed 250 g fresh lettuce, and blood was sampled before (0 h) and 2, 3 and 6 h after consumption. The protocol was repeated 3 d later with the same lettuce stored at 5°C under MAP conditions (O_2–N_2 (5:95, v/v)). Results showed that after ingestion of fresh lettuce, plasma total radical-trapping antioxidant potential (TRAP), measured as area under the curve, was significantly higher (1·3 (SEM 0·3) mmol/l per 6 h; $P<0.05$) than the value obtained with MAP-stored lettuce (0·1 (SEM 0·2) mmol/l per 6 h). Plasma TRAP, quercetin and *p*-coumaric acid were significantly different from baseline values ($P \leq 0.05$) 2 and 3 h after fresh lettuce ingestion. Caffeic acid increased significantly at 3 h ($P<0.05$). Plasma β-carotene levels increased significantly at 6 h ($P<0.05$). Vitamin C concentrations (mg/l) rose from 10·9 (SEM 2·0) to 12·7 (SEM 3·0) ($P<0.001$), 12·7 (SEM 2·0) ($P<0.01$) and 12·9 (SEM 3·0) ($P<0.05$) at 0, 2, 3 and 6 h respectively. No changes were observed after ingestion of MAP-stored lettuce for all the measured markers. Our present results showed that ingestion of MAP-stored lettuce does not modify plasma redox status in healthy subjects. Further research is needed to develop post-harvesting techniques able to preserve the bioactive molecule content of plant food.

Plasma antioxidant capacity: Modified-atmosphere packaging: Vegetables: Bioavailability

The existing scientific evidence regarding a primary role for fruits and vegetables in preventing degenerative diseases is consistent and trustworthy (Gey *et al.* 1987; Block *et al.* 1992). However, the exact mechanism for the link between plant food consumption and the decreased mortality rates for these diseases has not yet been fully elucidated. The precise nature of the compounds that may be involved in this protective function and the mechanisms responsible for it are still unclear. However, in recent years, several antioxidant nutrients present in plant food, such as vitamin C and vitamin E, have been suggested to exert a protective role against the diseases mentioned earlier (Halliwell, 1996). The mechanism by which they are supposed to act in man is based on their direct or indirect modulation of *in vivo* oxidative stress. Recent increasing evidence supports the hypothesis that other hitherto scarcely studied

molecules, such as phenolic compounds, could also play an essential health-promoting role (Duthie *et al.* 2000).

Over the last few decades, nutrition policies have strongly promoted the consumption of a diet containing >400 g fresh vegetables and fruits/d as a nutritional goal for health promotion. On the one hand, consumer response to healthy eating campaigns has created a sustained and increasing demand for foods that are 'healthier', free from contaminants, pesticides or additives. On the other hand, sociological factors, such as the lack of time necessary for purchasing fresh products from the market place, cooking them in a domestic setting and serving them according to traditional methods, hinder the conversion to a healthier, more natural way of living. These conditions have stimulated an increasing demand for 'ready-to-eat' vegetables, leading to a flourishing market of pre-packed fresh products,

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packaged under different conditions to extend shelf-life. The most studied and widely used packaging method for storing raw fruits and vegetables is modified-atmosphere packaging (MAP) (Kader *et al.* 1989; Powrie & Skura, 1991). The aim of MAP is to create an optimal gas balance inside the package, where the respiration activity of a product is as low as possible and the concentrations of O_2 and CO_2 are not detrimental to the product. In the past few years, 'ready-to-use' MAP-stored lettuce, one of the most widely consumed dietary vegetables, has become very popular and fresh-cut lettuce stored in polypropylene film is available in many supermarkets. However, due to the high respiration rates and the browning processes during storage, the antioxidant molecules present in vegetables could become oxidised (Brecht, 1980). Therefore, it appears important to assess if vegetables retain their antioxidant activity *in vivo* and whether bioavailability of their bioactive compounds is somehow modified by MAP storage conditions. In the present study, we assessed whether post-harvest MAP storage affected the *in vitro* and *in vivo* antioxidant properties of lettuce (*Lactuca sativa*). In addition, we determined whether the plasma redox status of human subjects responds to the acute ingestion of fresh lettuce, identifying the bioactive antioxidant molecules involved.

