ELSEVIER

Food Chemistry **54** (1995) 101–111

0308-8146/95/$9.50

Analytical, Nutritional and Clinical Methods Section

Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK

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(Received 22 September 1994; revised version received and accepted 25 November 1994)

This study further examines the factors which affect the chromatographic response of carotenoids and contribute to analytical variation and inaccuracies in their quantitative determination. A method for the analysis of carotenoids in vegetables and fruits is described and data are presented for the carotenoid content of vegetables and fruits commonly consumed in the UK. The addition of a solvent modifier (triethylamine) to the mobile phase was shown to improve the recovery of carotenoids from the column from around 60% to over 90%. The linearity and reproducibility of the chromatographic response was investigated and the robustness and reproducibility of the method was measured using a reference vegetable material developed in the laboratory. Short and longer term reproducibility showed an average CV of around 8% for all carotenoids. Analysis showed that good sources (>1000 μg/100 g) of lutein were broccoli, butterhead lettuce, parsley, peas, peppers, spinach and watercress; of lycopene: tomatoes and tomato products; and of β-carotene: broccoli, carrots, greens, butterhead lettuce, mixed vegetables, parsley, spinach and watercress. There was little or no loss of carotenoids on cooking, green vegetables showed an average increase in lutein levels of 24% and in β-carotene levels of 38%. This study and previous studies in our laboratory have demonstrated that a number of factors affect the validity of the 'peak response' and are likely to contribute to within and between laboratory variation. It is suggested that the development and use of standard reference materials would significantly improve the quality of data.

INTRODUCTION

With the increased interest in the possible link between carotenoid intake from foods and health, the need for reliable data on the individual carotenoid content of those foods has become increasingly important. Historically much of the carotenoid data has been obtained by measuring total absorption at a specified wavelength, or more usually by open column chromatography followed by spectrophotometric quantification as in the AOAC method. More recently HPLC methods have enabled the more discrete separation of carotenoids, although in currently available tables of food composition data are still usually expressed as β-carotene, β-carotene equivalents, or retinol equivalents. (The Composition of Foods, McCance & Widdowson, 5th edn, 1991; Food Composition and Nutrition Tables, Souci, Fachmann & Kraut, 1987.)

In Vegetables, Herbs and Spices (1991), a supplement to the 4th edn of The Composition of Foods (McCance & Widdowson), separate values are given for β-cryptoxanthin, α-carotene and β-carotene in a selection of vegetables. Heinonen *et al.* (1989) have reported data for lutein + zeaxanthin, β-cryptoxanthin, lycopene, and γ-, α- and β-carotene in Finnish foods. Similarly, Granado *et al.* (1992) reported data on the carotenoid levels in raw and cooked Spanish vegetables, giving separate values for lutein and zeaxanthin. Mangles *et al.* (1993) have compiled a carotenoid database of 120 foods from published data to which they attach confidence levels.

The development of more sensitive methods, such as HPLC, whilst enabling better separation of individual

carotenoids means that any artefacts produced during extraction, purification and chromatography will be more readily detected. We have previously reported on some of the problems associated with the analysis of carotenoids by HPLC (Scott 1992; Scott & Hart 1993). This paper reports the further development and evaluation of methodology, and describes a method for the analysis of carotenoids in vegetables and fruits. Data are presented for the wider carotenoid content of vegetables and fruits commonly consumed in the UK.

These data have been used in a complementary study to assess the correlation between intake of carotenoids and their concentrations in blood plasma.

MATERIALS AND METHODS

Selection of food items

Items were selected from food frequency/weighed intake data from 162 women, aged between 50 and 65 years, who were taking part in a study to evaluate methods of assessing food intake (Bingham *et al.* 1994). Intake was measured during four successive days at four time points during one year (16 days in total).

Sampling and preparation of food items

Four individual samples of each item were purchased from various retail outlets in the Norwich area between April and October 1993. Frozen materials were stored at −18°C, fruits and salad vegetables were stored at 4°C and other vegetables and canned foods stored at room temperature, for up to 3 days prior to preparation.

Vegetables and fruits were prepared as appropriate by removal of the outer leaves, peeling, coring, etc. Large items, such as cabbage, were quartered, cut and mixed, small items were cut and mixed, and frozen or canned items were mixed. Sub-samples of 100 g were taken from each of the four individual samples of each food item. These were bulked to give a composite sample of 400 g. Where an item was to be 'cooked', an additional 400 g bulked sample was prepared. Exceptions to this were lettuce (300 g), parsley (100 g), spinach (300 g), and watercress (100 g). Items were cooked using typical 'in home' cooking methods (see Appendix I). Frozen or canned products were cooked according to manufacturers instructions. Immediately after preparation of the composite raw sample or after cooking the sample was frozen in liquid nitrogen and ground under liquid nitrogen in a Waring blender. The ground sample was stored in an air-tight bottle flushed with nitrogen at −18°C for up to 3 days prior to analysis. All manipulations were carried out under gold fluorescent lighting.

Due to the high level of carotenoid contribution and frequency of consumption, the four individual samples of frozen peas, frozen carrots, fresh carrots and fresh tomatoes were also analysed individually. A further six varieties of tomatoes were obtained and analysed to assess differences between varieties.

Chromatography

The HPLC system comprised of a dual piston solvent delivery pump (LKB, now Pharmacia Biotech, St Albans, UK). The column system consisted of a 10 mm, 5 μm ODS2 metal-free guard column, with a 100 × 4·6 mm, 5 μm ODS2 metal-free column (Alltech Associates, Carnforth, UK) connected to a 250 × 4·6 mm, 5 μm Vydac 201TP54 analytical column (Separations Group, Hesperia, Cal., USA) modified by the replacement of metal frits with 'bio-compatible' Teflon frits. Column connections were made with PEEK (poly ether ether ketone) tubing (Alltech Associates.). The column temperature was maintained at 22·5° ± 0·1°C using a column water jacket (Alltech Associates), with a thermostatically controlled circulating water bath (Grant Instruments Ltd, Cambridge, UK).

