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ORIGINAL COMMUNICATION

Identification and quantitation of major carotenoids in selected components of the Mediterranean diet: green leafy vegetables, figs and olive oil

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Objective: To characterize the carotenoid content of selected components of the Mediterranean diet commonly eaten by Greek migrants to Melbourne, a population group maintaining a traditional dietary regimen, and who exhibit relatively high circulating carotenoid concentrations and low cardiovascular disease mortality.
Design and specimens: Opportunistic sampling of wild (sow thistle, amaranth, purslane and dandelion, collected from roadsides and home gardens) and commercially available (chicory, endive) green leafy vegetables and figs in season. Foods were selected on the basis that they are commonly eaten by Greek migrants but not by Anglo-Celtic persons, and had not previously been well-characterized with respect to their carotenoid contents. Extra virgin, cold-pressed olive oil and 'extra light' olive oil were obtained from commercial sources. Specimens were extracted with tetrahydrofuran (or chloroform:methanol for olive oil) and carotenoid contents were quantified using HPLC with UV detection. Two to six specimens of greens and figs were analysed. Dietary intake was assessed by food frequency questionnaire.
Results: Wild green vegetables contained high concentrations of lutein (sow thistle > amaranth > purslane > dandelion) and β-carotene (sow thistle > amaranth > purslane = dandelion). Sow thistle and amaranth contained lutein (15 and 13 mg/100 g, respectively) and β-carotene (3.3 and 4.0 mg/100 g, respectively) at concentrations greater than that seen in the commercially available species of chicory and endive. Figs contained all major carotenoids appearing in plasma, albeit at low concentrations. Extra virgin cold-pressed olive oil contained substantial quantities of lutein and β-carotene, but the more-refined 'extra light' olive oil did not.
Conclusions: These components of the traditional Mediterranean diet contribute to the higher circulating concentrations of carotenoids in Greek migrants compared to Anglo-Celtic Australians.
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Introduction

The Seven Countries Study showed that Mediterranean populations, particularly in Crete, had the lowest mortality from coronary heart disease (CHD) in Europe (Keys *et al*, 1986). The Cretan diet of the 1950s (Kromhout *et al*, 1989) is often considered the archetypal Mediterranean diet. Interestingly, this period coincides with a major migration from Greece to Australia. Greek migrants to Melbourne also exhibit relatively low coronary mortality, despite high prevalences of risk factors such as obesity, diabetes, hypertension and smoking (Young, 1992; Australian Bureau of Statistics,

1998), and this population has maintained many aspects of the traditional Cretan diet (Kouris-Blazos *et al*, 1996). Dietary antioxidant compounds are believed to provide at least part of the protection associated with diets rich in vegetables, fruits and olive oil, such as the Cretan Mediterranean diet (Simopoulos & Sidossis, 2000; Visioli, 2000). Some epidemiological studies have identified an inverse relation between dietary intake or plasma concentrations of carotenoids from fruit and vegetables and CHD incidence (Gey *et al*, 1993; Kohlmeier *et al*, 1997).

We have previously identified higher circulating concentrations of certain carotenoids and some unidentified compounds among Greek migrants compared to Australian-born people in the Melbourne Collaborative Cohort Study (MCCS; Su *et al*, 1998). It is possible that the higher concentrations of some of these carotenoids among Greek migrants were due to greater intake of certain components of the Mediterranean diet, such as wild dark green vegetables, figs, olives, vine leaves and other green leafy vegetables which are not normally consumed by Australians of Anglo-Celtic origin. These foods have not been well-characterized with respect to their carotenoid contents. The aim of this study was to separate and quantify carotenoids in several wild green vegetables, figs and olive oil that are part of the traditional Greek diet and which may contribute to high circulating carotenoid levels in Greek migrants.