Materials and methods

Subjects and protocol

Eleven healthy volunteers (six men and five women) aged 29–45 years were recruited from the Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione (INRAN; Rome, Italy) staff. Subjects were non-smokers, normolipidaemic and taking no drug or vitamin supplements, with a mean weight of 63·8 (SEM 10·5) (range 48·0–86·0) kg and BMI 22·2 (SEM 2·2) (range 19·4–27·5) kg/m^2. Each subject signed a consent form approved by the Human Subjects Committee of INRAN. On two different days, subjects consumed fresh lettuce (phase 1) and stored lettuce (phase 2). The first day of the present study (phase 1), a butterfly needle was inserted into a forearm vein of the subjects after they had fasted for 12 h and baseline blood samples were collected. After blood collection, subjects consumed 250 g fresh lettuce and venous blood samples were collected at 2, 3 and 6 h into vacutainer tubes containing heparin or Na$^+$ EDTA as anticoagulant. Blood samples were immediately centrifuged at 3000 *g* for 10 min (4°C), plasma was separated and placed at −80°C for analyses. Total radical-trapping antioxidant potential (TRAP) was measured within 3 h of the blood collection. A portion of plasma was immediately stabilised with metaphosphoric acid (0·75 mol/l; BDH Laboratory Supplies, Poole, Dorset, UK) and stored at −80°C for vitamin C analysis. The protocol was repeated after 3 d (phase 2); this time, each subject (*n* 8) received 250 g of the same lettuce used in phase 1 stored at +5°C under MAP conditions.

Preparation of plant material

Lettuce (*Lactuca sativa*, 7 kg), obtained from a local agricultural cooperative, was harvested on the same day, the outer wrapped leaves of lettuce were discarded, the edible part was washed and chopped into small pieces and divided into two portions: one was given to the subjects (phase 1) and the other was stored under MAP conditions at +5°C until administration (phase 2). The lettuce (250 g) was put into 30 µm polypropylene film bags and subjected to the MAP treatment. MAP packaging was achieved by flushing O_2–N_2 (5:95, v/v) for 10 s into the bags before heat sealing. The gas content in each bag of lettuce was analysed with a Servomax 1100A O_2 analyser (Taylor Instruments Analytic Ltd, Crowborough, Sussex, UK) and Morgan 8010 CO_2 analyser (PK Morgan, Chatham, Kent, UK). Lettuce samples were also used for food composition analysis and for assessing the effect of time on the *in vitro* antioxidant capacity of fresh and MAP-stored lettuce.

Chemicals

All solvents and reagents were HPLC or Optima grade; common reagents were purchased from Sigma (St Louis, MO, USA), Carlo Erba (Milan, Italy) and BDH Laboratory Supplies, and were the highest grade available.

Phenolic hydrolysis and extraction from food and plasma

Food polyphenols were hydrolysed to obtain aglyconic forms and extracted as previously described (Hertog *et al.* 1992) with some modifications. Briefly, one whole plant of lettuce was stripped of the outer wrapped leaves and divided longitudinally in two equal parts. Each part was washed, dried and chopped into small pieces by hand. One part was homogenised and analysed, while the other was subjected to MAP treatment as described earlier (p. 616) and homogenised just before analysis. Polyphenols were extracted from 3 g homogenised lettuce with ethyl acetate after acidic hydrolysis with HCl–methanol (1:1, v/v) at 90°C for 2 h exactly as described by Hertog *et al.* (1992).

Plasma circulating phenols (caffeic acid, *p*-coumaric acid and quercetin) were detected in their free form after enzymatic and acidic hydrolysis of the conjugated forms. To perform the enzymatic hydrolysis, 0·5 ml enzymatic solution containing $5·5 × 10^5$ U sulfatase and $1·0 × 10^7$ U β-glucoronidase (sulfatase S 3009 type H-5 from *Helix pomatia*, also containing β-glucoronidase; Sigma)/litre 0·2 M-acetate buffer (pH 5) were added to 0·5 ml plasma. The mixture was then incubated at 37°C for 45 min. Immediately afterwards, acidic hydrolysis was performed by incubating the mixture with 1 ml 3 M-HCl–MeOH (1:1, v/v) for 30 min at room temperature. Phenols were extracted by adding 2 ml ethyl acetate, followed by 3 min stirring and sonication for 1 min before centrifugation at 1700 *g* for 5 min. The extraction procedure was repeated twice and the two organic layers were combined and evaporated under an N_2 flow. The residue was dissolved into 250 µl mobile phase (phosphate buffer (pH 2·8)–methanol (1:1, v/v)).