The mobile phase consisted of acetonitrile, methanol, dichloromethane(MeCN : MeOH : DCM)75 : 20 : 5 v/v/v, containing 0·1% butylated hydroxytoluene (BHT) as an antioxidant and 0·05% triethylamine (TEA). The methanol contained 0·05 M ammonium acetate. All reagents were Fisons (Loughborough, UK) HPLC grade, except MeOH from BDH Ltd, (Lutterworth, UK) and BHT from Sigma (Poole, UK). The prepared mobile phase was filtered through a 0·5 μm PTFE membrane filter and degassed using ultrasonic agitation. The solvent reservoir was equipped with a helium degassing facility. The flow rate was 1·5 ml/min.

Samples were injected via a model 7125 Rheodyne syringe loading sample injector fitted with a 50 μl loop (Rheodyne, Cotati, Cal., USA).

Peak responses were measured at 450 nm using a variable wavelength UV/Vis monitor (LKB, now Pharmacia Biotech), with an output to a chromatographic data handling system (Philips, Cambridge, UK or Autochrom, Millford, Mass., USA) which permitted manual manipulation of peak integration. A diode array detector (Philips) was coupled to the UV/Vis monitor to assess peak homogeneity and confirm spectral identity of carotenoids.

Sample extraction

Duplicate 10 g aliquots of the ground sample were placed in conical flasks together with 1 g solid magnesium carbonate (Fisons) to neutralize any organic acids. Fifty ml of tetrahydrofuran and methanol (1 : 1 v/v THF : MeOH) were added along with an internal standard (β-apo-8'-carotenal or echinenone) appropriate for the type of sample. Carotenoids were extracted from the food matrix by homogenizing for 1 min using an ultra-turrax homogenizer (IKA, Staufen, Germany). The resulting suspension was filtered through a glass fibre filter pad (GF/A Whatman, Maidstone, UK) in a buchner funnel under vacuum. The flask and homogenizer were washed with 50 ml THF/MeOH and the washing filtered. The filter pad was washed with two further 50 ml aliquots of THF : MeOH. The combined THF : MeOH filtrates were transferred to a separating

funnel. Fifty ml petroleum ether (40–60° fraction, containing 0·1% BHT) and 50 ml 10% sodium chloride solution were added and mixed by careful shaking. The lower THF/MeOH/aqueous phase was drawn off and the upper petroleum ether phase was transferred to a 250 ml evaporating flask. The THF/MeOH/aqueous phase was extracted two more times with 50 ml aliquots of petroleum ether. The petroleum ether phases were combined in the flask and evaporated at 35°C in a rotary evaporator to near dryness. Ten to fifteen ml of petroleum ether was added and the residue redissolved by ultrasonic agitation, transferred to a 25 ml evaporating flask and evaporated just to dryness. The residue was redissolved, by ultrasonic agitation, to a final volume of 5 ml in DCM. If necessary this extract was saponified as described below, otherwise it was diluted with the mobile phase to a suitable concentration for HPLC analysis and filtered through a 0·45 μm PVDF syringe filter (Gelman). All manipulations were carried under gold fluorescent lighting (Thorn, UK).

Saponification

This procedure was used with the peppers and fruit samples only. Four ml of the DCM extract from the extraction procedure was saponified with an equal volume of 10% potassium hydroxide in MeOH (under nitrogen, in the dark) for 1 h at room temperature. The carotenoids were extracted from the KOH/methanolic phase by careful shaking with 20 ml petroleum ether (40–60° fraction, containing 0·1% BHT), and 20 ml 10% sodium chloride solution in a separating funnel. The lower KOH/MeOH/aqueous phase was removed to another separating funnel and was extracted two more times with 20 ml aliquots of petroleum ether. The petroleum ether phases were combined in a separating funnel and washed with water until washings were neutral to pH paper. The petroleum ether phase was transferred to a 100 ml round-bottomed flask and dried in a rotary evaporator at 35°C. Ten to fifteen ml of petroleum ether were added and the residue redissolved by ultrasonic agitation, transferred to a 25 ml evaporating flask and evaporated just to dryness. The residue was redissolved by ultrasonic agitation in 4 ml DCM, diluted with mobile phase to a suitable concentration for HPLC analysis and filtered through a 0·4 μm PVDF syringe filter. All manipulations were carried out under gold fluorescent lighting.

Internal standards

An internal standard was used to assess losses during the extraction procedures. One of two standards was used depending on the carotenoid profile of the sample. β-apo-8'-carotenal was used where possible, but since β-apo-8'-carotenal co-elutes with chlorophyll B, for green vegetables echinenone was used. Both standards showed similar recoveries during the extraction procedure.

Preparation of standard carotenoid solutions and calibration

Lutein, lycopene, α-carotene and β-carotene were obtained from Sigma Chemicals, β-apo-8'-carotenal from Fluka Chemicals (Gillingham, UK). Zeaxanthin, β-cryptoxanthin and echinenone were a gift from Hoffman La Roche (Basel, Switzerland).

Lutein, α-carotene, β-carotene and β-apo-8'-carotenal were dissolved in chloroform and made to volume with hexane to give a final solvent ratio of 1:9 v/v. Echinenone and β-cryptoxanthin were dissolved in 1:1 v/v chloroform/hexane. Zeaxanthin and lycopene were dissolved in chloroform. All solvents contained 0·1% BHT. With the exception of lycopene, all solutions were stored in air-tight screw-topped brown bottles under nitrogen at −18°C. To avoid degradation, the lycopene solution was divided into 1 ml aliquots in brown glass vials, dried under nitrogen and sealed prior to storing at −18°C. When required for use 1 ml of chloroform was added to redissolve.