Methods

Dietary intake

The dietary intake of wild and commercially-available green leafy vegetables, figs and olive oil was determined by food frequency questionnaire (Ireland *et al*, 1994) among Greek migrants and Australian-born (largely Anglo-Celtic) persons recruited from the MCCS (Giles, 1990). The MCCS is a community-based prospective study, examining the role of nutrition in chronic disease, which includes large numbers of southern European migrants. Data are expressed as serves per person per week (or mL per week for olive oil). A 'serving' of vegetables was defined as 'half a cup of cooked vegetables or one cup of salad vegetables'. A serving of figs was 140 g.

Reagents and materials

Magnesium carbonate, anhydrous sodium sulphate, butylated hydroxytoluene (BHT) and the reference samples of all-*trans*-β-carotene, all-*trans*-α-carotene, and *trans*-lycopene were purchased from Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia). Lutein, zeaxanthin and β-cryptoxanthin were gifts from Hoffmann-LaRoche (Basel, Switzerland). β-Apo-8-carotenal (*trans*) (Fluka Chemical Corp.) was used as internal standard. HPLC-grade solvents and other reagents were obtained locally from commercial suppliers (Mallinkrodt, Selby-Biolab; Melbourne, Australia).

Raw vegetables and fruits and olive oil

The wild dark green vegetables (sow thistle, dandelion, amaranth, purslane) were collected from roadsides and gardens of Melbourne during February (summer). Sources were those normally accessed for collection of plants for consumption. Commercial leafy green vegetables (chicory, endive) were obtained from Oakleigh market (Victoria, Australia). Fresh figs were collected from a local resident's garden in April. All samples were stored in plastic bags and kept in a darkened refrigerator at 4°C until analysis (within 24 h). Samples were collected as normally consumed, and the whole sample analysed. In the case of wild greens, the edible portion includes only the leaves. Extra virgin olive oil (naturally cold pressed) was supplied by Picuba Foods, Marrickville, NSW (Minos Extra Virgin olive oil, imported from Greece). 'Extra light' olive oil was obtained from a local supermarket (Bertolli 'Extra Light'; imported from Italy by Bertolli Australia Pty Ltd).

Preparation and extraction of food samples

Wild green vegetables and figs were extracted by a modification of published methods (Khachik *et al*, 1992; Nyambaka & Ryley, 1995). Two to six specimens of each plant species were collected, homogenized as a single sample, and two aliquots analysed (each in duplicate). The internal standard was prepared by dissolving β-apo-8′-carotenal to a concentration of 0.7 μg/ml in hexane with 0.01% of BHT. Duplicate 1 g food samples were homogenized in a Waring blender or food processor (Magimix, Compact 3100, Australia), 1 ml of the internal standard was added, followed by magnesium carbonate (0.1 g) and tetrahydrofuran (THF, the commonly reported solvent for extraction, 10 ml) containing 0.01% BHT. The solution was kept in the dark for 30 min. Homogenization was carried out under red lighting and with aluminium foil around the glassware to prevent the degradation and isomerization of carotenoids during the extractions. The extract was filtered under suction, and the solid materials were extracted repeatedly with THF (10 ml) until the resulting filtrate was colourless. The combined extract was concentrated to a volume of approximately 2 ml on a rotary evaporator at 35°C and then partitioned by addition of chloroform (30 ml) and water (20 ml). The solutions were transferred to a separation funnel and washed with water (2×20 ml) to remove the water-soluble materials. The organic phase was collected and 10 ml chloroform:methanol (2:1) added to the water phase for further extraction. The organic layers were combined, dried over anhydrous sodium sulphate, and 2.5 ml of solution evaporated to dryness under a nitrogen stream. The residue was reconstituted with 200 μl of solvent (acetonitrile:methanol:chloroform, 35 μl:35 μl:30 μl) and 50 μl samples injected onto the HPLC column.