Chromatography

Quantitative analysis of caffeic acid, *p*-coumaric acid and quercetin was performed by a HPLC system comprising

an ESA MODEL 540 refrigerated autoinjector (4°C), an ESA MODEL 580 solvent delivery module with two pumps, an ESA 5600 eight-channel coulometric electrode array detector and the ESA coularray operating software, which controlled all the equipment and carried out the data processing (ESA, Chelmsford, MA, USA). Phenols in the extracts were separated by a Supelcosil LC-18 (particle size 5 μm) column (25·0 × 4·6 cm) with a Perisorb Supelguard LC-18 (Supelco, Bellefonte, PA, USA) at the temperature of 30°C maintained by a column heater. Injection was performed with an autoinjector (100 μl fixed loop) and the volume injected was 30 μl. The mobile phase comprised two solvents: a sodium phosphate monobasic solution (0·01 M brought to pH 2·8 with orthophosphoric acid (850 ml/l) (buffer, solvent A)) and methanol (solvent B). Solvent A was filtered through a 0·2 μm membrane filter. The linear gradient applied consisted of 87 % solvent A, decreasing linearly to 60 % over 13·5 min, to 10 % after a further 26·5 min, reaching the final conditions of 0 % 3·0 min later. The elution program returned to 87 % of solvent A over 3 min, maintaining these conditions for 4 min before the subsequent injection. The flow rate of the eluent was constant at 1 ml/min and the setting potentials were: 60, 120, 200, 340, 580, 620, 760 and 900 mV (v. Palladium reference electrode). The phenols peaks were determined by matching sample peaks with standard peaks on the basis of their retention time ($\pm 4 \%$) and on the accuracy ratio between adjacent channels ($\pm 30 \%$) (Svendsen, 1993).

The method of the external standard was used for the calibration. Standard stock solution containing caffeic acid, p-coumaric acid and quercetin in methanol was stored at −20°C. The stability of the standard was checked every day by u.v. spectroscopy for all the period of the present study. The calibration graphs for each polyphenol were obtained by a least squares linear fitting of the peak height (nA) of the dominant channel v. phenol concentration (mg/l). Standard curves were constructed every day of the present study to check the daily instrumentation performance.

Method validation

Since the method used for polyphenols determination in food was validated by the authors (Hertog *et al.* 1992) only for flavonoid class and using lyophilised plant material, we performed a validation study to verify the method suitability. Precision was estimated as the CV of intra- and inter-day replicate analyses. For both hydroxycinnamic acids and quercetin, a CV <8 (n 10) and <10 % (n 20) was found for intra- and inter-day precision respectively.

Due to the lack of blank matrix, the method of standard addition was used in order to evaluate intra- and inter-day method bias by comparing the slopes of the standard addition line with the calibration line. Standard addition was performed by preparing four different solutions of p-coumaric acid, of caffeic acid and of quercetin: 0·25, 0·5, 1·0 and 2·0 g/l methanol. Each solution (200 μl) was used to spike a sample of homogenised lettuce (3 g) that was processed exactly as described earlier. The procedure

was repeated in triplicate on two different occasions. An intra-day bias <7 % and inter-day bias <12 % was found for each compound tested.

The same experiment conducted to calculate bias was used to deduce recovery data. Relative recovery was calculated by dividing the difference between the responses of spiked and non-spiked processed samples by the response of pure unprocessed standard. A recovery >90 % was calculated at each concentration for both hydroxycinnamic acids and quercetin.

To evaluate accuracy, precision and absolute recovery of the method used to analyse polyphenols in plasma, we analysed spiked samples in a concentration range bracketing the interval in which experimental values fall, in replicates of four, on two separate occasions. Spiked samples were prepared at four concentrations of each standard (quercetin, caffeic and p-coumaric acid) ranging between 12 and 100 μg/l, adding a suitable volume of a solution containing 0·5 mg standard/l methanol and 500 μl previously analysed plasma to ascertain the absence of quercetin, caffeic and p-coumaric acid over limit of detection (calculated as the amount of polyphenol resulting in a peak height of three times the standard deviation of the baseline noise). Spiked samples were processed and analysed exactly as described earlier. For all polyphenols tested, a CV <5 % was found for intra-day precision, while a CV <8 % was calculated for inter-day precision. Bias <10 % and recovery >8 % was found for each compound.

Measurement of the total antioxidant capacity of plasma and lettuce: total radical-trapping antioxidant potential assay

The method (Ghiselli *et al.* 1995) is based on the protection afforded by plasma or other substrate, against the decay of a fluorescent target, R-phycoerythrin during a controlled peroxidation reaction. In the present study, plasma or lettuce extract was added to the reaction mixture, made up to a final volume of 2·0 ml and pre-incubated at 37°C for 5 min in 10 mm quartz fluorometer cells. The oxidation reaction was started by adding a peroxyl radical generator (2,2′-azobis(2-amidinopropane) dihydrochloride) to a final concentration of 5·0 mM. The results were standardised using 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, a water-soluble analogue of α-tocopherol, and expressed as μmol peroxyl radicals trapped per litre plasma or per g lettuce.