Prior to measuring absorbance, the stock solutions were brought to room temperature and filtered through a 0·45 μm PVDF syringe filter (Gelman, Northampton, UK). An aliquot of the solution was evaporated under nitrogen and diluted in the appropriate solvent to give an approximate absorbance reading of 0·5 AU and the exact absorbance measured using a Perkin–Elmer Lambda 3 spectrophotometer equipped with a wavelength scanner to assess the spectrum. The concentrations were calculated using the appropriate extinction coefficients, shown in Table 1.

Individual working solutions of around 0·5–1·0 μg/ml were prepared from stock solutions by evaporating an aliquot under nitrogen and making to volume with mobile phase and their 'purity' assessed by HPLC analysis. 'Purity' of a carotenoid was expressed as the peak area of that carotenoid as a percentage of the total area of the chromatogram. The concentration calculated from the absorbance reading was corrected accordingly. The concentration of the stock standard solutions and their purity were measured each time a new working standard was prepared. A mixed working standard solution was prepared, in mobile phase, from individual stock solutions.

Calculation of carotenoid concentration

The concentration of carotenoids (μg/100 g) were calculated using response factors relative to β-cryptox-

Table 1. Extinction coefficients

	Solvent	λ	$E^{1\%}_{1\,cm}$	$E^{mM}_{1\,cm}$	M.Wt
Lutein	EtOH	445	2550	145·1	569
Zeaxanthin	Hexane	451	2480	141·1	569
β-cryptoxanthin	Hexane	451	2460	136·0	553
Lycopene	Hexane	472	3450	185·3	537
α-carotene	Hexane	444	2800	150·3	537
β-carotene	Hexane	450	2560	137·4	537

anthin. A working solution of β-cryptoxanthin was analysed with each batch of samples on the day of analysis (see Results and Discussion).

Calculation of relative response factor (RF)

$$RF = \frac{\text{Peak area of carotenoid working solution} \equiv 1\ \mu g/ml}{\text{Peak area of } \beta\text{-cryptoxanthin working solution} \equiv 1\ \mu g/ml}$$

Response factors used were: lutein 1·068, zeaxanthin 1·150, lycopene 0·996, α-carotene 1·114, β-carotene 1·004.

Calculation of carotenoid concentration in samples

$$\text{Concentration of carotenoid A } (\mu g/ml \text{ of extract}) = \frac{\text{area of peak A of diluted extract}}{\text{area of } \beta\text{-cryptoxanthin} \equiv 1\ \mu g/ml} \times RF\ (A) \times \text{dilution of extract} \times \frac{100}{\%\text{ rec int std}}$$

$$\text{Concentration of carotenoid A } (\mu g/100\ g) = \frac{\text{concentration of caretenoid A } (\mu g/ml \text{ of extract})}{\text{concentration of food sample in extract } (g/ml)} \times 100$$

Effect of solvent modifiers, recovery determination

For recovery studies with triethylamine (TEA), with the column system *in situ*, a mixture of lutein and β-carotene was injected and the total area of the chromatogram integrated. The column system was replaced with 200 cm of PTFE tubing (0·5 mm i.d.) and the mixture of lutein and β-carotene injected and the response measured again. The total response without the column system was taken to represent 100% recovery. 0·1% TEA was added to the mobile phase and the column system thoroughly equilibrated. The exercise was repeated with and without the column system in place. Column recovery was expressed as the percentage of the total response obtained without the column.

The columns were subsequently thoroughly re-equilibrated with mobile phase without TEA and the effect of TEA on the recovery of a mixed working solution of lutein, zeaxanthin, β-cryptoxanthin, lycopene, and α- and β-carotene measured under the conditions described above. In addition the response of individual carotenoids in the mix was measured in the presence and absence of TEA.

Further comparison was made between 0·1% and 0·05% TEA and the combined effect of 0·05% TEA plus 0·05 M ammonium acetate (AA).

RESULTS AND DISCUSSION

Method development and evaluation

We have previously discussed a number of factors which affect the chromatographic responses of carotenoids and contribute to analytical variations and inaccuracies in their quantitative determination (Scott, 1992; Scott & Hart, 1993). The following gives the results of further method development and assesses the 'robustness' of the method used.

Solvent modifiers

Carotenoids may undergo losses or degradation on the column. Studies have indicated that solvent modifiers improve the recovery of carotenoids from the column and reduce or eliminate on-column degradation. Lauren *et al.* (1987) reported that the addition of 0·1% *n*-decanol to the mobile phase conditioned new columns and avoided problems with trace solvent impurities. In the absence of *n*-decanol activation of residual silanol groups in the reversed phase packing material caused deterioration in column behaviour. However, Epler *et al.* (1993) did not observe any improvement in the performance of columns with poor recoveries when *n*-decanol was added to acetonitrile based mobile phases. Kamber and Pfander (1984) found it was necessary to add *N,N*-diisopropyl-ethylamine, a nucleophilic base, to obtain good recoveries from silica columns.

Work in our own laboratory and that reported by Epler *et al.* (1993) showed that the addition of triethylamine (TEA) to the mobile phase could increase the recovery of carotenoids. Under the conditions described in the Methods we found the recovery of a mixture of lutein and β-carotene increased from 60·6 to 97% on the addition of 0·1% TEA to the mobile phase.