For analysis of olive oil, 1 g of olive oil and 1 ml of the internal standard were mixed, followed by magnesium carbonate (0.1 g) and 10 ml chloroform:methanol (2:1). The

solution was kept in the dark for 30 min, transferred to the separating funnel and washed with water (2×20 ml) to remove the water-soluble materials. The organic phase was collected and 10 ml chloroform:methanol (2:1) was added to the water phase for further extraction. The organic layers were combined, dried and reconstituted as described above.

Standard curve and chromatography

An UV–visible spectrophotometer (Hewlett-Packard) was used for the determination of the concentration of standard solutions. Internal standard β-apo-8-carotenal (*trans*) was prepared with hexane containing 0.01% butylated hydroxytoluene (BHT) in a brown bottle. For the stock solution of β- and α-carotene, *trans*-lycopene, lutein, zeaxanthin and β-cryptoxanthin, n-hexane (0.01% BHT) was used as a solvent. The dissolution of *trans*-lycopene was made in 1 ml of dichloromethane, which was then diluted with n-hexane containing 0.01% BHT. α- and γ-tocopherol were dissolved in ethanol. Standard curves for analytes were generated based upon concentration *vs* peak-area ratio with internal standard. The calculation of unidentified compound concentrations was performed using the lutein/zeaxanthin standard curve.

The chromatographic system used included: the Alliance 2690 separations module, 996 Photodiode Array Detector and Millennium Chromatography Manager PDA Software (Waters Australia, Melbourne, Australia); a Spherisorb ODS-2 column (250×4.6 mm; Goldpak, UK), HP BIO cartridge holder and biocompatible guard cartridge (10×4.3 mm C_{18}; Activon, Melbourne, Australia). A mobile phase of acetonitrile:methanol:chloroform (45:45:10), containing 0.05% ammonium acetate in the methanol and 0.1% triethylamine in the acetonitrile, at a flow-rate of 1.0 ml/min was used. Run time was 24 min. Individual carotenoids (lutein, zeaxanthin, β-cryptoxanthin, lycopene, β- and α-carotene) were monitored at 450 nm. Within-day coefficient of variation was 8.7, 7.8, 10.3, 8.4 and 6.9% for lutein, cryptoxanthin, lycopene, α-carotene and β-carotene, respectively. Limit of detection, defined as a peak height three times the size of the mean baseline noise (Shah *et al*, 1991), was less than 1 ng (corresponding to a concentration in food of approximately 0.4 μg/100 g) for all analytes.

Results

Dietary intake

Table 1 shows dietary intake of green leafy vegetables, figs and olive oil in Greek- and Australian-born persons recruited from the MCCS. Green leafy vegetables (including the wild and commercially available species analysed here) were consumed approximately twice as frequently among Greek-born compared with Australian-born persons. None of the Australian-born persons surveyed reported eating figs. Olive oil consumption was over three times greater among Greek-born compared to Australian-born persons.

Table 1 Dietary intake of selected components of the Mediterranean diet by Greek- and Australian-born persons in the Melbourne Collaborative Cohort Study. Data are expressed as servings/person/week, except olive oil which is mL/person/week

	Dietary intake	
	Greek-born (n = 221)	*Australian-born (n = 229)*
Green leafy vegetables	6.2 (4.1, 10.0)	3.5 (1.5, 6.0)
Figs	0.1 (0.0, 1.1)	0.0
Olive oil	233 (153, 311)	74 (0, 117)

Median (inter-quartile range).

Detection and quantitation of carotenoids and tocopherols in green vegetables, figs and olive oil

Figure 1 shows chromatographic profiles of wild green vegetables (sowthistle, amaranth, purslane and dandelion). Lutein and β-carotene were the major identified peaks. Chromatograms of commercially available species (chicory and endive) were qualitatively similar (not shown). There were high concentrations of lutein and β-carotene in wild dark green vegetables (Table 2). Sow thistle and amaranth had higher lutein levels than did the samples of chicory and endive examined. Purslane and dandelion also contained substantial quantities of lutein. The carotenoids present in figs included lutein, cryptoxanthin, lycopene, β-carotene and α-carotene. Lycopene was the most abundant carotenoid (Table 2), followed by lutein and β-carotene. Extra virgin olive oil contained some lutein and β-carotene. Other carotenoids were not detectable. Extra light olive oil did not contain detectable levels of carotenoids but had a higher α-tocopherol content than did the extra virgin olive oil.