The edible portion of lettuce was homogenised in a blender after the addition of deionised water (1:2, w/v) (Cao *et al.* 1996). The homogenate was then centrifuged at 34 000 *g* for 30 min (4°C). The supernatant fraction (water-soluble) was recovered and used directly for the TRAP assay. The residue was washed twice with deionised water and further extracted using pure acetone (1:4, w/v), and shaking at room temperature for 30 min. The acetone extract (liposoluble fraction) was recovered after centrifugation (34 000 *g*, 10 min, 4°C), and the supernatant fraction was used for the TRAP assay. The TRAP value of the lettuce was obtained by adding

together the TRAP value of the water- and liposoluble fractions.

Other measurements

The total polyphenol content in lettuce was quantified following a colorimetric assay with Folin–Ciocalteu reagent (Swain & Hillis, 1959). Vitamin C was extracted according to Margolis *et al.* (1990) and Margolis & Schapira (1997) respectively for food and plasma. Plasma and food vitamin C was measured according to Margolis & Schapira (1997) with slight modifications. Vitamin C signals were detected using the coulometric electrode array detector previously described for phenolic determination. The setting potential was 0, 100, 200, 300 and 400 mV (*v.* Palladium reference electrode). The flow rate was 0·8 ml/min and the volume injected was 30 μl. Carotenoids (α-carotene, β-carotene and lycopene) were determined exactly as described by Maiani *et al.* (1995) for plasma and by Sharpless *et al.* (1999) for food. Carotenoid separation was performed by injecting 50 μl reconstituted extract on a Waters Nova Pack C_{18} column (particle size 4 μm; 39 × 150 mm) (Waters Millipore Corporation, Milford, MA, USA) and eluting isocratically mobile phase (methanol–acetonitrile–tetrahydrofuran (50:45:5, by vol.) at 1 ml/min. Carotenoid extracts were analysed by an HPLC system, Perkin-Elmer ISS 200 with four pumps (Perkin-Elmer, Norwalk, CT, USA), coupled with a programmable sample injector. Carotenoid peaks were detected at 450 nm with a variable spectrophotometric detector (Perkin-Elmer LC-95; Perkin-Elmer) connected to a personal computer Pe-Nelson mod 1020 (Perkin-Elmer).

Statistics

All variables were tested for normality with the Kolmogorov–Smirnoff test. For normal variables (quercetin, *p*-coumaric acid, α-carotene, vitamin C and lycopene), a repeated-measures ANOVA was used to assess changes over time and paired *t* tests to test each pairwise comparison. When the variable distribution differed significantly from normal (TRAP, caffeic acid and β-carotene), the Friedman test was used to assess changes over time and the Wilcoxon test for *post-hoc* comparisons. All data were entered in an Excel database and analysed with STATISTICA for Windows (release 4.5, 1993; StatSoft Inc., Vigonza, PD, Italy). Values are reported as mean values with their standard errors, unless otherwise noted. Statistical significance was set at the 0·05 level. All *P* values are two-tailed.

Results

Modified-atmosphere packaging storage and lettuce antioxidant properties *in vitro*

The changes in the concentration of vitamin C, *p*-coumaric acid, caffeic acid, quercetin, β-carotene and TRAP in lettuce initially and after 3 and 7 d storage in air or under MAP conditions at +5°C are described in Table 1. The TRAP value of air- and MAP-stored lettuce rapidly decreased (36 and 33 % respectively) during the first 3 d of storage, followed by a very slow decay after 7 d (42 and 45 % respectively). The decrease of the concentrations of phenolics paralleled the TRAP time course, i.e. a quick decay during the first 3 d and a less pronounced loss during the next 4 d. β-Carotene and vitamin C content decreased dramatically during both air and MAP storage, reaching less than one-half of the initial values after 3 d and almost disappearing after 7 d (Table 1).

Analysis of lettuce antioxidant profile and total antioxidant potential

The bioactive molecule content of fresh lettuce administered to the subjects, (total phenolics, *p*-coumaric acid, caffeic acid, quercetin, vitamin C and β-carotene) and *in vitro* TRAP value are reported in Table 2. Results are given as total content in the dose (250 g) administered to the subjects.