The recovery of a mixture of lutein, zeaxanthin, β-cryptoxanthin, lycopene and α- and β-carotene increased from 68·1% without TEA to 92·2% with TEA. The percentage increase in the response of the individual carotenoids as a result of the addition of TEA was lutein 18%, zeaxanthin 33%, β-cryptoxanthin 33%, lycopene 53%, α-carotene 30% and β-carotene 42%. The differences in the recovery of individual carotenoids suggests that on-column losses vary for different carotenoids. We would suggest however that the magnitude of the effect on the individual carotenoids may have been affected by the conditioning of the columns with TEA in the initial part of the exercise with lutein and β-carotene prior to the subsequent reconditioning of the columns without TEA and the measurement of the effect of TEA on the mixture of all carotenoids. The possibility being that the initial effect of TEA had not been completely reversed. Nevertheless the beneficial effect of TEA was still amply demonstrated (see Fig. 1).

The addition of TEA also had the effect of reducing retention times but the main carotenoid peaks were still baseline resolved. The subsequent addition of 0·05% TEA did not substantially alter the responses or elution times compared to 0·1% TEA.

Ammonium acetate (AA) has also been reported to improve column recovery. Epler *et al.* (1993) tested the effect of AA, with and without TEA, on five different columns, the recoveries of which without modifier varied from 1% to 48%. The column with the highest initial recovery was shown to have the lowest trace metal

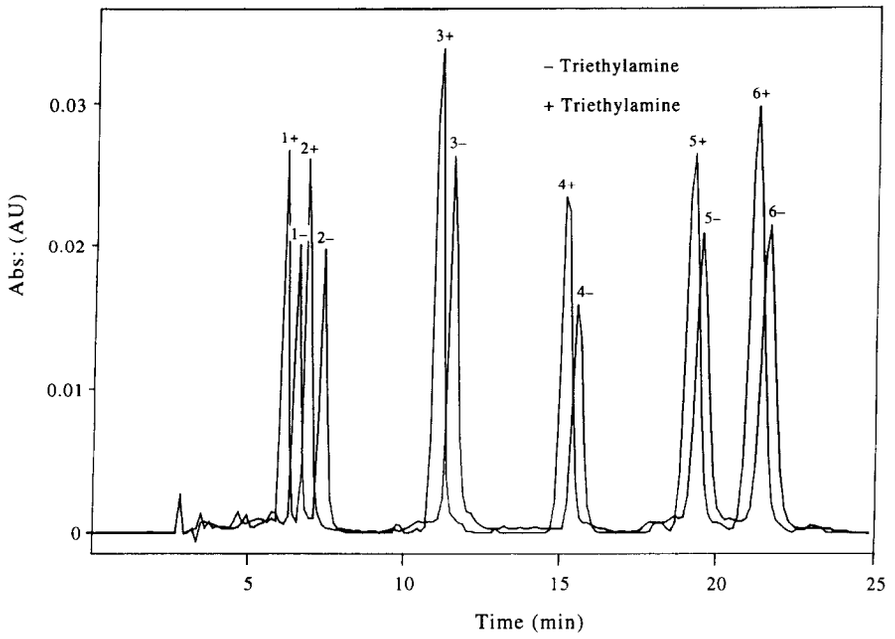

Fig. 1. The effect of the addition of triethylamine to the mobile phase on the recovery of carotenoids from the column. Elution profile of lutein (1), zeaxanthin (2), β-cryptoxanthin (3), lycopene (4), α-carotene (5) and β-carotene (6).

activity. Addition of AA (0·05 M added to MeOH used in the mobile phase) increased the recovery from all columns, but the percentage increase varied. Addition of 0·05% TEA to the mobile phase containing AA further increased the recovery of all columns to around 100%. Handleman *et al.* (1992) also reported that the use of AA reduced on-column degradation, which appeared most severe when small quantities of carotenoids were chromatographed. In our study, the addition of 0·05 M AA to a mobile phase containing 0·05% TEA did not significantly improve the recovery of the particular column tested. It did, however, slightly alter retention times, slightly reducing the separation of lutein and zeaxanthin, while increasing the separation between α- and β-carotene. Because of the obvious effect of TEA and the additional beneficial effect of AA found by Epler *et al.* we routinely add both to the mobile phase. The exact action of these solvent modifiers is unclear, but it is suggested that the improvement in recovery is caused by the buffering of acidity in the mobile phase, or more likely the acidity of the free silanols in the stationary phase or by preventing reactions with free metal ions.

Linearity of response

The response of lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene was linear from 0·05 to 4 μg/ml (r = 0·999–1·000). Estimated limits of detection are: Lutein 0·010 μg/ml; Zeaxanthin 0·015 μg/ml; β-cryptoxanthin 0·025 μg/ml; Lycopene 0·035 μg/ml; α-carotene 0·040 μg/ml; and β-carotene 0·045 μg/ml.

Repeatability and reproducibility of response

The response of five separate mixed working standard solutions prepared at the same time from stock solutions showed an average CV in response for all carotenoids of 4·1%.

The reproducibility of preparation (including calculation of concentration by absorbance and purity measurement) and response assessed from three sets of individual carotenoid working standards, prepared at different times over a period of six months, showed an average CV for all carotenoids of 4·2%. Under certain conditions, solutions of lycopene are unstable, even in the short term and large losses are observed. However we have previously reported (Scott, 1992) that a working solution of lycopene stored at −18°C in mobile phase plus BHT showed no apparent loss over a 20 day period. The response of two separate mixed working solutions was monitored over periods of 12 and 14 weeks respectively to assess the stability of the solutions and the longer term reproducibility of response. Table 2 shows that during these periods, the response of all carotenoids, excepting lycopene, had an average CV of 1·7%, indicating no losses. That for lycopene was variable, one solution having a CV of 55·5%, the response falling by 50% over the first 5 weeks, the other only 3·9%. These results indicate that the response of the stable carotenoids was highly reproducible in the longer term.