Characterization and quantitation of unidentified compounds

There were a number of unidentified compounds present in all the green leafy vegetables. The major peaks common to all the green leafy vegetables examined eluted at 4.3, 4.8 and 11 min and a fourth occurred at 15 min in all species (including chicory and endive) except purslane (Figure 1). Purslane exhibited a major peak at 18 min which did not appear in other species (Figure 1). Figure 2 shows UV–visible spectra in acetonitrile:methanol:chloroform (45:45:10) of major unidentified peaks. We have not observed any of these compounds in plasma samples, including those from over 200 Greek-born residents of Melbourne (Su *et al*, unpublished data). Table 3 shows the absorption maxima and concentrations of unidentified compounds in wild greens and green leafy vegetables. Compound U5 occurred in substantial quantities in all species but purslane. The unknown compounds present in green leafy vegetables did not appear in detectable quantities in figs or olive oil.

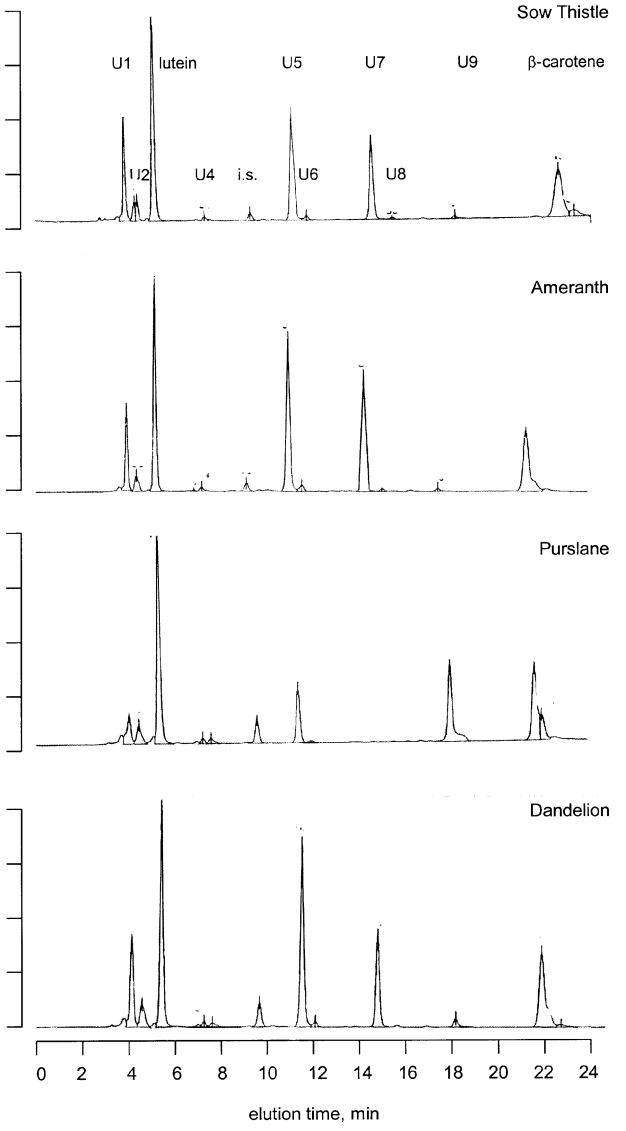

Figure 1 Chromatographic profiles of wild green vegetables. i.s., internal standard; U1 – U9, unidentified compounds 1 – 9.