Table 1. Changes in the concentration of vitamin C, *p*-coumaric acid, caffeic acid, quercetin, β-carotene and total radical-trapping antioxidant potential (TRAP) in lettuce after 3 and 7 d of storage in air or under modified-atmosphere packaging (MAP) at 5°C†

(Mean values and standard deviations for two experiments in triplicate)

	TRAP‡		Coumaric acid‡		Caffeic acid‡		Quercetin‡		β-Carotene‡		Vitamin C‡	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Baseline	100	26	100	5	100	5	100	14	100	27	100	30
Air												
Day 3	67*	11	79*	5	77*	1	43*	16	37*	8	39*	4
Day 7	55*	4	60*	14	60*	3	28*	10	23*	7	14*	4
MAP												
Day 3	64*	3	56*	11	67*	3	36*	12	42*	16	37*	5
Day 7	58*	5	47*	8	63*	1	31*	11	17*	7	24*	5

Mean values were significantly different from baseline value: *$P \leq 0.05$.
† For details of procedures, see p. 616.
‡ Results are expressed as % baseline value.

Table 2. Total radical-trapping antioxidant potential (TRAP), phenolic compounds, ascorbic acid and carotenoid concentrations in 250 g fresh lettuce (as is basis)*

(Mean values with their standard errors for three experiments in triplicate)

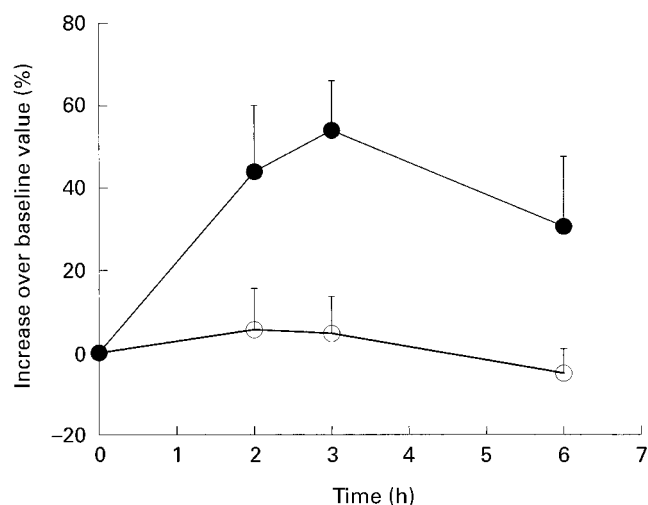| Compound	Concentration	
	Mean	SE
Total phenolics (mg QE)	433·8	31·0
Coumaric acid (mg)	7·3	0·5
Caffeic acid (mg)	31·7	5·7
Quercetin (mg)	12·7	1·8
Vitamin C (mg)	26·7	2·0
β-Carotene (mg)	1279·4	66·3
TRAP (μmol)†	992·5	25·1

QE, quercetin equivalents.
* For details of procedures, see p. 616.
† TRAP values are expressed as μmol peroxyl radicals trapped by 250 g lettuce and represent the sum of the hydrosoluble and liposoluble fractions.

Plasma total antioxidant potential

In order to assess if post-harvest MAP storage affected the *in vivo* antioxidant properties of lettuce, we measured the area under the curve of the individual response of plasma TRAP values over the entire observation period. The mean value of area under the curve for the MAP-stored lettuce (0·1 (SEM 0·2) mmol/l per 6 h) was significantly lower (two-tailed paired *t* test, $P < 0.05$) than the value obtained for fresh lettuce (13·0 (SEM 27·0) mmol/l per 6 h). In order to give an idea of the order of magnitude of the increase of TRAP after fresh lettuce ingestion, % increase over baseline values (not significantly different between phase 1 and phase 2) are shown in Fig. 1. Fresh lettuce consumption significantly increased plasma TRAP values (Friedman test, $P < 0.01$). TRAP values (mmol/l) increased from a baseline level of 0·8 (SEM 0·1) to 1·0

(SEM 0·2) at 2 h (Wilcoxon test, $P < 0.05$), peaking at 3 h (1·1 (SEM 0·2); Wilcoxon test, $P < 0.001$) and beginning to decrease after 6 h (0·9 (SEM 0·09)) (Fig. 1). MAP-stored lettuce did not induce any changes in plasma TRAP during the period of observation (mmol/l): 0·9 (SEM 0·06), 1·0 (SEM 0·07), 1·0 (SEM 0·9), 0·9 (SEM 0·5) at 0, 1, 2 and 6 h respectively (Fig. 1).