Table 2. The reproducibility of the response of mixed carotenoid working solutions (% CV)

	A	Std B
Lutein	2·022	1·417
Zeaxanthin	1·670	1·790
Lycopene (trans)	55·5	3·880
α-carotene (trans)	1·945	1·836
β-carotene (trans)	1·290	1·488
n	16	14

Table 3. Short-term (7 days) and long-term (12 months) reproducibility in the analysis of a reference vegetable mix (% CV)

	Short term	Long term
Lutein	5·6	4·9
Zeaxanthin	6·8	7·5
Lycopene	11·8	10·8
α-carotene	7·9	7·6
β-carotene	9·4	8·1
Mean % CV	8·3	7·8

In addition to assessing the reproducibility of the chromatographic system using carotenoid solutions, the reproducibility of the whole method including extraction (but not saponification) was measured using a reference vegetable material developed in our laboratory. The mix consisted of sweetcorn, tomatoes and carrots, providing sources of lutein, zeaxanthin, lycopene, α-carotene and β-carotene. Short-term reproducibility, measured by analysing 20 samples of the mix in duplicate over a period of 7 days, showed an average CV of 8·3%. Longer term reproducibility, measured by analysis of a further 20 samples, four on each of 5 occasions over a period of 12 months, showed an average CV of 7·8%. These are shown in Table 3.

Extraction

We have previously indicated that a 1:1 THF:MeOH solution was a suitable extraction solvent for vegetable material (Scott, 1992). This procedure has been further developed and is detailed in the Methods section. The efficiency of the extraction procedure was measured using samples of carrots, spinach and peas. After initial extraction, the extract was filtered and the absorbance of the subsequent washings was measured at 450 nm. After the third washing the filter pad was removed and re-extracted. Table 4 shows that over 90% of extractable material absorbing at 450 nm was extracted during the initial extraction and first washing. After the third washing less than 1% of the extractable material remained.

Recovery of an internal standard using this procedure was 87·8% (SD = 8·21, n = 127).

Saponification

Saponification of plant material is carried out to hydrolyse carotenoid esters and as a 'purification' step to remove chlorophylls. Where, however, the chromatographic system separates the chlorophylls from the carotenoids of interest then saponification is not necessary for most vegetables commonly consumed in the UK as the carotenoids occur in an unesterified form. However for certain vegetables such as peppers and fruits (this study) a mild saponification procedure was developed and used.

Table 4. Efficiency of extraction (% of total extractable compounds absorbing at 450 nm)

	Spinach	Peas	Carrots
Initial extraction	83·1	81·3	77·0
1st wash	14·0	15·0	16·5
2nd wash	1·6	2·1	4·0
3rd wash	0·4	1·3	1·7
Re-extraction	0·9	0·3	0·8
Extraction effic.	99·1%	99·7%	99·2%

Saponification procedures suggested in the literature vary widely in the concentrations of KOH, times and temperatures used, both in the presence and absence of antioxidants and nitrogen. Depending on the saponification conditions and the particular carotenoid, destruction or structural transformation may occur (Khachik, 1986; Miki *et al.*, 1990). Our studies indicate that when saponification was carried out subsequent to extraction, 5% methanolic KOH (final volume) for 30 min at room temperature under N_2 was sufficient to obtain the maximum value for the carotenoids of interest. However for practical reasons, for routine analysis, the time was extended to one hour. Recovery of an added internal standard averaged 73·8% (SD = 9·85, n = 22). Although this procedure appeared satisfactory for fruits and vegetables, preliminary trials with a 'higher fat' material indicated that a higher concentration of KOH may be necessary. Further studies are required to develop and evaluate routine saponification procedures for a wide range of food materials, particularly with regard to the potential losses of individual carotenoids and the effect of various antioxidants.

Quantification of data

A probable source of error and variance both within and between laboratories is in the preparation and calibration of stock and working standard carotenoid solutions, and their use in quantifying the carotenoid content of samples.

The solubility (dissolution) of carotenoids must be ensured, although we would recommend that all solutions should be filtered prior to establishing concentrations by absorbance at a specified wavelength. It should also be noted that absorption maxima and extinction coefficients used do appear to vary between laboratories, even with the same solvent. It is also an essential part of good laboratory practice that the spectrophotometer is regularly calibrated both for wavelength and response.

In our experience, stock solutions of lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene, but not lycopene, are stable over a relatively long period, but to ensure accuracy the absorption must be checked every time a new working solution is prepared. It is also important that the 'purity' of the carotenoid working solutions is measured and the concentrations corrected accordingly (see Methods). In this respect it should be noted that because of the 'impurities' within individual working solutions (e.g. α-carotene as a contaminant in β-carotene), the response of individual carotenoids within a 'mixed' working standard will

Table 5. The carotenoid content of vegetables (μg/100 g 'wet weight' as eaten)