Discussion

We have identified high concentrations of lutein and β-carotene in wild and commercially available green leafy vegetables commonly consumed as part of a Mediterranean diet among Greek-born people in Melbourne. We have also demonstrated that these carotenoids are present in appreciable amounts in extra virgin (but not more refined) olive oil, and that figs, another food consumed in significant quantities as part of this dietary regimen, contain all the carotenoids normally quantified in human plasma. Various studies have shown a significant correlation between habitual vegetable and fruit intake and plasma carotenoid concentrations (Martini *et al*, 1995; Scott *et al*, 1996). Lutein concentrations were particularly high in Greek people in the MCCS (Su *et al*, 1998), and these lutein-rich green leafy vegetables are not commonly consumed by Anglo-Celtic people in Australia. Lutein appears to be readily bioavailable from green vegetables, in contrast to β-carotene (van het Hof *et al*, 1999). The use of olive oil in food preparation aids absorption of lipid-soluble compounds such as carotenoids, particularly those

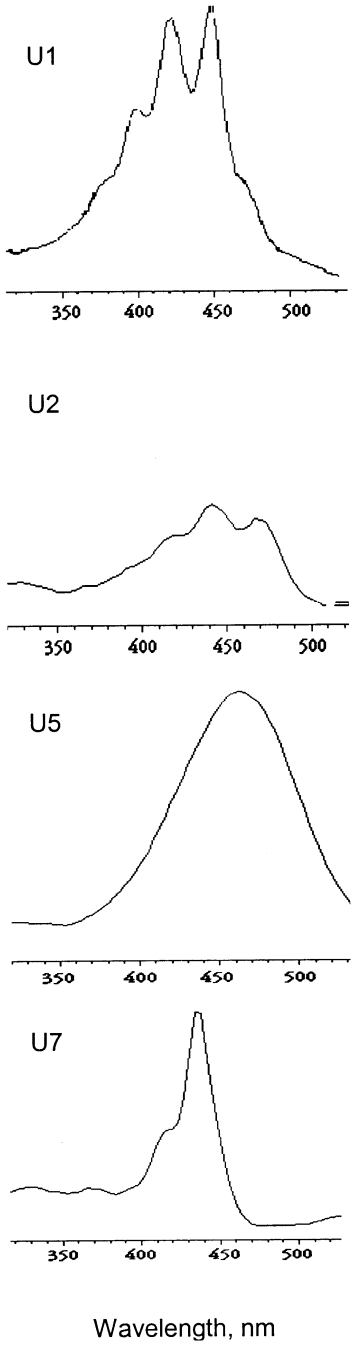

Figure 2 Absorption spectra of major unidentified peaks in wild green vegetables.

Table 2 Carotenoid and tocopherol contents of selected components of the Mediterranean diet (mg/100 g)

	Lutein	*β-Carotene*	*α-Carotene*	*Cryptoxanthin*	*Lycopene*	*α-Tocopherol*	*γ-Tocopherol*
Sow thistle (*Sonchus oleraceus*[a])	15.2	4.0	ND[c]	ND	ND	—	—
Amaranth (*Amaranthus sp.*[a,b])	13.0	3.3	ND	ND	ND	—	—
Purslane (*Portulaca oleraceae*[a,b])	7.0	1.6	ND	ND	ND	—	—
Dandelion (*Hypochoeris radicata*[a,b])	6.0	1.6	ND	ND	ND	—	—
Chicory (*Cichorium intybus*)	9.1	2.3	ND	ND	ND	—	—
Endive (*Cichorium endivia*)	8.7	2.4	ND	ND	ND	—	—
Fig (*Ficus carica*)	0.08	0.04	0.02	0.01	0.32	—	—
Olive oil, extra virgin	0.35	0.23	ND	ND	ND	9.86	1.00
Olive oil, 'extra light'	ND	ND	ND	ND	ND	12.9	1.00

[a]Identification by the National Herbarium of Victoria, Royal Botanic Gardens, Melbourne.
[b]Probable identification only.
[c]Not detectable.