Levels of plasma phenolic compounds, carotenoids and vitamin C

In the attempt to identify the molecules responsible for the *in vivo* redox activity of fresh lettuce, we measured plasma concentrations of the main antioxidants present in lettuce. For this purpose, we developed a selective and sensitive
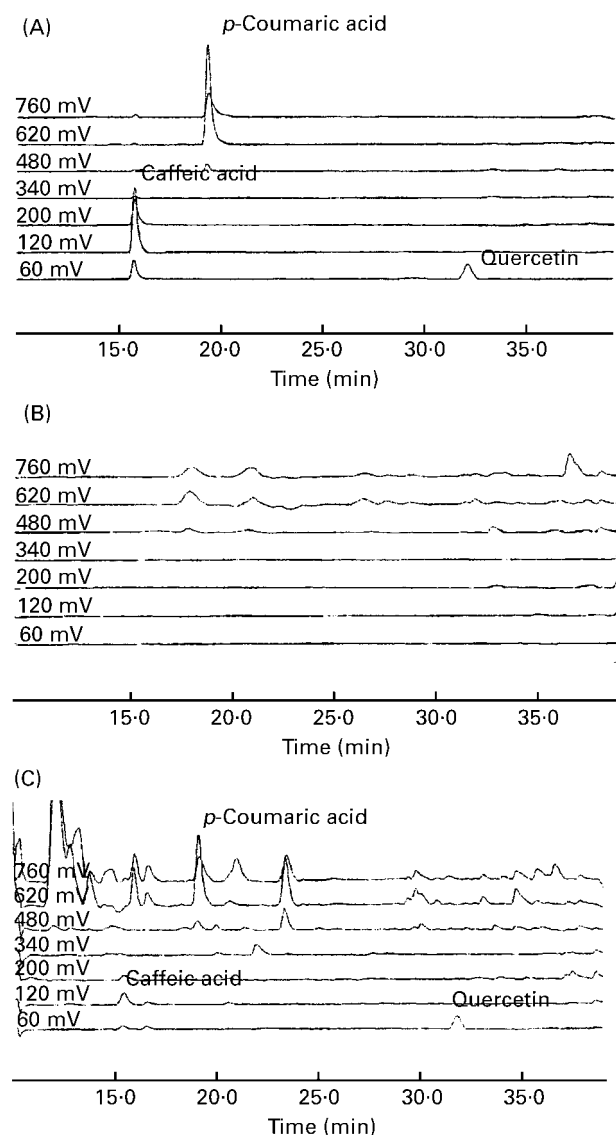

Fig. 1. Effect of acute ingestion of fresh (●; *n* 11) and modified-atmosphere packaging-stored (○; *n* 8) lettuce on plasma total antioxidant capacity. Values are means of the individual % increments over baseline values with standard errors shown by vertical bars. For details of subjects and procedures, see p. 616.

Fig. 2. Representative chromatograms of: (A) standards (caffeic acid, *p*-coumaric acid and quercetin) mixture; (B) plasma samples before consumption of fresh lettuce; (C) plasma samples 3 h after consumption of fresh lettuce, from one of the volunteers. For details of extraction procedures and chromatographic conditions, see p. 617. All the unnamed peaks are to be considered unknown.

method to measure specific phenolic acids (caffeic and *p*-coumaric acid) and the flavonoid quercetin in plasma, using HPLC with electrochemical detection. Typical HPLC chromatograms of standards (caffeic acid, *p*-coumaric acid and quercetin) mixture and of a plasma extract before and after fresh lettuce ingestion are shown in Fig. 2. The chromatogram shown in Fig. 2(C) shows a clear increase of caffeic acid at 15·58 min, in *p*-coumaric acid at 19·21 min and in quercetin at 31·88 min compared with the chromatogram obtained before fresh lettuce consumption (Fig. 2(B)). Mean plasma concentrations of caffeic acid, *p*-coumaric acid and quercetin after ingestion of fresh and MAP-stored lettuce are described in Table 3. Fresh lettuce consumption significantly increased caffeic acid (Friedman test, $P<0.01$), *p*-coumaric acid (ANOVA, $P<0.001$) and quercetin (ANOVA, $P<0.01$) concentrations. The concentration of phenolics at 2 h was significantly greater than baseline values for both *p*-coumaric acid and quercetin (Table 3). Quercetin, caffeic acid and *p*-coumaric acid concentrations reached a peak at 3 h, mirroring the time trend described for plasma antioxidant capacity. Ingestion of MAP-stored lettuce failed to modify plasma phenolic concentrations (Table 3), confirming the results obtained for TRAP values. Fresh lettuce ingestion significantly raised vitamin C concentration (ANOVA, $P<0.001$); the increase was observed 2 h ($P<0.001$), 3 h ($P<0.01$) and 6 h ($P<0.05$) after the ingestion of fresh lettuce (Table 4), paralleling TRAP results. In contrast, plasma concentrations of carotenoids were not modified by the ingestion of fresh lettuce, except for β-carotene (Friedman test, $P<0.01$) (Table 4). Ingestion of MAP-stored lettuce did not change either plasma concentrations of carotenoids or vitamin C (Table 4).