		Lutein	Zeax	β-cryp	Lyco	α-car	β-car	*cis* β-car
Brussels frozen	raw	610	—	—	—	—	441	112
Brussels frozen	cooked	621	—	—	—	—	411	144
Beans green frozen	raw	494	—	—	—	70	299	77
Beans green frozen	cooked	548	—	—	—	26	323	50
Beans baked T/S	canned	25	—	—	1659	—	30	nd
Beans broad	raw	506	—	—	—	—	215	46
Bean broad	cooked	620	34	—	—	—	358	48
Beans French	raw	479	—	—	—	—	306	77
Beans French	cooked	577	—	—	—	—	388	55
Beans runner	raw	555	—	—	—	33	277	66
Beans runner	cooked	632	—	—	—	Tr	490	48
Broccoli fresh	raw	1614	—	—	—	—	800	119
Broccoli fresh	cooked	1949	—	—	—	—	1125	256
Cabbage green	raw	80	—	—	—	—	51	8
Cabbage green	cooked	111	—	—	—	—	65	6
Cabbage Savoy	raw	103	—	—	—	—	50	nd
Cabbage Savoy	cooked	341	—	—	—	—	240	33
Carrots (May)	raw	170	—	—	—	2660	8521	76
Carrots (May)	cooked	149	—	—	—	2838	8831	228
Carrots (Sept)	raw	283	—	—	—	3610	10800	Tr
Carrots (Sept)	cooked	313	—	—	—	3767	10487	1815
Carrots frozen	raw	268	—	—	—	3268	8538	nd
Carrots frozen	cooked	300	—	—	—	3851	9907	nd
Cauliflower	raw	Tr	—	—	—	—	nd	nd
Cauliflower	cooked	Tr	—	—	—	—	nd	nd
Cucumber	raw	670	—	—	—	—	222	73
Greens	raw	3046	—	—	—	—	1663	286
Greens	cooked	4437	—	—	—	—	2700	405
Leeks	raw	161	—	—	—	—	69	18
Leeks	cooked	164	—	—	—	—	70	14
Lettuce iceberg	raw	110	—	—	—	—	74	17
Lettuce butterhead	raw	1611	—	—	—	—	1603	385
Marrow	raw	128	—	—	—	—	36	12
Marrow	cooked	135	—	—	—	—	44	9
Mixed veg frozen	raw	882	84	—	—	1045	3670	525
Mixed veg frozen	cooked	976	59	—	—	1045	3639	866
Parsley	raw	5812	—	—	—	—	3505	1018
Peas frozen	raw	1633	—	—	—	—	360	78
Peas frozen	cooked	1991	—	—	—	—	546	114
Pepper green	raw	660	—	—	—	—	235	63
Pepper green	fried	1023	—	—	—	—	496	47
Pepper green*	raw	1116	—	—	—	—	626	88
Pepper orange	raw	503	1608	90	—	167	219	197
Pepper orange*	raw	2492	8480	779	—	636	888	240
Sweetcorn frozen	raw	522	437	—	—	60	45	14
Sweetcorn frozen	cooked	444	375	—	—	33	24	nd
Spinach	raw	5869	—	—	—	—	3397	624
Spinach	cooked	7410	—	—	—	—	4461	1040
Spring onions	raw	255	—	—	—	—	112	30
Tomato	raw	78	—	—	2937	—	415	24
Tomato	cooked	120	—	—	3703	—	549	99
Tomatoes canned	in sauce	105	—	—	6205	—	353	70
Tomatoes canned	reheated	116	—	—	5613	—	258	Tr
Watercress	raw	10713	—	—	—	—	4777	1142
Cabbage Savoy	outside leaves	14457	—	—	—	—	10020	1829

* Saponified.
Tr = trace; — = not present or nd = not detected.

probably be higher than that of the individual solution alone. The corrected concentration calculated for individual solutions is therefore not necessarily appropriate for 'mixed' standard solutions. If a mixed standard is used for quantification of the sample the concentration of the standard carotenoids must be recalculated.

Whilst in our experience individual or mixed carotenoids are relatively stable, under strict quality control conditions either new solutions should be prepared regularly and compared or a system to monitor the reproducibility of response should be introduced.

By using relative response factors (i.e. the relative response of individual carotenoids to another specified carotenoid of equivalent mass/volume, e.g. 1 μg/ml), and by monitoring the reproducibility of the system using a 'mixed standard', we would suggest that accuracy can be improved and variations due to preparation, calibration and instability of working solutions reduced.

In our study, response factors relative to β-cryptoxanthin for individual carotenoids were established from the response equivalent to 1 μg/ml for individual carotenoids, to the response equivalent to 1 μg/ml of β-cryptoxanthin. Both the working solution of β-cryptoxanthin and a mixed standard were run with each set of samples. The absolute response of β-cryptoxanthin (peak area) was monitored for each run and compared with the 'norm'. If this absolute response varies by more than ±5% from that 'norm' a new β-cryptoxanthin working solution should be prepared and compared. The typical variation in response was <±2% (n = 24). The reproducibility of the chromatographic system was checked by comparing response factors calculated from the 'mixed' standard. Any undue variation would indicate changes in the chromatographic system or degradation of the 'mixed' standard solution.

Carotenoid composition of vegetables

Table 5 shows the carotenoid composition of vegetables commonly consumed in the UK, and where appropriate, the effects of cooking.

Good sources (>1000 μg/100 g) of lutein were broccoli, butterhead lettuce, parsley, peas, peppers, spinach and watercress, of lycopene; tomatoes and tomato products, of β-carotene; broccoli, carrots, greens, butterhead lettuce, mixed vegetables, parsley, spinach and watercress. However the figures quoted here must not be considered absolute values for all sources of these items. The content of particular items may be affected by variety, maturity, growing conditions, season of the year and which part of the item is consumed. Invariably outer leaves, skin, etc., contain higher levels of carotenoids than inner parts of the plant. Analysis of a sample of carrots showed that the outer part contained twice as much β-carotene as the inner part. The outer leaves of a savoy cabbage were found to contain 150 times more lutein and up to 200 times more β-carotene than the average for the inner portion. Consequently, the carotenoid content of the item as consumed will depend on the extent of the 'outer' material discarded during preparation.

To assess the variation in the lutein and β-carotene content between samples of the same item, the four individual samples of frozen peas, fresh carrots (May purchase) and frozen carrots were analysed separately.

The range for lutein and β-carotene in peas was from 1519 to 1929 μg/100 g and from 391 to 456 μg/100 g, in fresh carrots from 117 to 294 μg/100 g and 8021 to 8929 μg/100 g, frozen carrots from 161 to 531 μg/100 g and 6689 to 14640 μg/100 g, respectively.