Table 3 Chromatographic properties and concentration of unidentified compounds in green leafy vegetables

Peak	*U1*	*U2*	*U5*	*U7*
Retention time (min)	4.3	4.8	11	15
Absorption maxima (nm)	398, 422, 450	414, 438, 464	464	414, 436
Concentration (mg/100 g)				
Sow thistle	2.44	0.52	6.71	7.63
Amaranth	5.42	1.21	13.4	1.08
Purslane	1.19	0.70	1.74	ND
Dandelion	2.22	1.07	5.64	2.97
Chicory	2.66	1.21	8.77	4.16
Endive	3.60	0.80	6.80	4.80

less polar (Khachik *et al*, 1992), as well as being an important source of α-tocopherol in itself (and of carotenoids in the case of extra-virgin olive oil). Hence high intakes of these carotenoid-rich foods may be responsible for the relatively high circulating concentrations of carotenoids among Greek migrants to Melbourne (assumptions regarding bioavailability notwithstanding), and may contribute to protection from CHD mortality exhibited by this group.

The carotenoid profile of the wild greens was similar to that of the commercially available species chicory and endive. Sow thistle and amaranth contained lutein and β-carotene in amounts similar to that found in raw spinach (about 12 mg lutein + zeaxanthin/100 g and 6 mg β-carotene/100 mg; US Department of Agriculture, 1998). Figs contained all the carotenoids routinely quantified in human plasma, albeit in relatively low amounts. Few foods are known to contain such a range of carotenoids (US Department of Agriculture, 1998). A number of unidentified compounds were separated by this method and these were largely common to all the green leafy vegetables. These compounds were not observed in figs or olive oil. Spectral analysis showed that these compounds were not the same as those unidentified peaks appearing in human plasma (Su *et al*, 1999), as peaks with similar retention times or absorption maxima could not be identified in any plasma samples from a large number of Greek migrants to Melbourne. Hence, unidentified carotenoids present in green leafy vegetables do not directly contribute to the concentration of unidentified carotenoids present in human plasma, some of which occur at greater concentrations in Greek migrants than in Australian-born people in the MCCS. They may be unstable in cooking, may not be absorbed or may be metabolized in the human body.

We found extra virgin olive oil (cold pressed) to contain some lutein and β-carotene as well as substantial amounts of α- and γ-tocopherol. The more refined 'extra light' olive oil did not contain carotenoids in amounts detectable by our method. Lutein (0.3 mg/100 ml) and β-carotene (0.07 mg/100 ml) were identified in extra virgin olive oil using a colourimetric method (Ranalli *et al*, 1999b). These results were similar with respect to lutein, but the β-carotene content was less than we identified. This may be a real difference due to olive variety or seasonal/climatic effects, or it may be an artefact due to methodological differences. Other studies have identified a range of α-tocopherol contents consistent with that identified in the present study (García *et al*, 1996; Gutiérrez *et al*, 1999; Ranalli *et al*, 1999a).

A limitation of the present study is the small numbers of food samples analysed and the single time point of collection. Regarding time of collection, these foods are normally eaten only at certain times of the year when in season, especially in the case of amaranth, purslane and figs, and the samples used in the present study were collected at that time. Hence the measured carotenoid contents are relevant to those normally consumed. In order to properly assess the biological variability in carotenoid content of these foods, a systematic sampling strategy covering a range of geographic and (micro)climatic conditions is required, but was beyond the scope of the present study. The carotenoid profile of vegetables is much more complex than previously recognized, with the proportion and forms occurring as different carotenoids varying considerably according to growing conditions, storage and processing (Nyambaka & Ryley, 1995). It is noteworthy that the wild green vegetables described in this paper would typically be collected and eaten within several hours. Further detailed studies of these aspects are warranted.

In conclusion, we have identified high concentrations of lutein and β-carotene in wild green vegetables commonly consumed as part of a traditional Mediterranean diet. Figs and extra virgin olive oil are also important sources of carotenoids in such a dietary regimen.

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