Discussion

The effect of post-harvesting storage conditions on the loss of micronutrients is usually assessed utilising *in vitro* models (Gil *et al.* 1999). In the present study, we followed

a different and innovative approach, determining the biological effect of the stored food *in vivo* on the total antioxidant capacity of plasma. This biomarker has been shown to be strongly related to the dietary intake of fruits and vegetables: an association between dietary antioxidant capacity, intake of plant food and *in vivo* plasma antioxidant potential has been described (Cao *et al.* 1998). Furthermore, in a randomised clinical trial on 123 healthy individuals, a plant food-rich diet (with high antioxidant potential) was shown to reduce the free radical load *in vivo* and to increase plasma TRAP more than a diet low in plant food (four servings per d) (Miller *et al.* 1998). The importance of this information is underlined by previous studies showing that total antioxidant capacity of plasma is significantly lower (with regard to controls) in subjects affected by HIV infection (McLemore *et al.* 1998), diabetes (Ceriello *et al.* 1998), systemic inflammatory disorders (Tsai *et al.* 2000) and lung cancer (Erhola *et al.* 1997).

Our present results clearly show that ingestion of fresh lettuce increases the antioxidant capacity of plasma in healthy subjects, whereas the same lettuce, stored for 3 d under MAP conditions, fails to modify plasma antioxidant defences. Moreover, we showed that 3 d of MAP storage significantly affected the content of bioactive molecules in lettuce, leading to an overall decrease in its *in vitro* antioxidant capacity. Our present results are in agreement with Gil *et al.* (1999), who showed a marked decrease of the *in vitro* total antioxidant activity and ascorbic acid content in spinach stored under MAP conditions.

MAP technology has been shown to prevent ripening and browning, slowing respiration, retaining moisture and extending the shelf-life of the stored items (Kader *et al.* 1989). However, on the basis of our present results, MAP storage does not preserve the bioactive antioxidant compounds present in the lettuce. Under MAP conditions, plant food inside the package is exposed to a low O_2 atmosphere (5 %) with a consequent decrease in O_2 availability for the plant cells. The biochemistry of cells and

Table 3. Plasma concentrations of caffeic acid, *p*-coumaric acid and quercetin before and after ingestion of 250 g fresh or modified-atmosphere packaging (MAP)-stored lettuce†

(Mean values with their standard errors for eleven subjects for fresh lettuce and eight subjects for MAP lettuce)

	Time (h)							
	0		2		3		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Caffeic acid (μg/l)								
Fresh	34·0	14·0	52·0	16·0	63·0*	18·0	44·0	16·0
MAP	22·0	4·0	24·0	5·0	22·0	4·0	16·0	4·0
p-Coumaric aicd (μg/l)								
Fresh	46·0	13·0	78·0**	11·0	85·0**	11·0	46·0	12·0
MAP	42·0	17·0	32·0	13·0	35·0	14·0	32·0	14·0
Quercetin (μg/l)								
Fresh	46·0	7·0	59·0**	6·0	66·0**	6·0	54·0	6·0
MAP	23·0	7·0	25·0	7·0	24·0	6·0	26·0	8·0

Mean values were significanlty different from values at 0 h (two-tailed paired *t* test for *p*-coumaric acid and quercetin and Wilcoxon test for caffeic acid): *$P<0.05$, **$P<0.01$.
† For details of subjects and procedures, see p. 616.

Table 4. Plasma concentrations of vitamin C and carotenoids before and after ingestion of 250 g fresh or modified-atmosphere packaging (MAP)-stored lettuce†

(Mean values with their standard errors for eleven subjects for fresh lettuce and eight subjects for MAP lettuce)

	Time (h)							
	0		2		3		6	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Vitamin C (mg/l)								
Fresh	10·9	2·0	12·7***	3·0	12·7**	2·0	12·9*	3·0
MAP	11·5	2·7	11·0	2·2	10·4	3·2	12·0	3·4
α-Carotene (μg/l)								
Fresh	44·0	10·0	44·0	10·0	45·0	10·0	53·0	11·0
MAP	38·0	11·0	32·1	10·0	38·0	11·0	32·0	11·0
β-Carotene (μg/l)								
Fresh	333	74	310	70	346	73	402*	86
MAP	360	69	321	65	377	67	331	74
Lycopene (μg/l)								
Fresh	333	40	304	29	350	32	291	30
MAP	204	30	212	35	224	37	200	21