Table 6. The content of lutein, lycopene and β-carotene in 10 varieties of tomatoes (μg/100 g)

	Lutein	*trans*-Lycopene	Total Lycopene	*trans*-β-Carotene
Red varieties				
Cherry	101	2686	3780	473
'Large'	68	1915	2270	349
'Salad'	78	2158	2547	509
Flavourtop	48	4958	5653	428
Tigerella	191	1223	1582	1702
Ida F1 hybrid	103	1324	1711	964
Shirley F1	79	2079	2347	771
Craig	149	2948	3907	1093
Moneymaker	59	3475	4255	427
Allicanti	91	3659	4037	525
Beefsteak	89	2729	4833	883
Yellow varieties				
Sungold	204	390	528	2232
Gold sunrise	107	21	21	93

The variation between 10 varieties of fresh tomatoes is shown in Table 6. Figure 2 shows the typical carotenoid profiles of peas, carrots and tomatoes.

Effect of cooking

Table 7 shows the percentage change in the lutein and β-carotene content on cooking of vegetables.

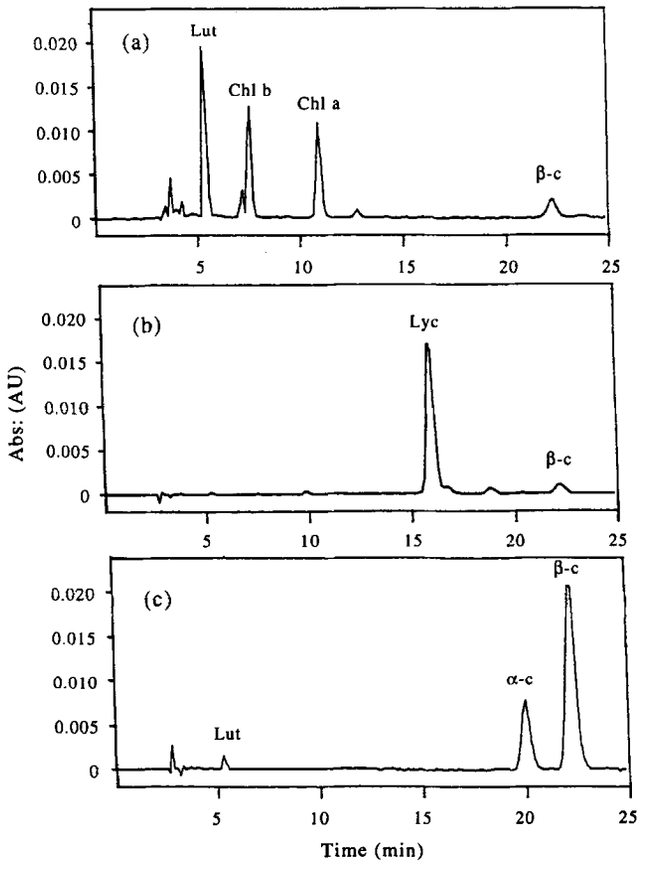

Fig. 2. The carotenoid profile of pea, tomato and carrot. (a) pea, (b) tomato, (c) carrot. Lut = Lutein, Lyc = Lycopene, α-c = α-carotene, β-c = β-carotene. Chl a = Chlorophyll *a*, Chl b = Chlorophyll *b*.

Table 7. The percentage change in the lutein and β-carotene content of vegetables on cooking

	Lutein	β-carotene
Brussels sprouts	+18	-6
Beans green	+11	+8
Beans broad	+22	+66
Beans french	+20	+26
Beans runner	+14	+77
Broccoli	+21	+41
Cabbage green	+39	+27
Cabbage Savoy	+231	+380
Carrots May	-12	+4
Carrots Sept	+10	-3
Carrots frozen	+12	+4
Greens	+46	+62
Leeks	+2	+1
Marrow	+5	+22
Mixed vegetables	+10	-1
Peas	+22	+52
Pepper green	+55	+111
Spinach	+26	+31
Tomatoes	+54	+11

Whilst some authors have described losses from vegetables subjected to different 'cooking' procedures, our findings are in general agreement with those reported by Granado *et al.* (1992). We have noted little or no loss. In fact, when compared on a wet weight basis, green vegetables (excluding savoy cabbage), peas and beans showed an average increase of 24% lutein and 38% β-carotene. Cooking has been reported to increase the chemical extractability of carotenoids and this may be a factor in reports that cooking increases the bioavailability of carotenoids to humans.

Table 8 shows the carotenoid composition of fruits. Again the content of particular fruits may be affected by variety, season, etc., and the part eaten. The skin (1·5 mm layer) of eating apples contained around five times more lutein and β-carotene than the flesh alone.

Published data on the wider carotenoid composition of vegetables and fruits is limited and absolute comparison of our data with other published data is difficult because of the variations previously discussed. This includes the reliability of data. Mangles *et al.* (1993), in the discussion of the limitations of the quality and quantity of data used in their data base, state that only 9% of foods could be given an 'A' confidence code (i.e. the user can have considerable confidence in the value). The limited quantity of data was illustrated by the fact that although 61% of foods had more than one acceptable value for β-carotene, only 20% had more than one acceptable value for β-cryptoxanthin. In addition, limited quality control information in published reports make it difficult to compare the relative quality of analytical data. Certainly an area which may improve the quality of data would be the development and use of a standard reference material for the analysis of carotenoids. The study by Heinonen *et al.* (1989) on Finnish foods is possibly the most comprehensive yet published for European type foods. Where comparison can be made, our data is in very good general agreement with that of Heinonen *et al.*, both in relative and absolute terms.

CONCLUSIONS

The development of more sensitive methodology, such as HPLC, means that artefacts produced during extraction, purification and chromatography will be more readily detected. The isolation of carotenoids from natural products involves extraction and chromatography with organic solvents. Carotenoids are sensitive to light, heat, air and active surfaces. Their isolation and analysis may be accompanied by degradation, structural rearrangement, formation of stereoisomers, and other physicochemical reactions. This study has demonstrated the need for careful evaluation of analytical procedures and validation of carotenoid responses in order to avoid factors which cause variation and inaccuracies in the quantitative determination of carotenoids.