Mean values were significantly different from values at 0 h (two-tailed paired t test for vitamin C and Wilcoxon test for β-carotene): *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

† For details of subjects and procedures, see p. 616.

cellular functions changes dramatically under hypoxic conditions and, paradoxically, cells become more susceptible to oxidative injury at low P_{O_2}. One of the factors contributing to hypoxic injury is 'reductive stress', in which the concentration of potential electron donors is increased (lack of O_2 to act as an electron acceptor results in increased reduction of all electron carriers in the chain). The accumulation of reducing equivalents can result in enhanced formation of reactive oxygen species either by direct electron donation to O_2 or by redox cycling (a process whereby a low-molecular-mass 'mediator' accepts one electron from a reduced cellular component and transfers that electron to produce O_2^-) (Jones, 1985).

The 'reductive/oxidative stress' originating from hypoxia changes membrane potentials and cells become more sensitive to redox insults (Jones, 1985). We hypothesise that this overproduction of free radicals induced by hypoxic conditions could be responsible for the loss of antioxidant activity and for the decrease in antioxidant molecules in the MAP-stored lettuce.

The role played by the single compounds in the antioxidant effect of plant food has not yet been clarified. In the present study, we measured plasma concentrations of quercetin, caffeic acid, p-coumaric acid, carotenoids and vitamin C, and showed that peak concentrations of these components, except for carotenoids, coincided with the peak in plasma antioxidant capacity. Miller et al. (1998) showed that a diet rich in fruits and vegetables for a period of 8 weeks favourably affected serum antioxidant capacity compared with a control diet low in plant foods, an increase partially explained on the basis of a rise in the concentration of carotenoids. In our present study, the increase in plasma concentration of β-carotene was not able to modify plasma antioxidant capacity significantly. These results are in agreement with those of Pellegrini et al. (2000), who found that the daily ingestion of 25 g tomato puree for 14 d significantly increased lycopene and β-carotene plasma concentrations in eleven healthy

female subjects without significant changes in the antioxidant capacity of plasma. Moreover, Borel et al. (1998) reported a lack of correlation between clinical markers of redox status and concentrations of single carotenoids in healthy subjects. Carotenoids seem not to play a central role in the modulation of plasma antioxidant capacity, although we cannot rule out the possibility that they could be involved in the protective effect of plant food through a different mechanism.

For a long time, the absorption of phenolics from the diet was considered to be negligible and their mechanisms of absorption, distribution, metabolism and excretion were scarcely studied. In recent years, evidence describing the absorption of single phenolic compounds in body fluids after ingestion of onions, red wine, dealcoholised red wine, green tea and chocolate is mounting (Maiani et al. 1997; Serafini et al. 1998; McAnlis et al. 1999; Richelle et al. 1999; Bell et al. 2000). Previous studies (Serafini et al. 1998; McAnlis et al. 1999) showed that the increase in quercetin and total phenolic concentration following an onion meal and dealcoholised red wine ingestion was paralleled by an increase in total antioxidant capacity. In contrast, Caccetta et al. (2000) described how plasma absorption of caffeic acid and 4-O-methylgallic acid was not sufficient to produce an effect on the ex vivo oxidation of serum following red wine ingestion.

Our present results show that the increase of plasma levels of caffeic acid, p-coumaric acid and quercetin mirror the increase in plasma antioxidant capacity. However, the in vitro addition of the same phenolic concentrations to a plasma sample (results not shown) does not account for the increase in TRAP shown in vivo. Other factors, other than the plasma concentration of the original compounds present in the food matrix, should play a role in the antioxidant effect of phenolic compounds. The existence of antioxidant metabolites has been reported (Moon et al. 2001) and, although the conjugation with glucuronide and sulfate is a step toward

detoxification, intermediate products seem to retain their antioxidant activity (Terao, 1999). Moreover, synergistic redox interactions between phenolic compounds and the antioxidant network (Laranjinha & Cadenas, 1999) could contribute to explaining in part the increase in plasma total antioxidant potential following plant food ingestion. Further research is needed to elucidate the contribution of phenolic compounds to the dietary modulation of antioxidant defences.

Conclusion

The demonstration that fresh lettuce ingestion is able to raise plasma antioxidant defence sheds further light on the beneficial effect of plant food consumption. The lack of antioxidant effect after consumption of MAP-stored lettuce suggests that until MAP storage conditions are optimised, the consumption of 'fresh' plant food should be encouraged. The development of innovative storage techniques able to preserve the bioactive molecule content of plant food could be a formidable step in promoting the consumption of fresh-cut produce in western countries.

Acknowledgements

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