These factors can be summarized as follows; the use of a suitable column and solvent system to obtain discreet separation of carotenoids, the use of metal-free columns or 'biocompatible' column frits, the use of solvent

Table 8. The carotenoid content of fruits (μg/100 g 'wet weight', as eaten)

	Lutein	Zeax	β-cryp	Lyco	α-car	β-car	*cis* β-car
Apples eating	84	13	11	—	nd	35	nd
Apricots dried	44	23	75	—	nd	607	407
Apricots fresh	101	31	231	—	37	1458	308
Apricots tinned	59	16	32	—	nd	713	166
Bananas	33	4	nd	—	50	39	nd
Mandarins	50	142	1774	—	12	274	11
Orange Valencia	64	50	83	—	nd	14	nd
Satsumas	44	41	1180	—	nd	23	nd
Peach	78	42	86	—	Tr	81	22
Strawberry	6	nd	nd	—	nd	11	nd
Apple peel (1·5 mm)	434	16	20	—	nd	190	39

All samples were saponified.
nd = not detectable; Tr = trace; — = not present or nd = not detected.

modifiers, and the use of a temperature controlled system to obtain repeatable time elution profiles. Extreme care must be taken to ensure accuracy in measuring the concentration of stock carotenoid solutions. Quality control systems should be used to monitor the day-to-day reproducibility of the chromatographic system. We would suggest that the universal availability of a suitable reference material would significantly enhance the evaluation of the quality of data, both within and between laboratories.

We are currently involved in preliminary studies being carried out by the European Community Bureau of Reference and the National Institute of Standards and Technology (USA), to evaluate potential reference materials for the analysis of carotenoids in foods.

ACKNOWLEDGEMENTS

The food items analysed were selected from a study to validate methods of measuring dietary intake, (Dr S. A. Bingham, Dunn Clinical Nutrition Centre, Cambridge, UK). This study was partly supported by the Ministry of Agriculture Fisheries and Food. The authors would like to acknowledge the contribution of Mr Ken Day and Jennie Macdairmid, and the assistance of Margaret Buxton, Janet Newman and Lionel Perkins during the course of this study. This study was supported by the Ministry of Agriculture Fisheries and Food.

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APPENDIX I

Cooking methods

Preparation and cooking

Brussels sprouts	Frozen. Boiled in water for 15 min
Green beans	Frozen. Boiled for 10 min
French beans	Remove and discard ends, boiled whole in 4 cm water for 7 min in a covered pan
Runner beans	Remove and discard ends, cut into 3 cm pieces, boil in 4 cm water for 10 min in a covered pan
Broad beans	Beans removed from pods and boiled 4 cm water 20 min in a covered pan
Broccoli	Boiled in 4 cm water for 10 min in a covered pan
Green cabbage Savoy cabbage Spring greens	Outer leaves and stalk removed and discarded, shredded and boiled for 12 min
Spring onions	Roots, outer sheath and green material above first leaf division discarded
Carrots	Fresh: Ends and periderm (1·5 mm) removed and discarded. Boiled for 15 min Frozen: Placed in boiling water and boiled 10 min
Cauliflower	All leaves discarded. Quartered, and boiled in 4 cm of water for 10 min in a covered pan
Leeks	Removed outer two sheaths, and leaf material above the point where the outer sheath splits and discard Remaining portion sliced and boiled for 10 min
Marrow	Peeled and de-seeded. Boiled in 2 cm water for 10 min in a covered pan
Peas	Frozen. Placed in boiling water for 5 min
Pepper	Top and seeds discarded. Fried in 15 ml sunflower oil for 20 min
Sweetcorn	Frozen. Placed in boiling water for 7 min
Spinach	Stalks removed. Leaves boiled in 2 cm of water for 10 min in a covered pan
Tomato	Fresh: Fried in 15 ml sunflower oil for 10 min. Tinned: All contents of tin brought to the boil

Boiling was carried out in an aluminium saucepan of 20 cm diameter and 12 cm depth.

APPENDIX II

Taxonomic names

Common name	Taxonomic Name
Brussels sprout	*Brassica oleracea* var. *gemmifera*
Green beans	*Phaseolus vulgaris*
Baked beans	*Phaseolus vulgaris*
French beans	*Phaseolus vulgaris*
Runner beans	*Phaseolus coccineus*
Broad beans	*Vicia faba*
Broccoli	*Brassica oleracea* var. *botrytis*
Green cabbage	*Brassica oleracea* var. *capitata*
Savoy cabbage	*Brassica oleracea* var. *bullata*
Carrot	*Daucas carota*
Cauliflower	*Brassica oleracea* var. *botrytis*
Cucumber	*Cucumis sativus*
Spring greens	*Brassica oleracea* var. *capitata*
Spring onions	*Allium cepa*
Leeks	*Allium ampeloprasum* var. *porrum*
Lettuce	*Lactuca sativa*
Marrow	*Cucurbita pepo*
Parsley	*Petroselinum crispum*
Pea	*Pisum sativa*
Pepper	*Capsicum annum* var. *grossum*
Sweetcorn	*Zea mays*
Spinach	*Spinacia pumila*
Tomato	*Lycopersicon esculentum*
Watercress	*Nasturtium oficinale*
Apple	*Malus pumila*
Apricot	*Prunus armeniaca*
Banana	*Musa* spp.
Mandarin	*Citrus reticulata*
Orange	*Citrus sinensis*
Satsuma	*Citrus unshiu*
Peach	*Prunus persica*
Strawberry	*Fragaria* spp.