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PII: S0308-8146(98)00011-9

Food Chemistry, Vol. 63, No. 4, pp. 577–584, 1998

Printed in Great Britain
0308-8146/98 $19.00 + 0.00
Analytical, Nutritional and Clinical Methods Section

Evaluation of extraction method for the analysis of carotenoids in fruits and vegetables

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(Received 11 July 1997; revised version received and accepted 18 November 1997)

This study evaluated a suitable extraction method for a wide range of sample matrices in carotenoid analysis. Using canned tomato juice as a representative sample, it is shown that two solvents of low biological hazard, ethanol and hexane are the most suitable for extracting carotenoids from the matrix. The use of double extraction, each with 35 ml of ethanol:hexane mixture (4:3, by volume), resulted in good recoveries of carotenoids (lycopene 96%, α-carotene 102% and β-carotene 93–100%). Coefficients of variation conducted on different days were: lycopene 5% and β-carotene 7%. An application of the established method to various kinds of fruit and vegetable matrices is also shown, using carrot and spinach as representative samples of root and leafy vegetables, for determining recoveries of added carotenoids. The average percent recoveries of added carotenoids from canned tomato juice, carrot and spinach were: 101, 99.8 and 101% for α-carotene (12.4, 24.8, 49.6 and 99.2 μg/10 ml of added α-carotene); and 98.1, 99.7 and 96.1 percent for β-carotene (25.5, 50.9, 101 and 201 μg/10 ml of added β-carotene). These similar recoveries over the explored concentration ranges confirm that the application of established extraction method is unaffected by differences in matrix composition of the samples.

INTRODUCTION

Fruits and vegetables contain different amounts and types of carotenoid and other components (Paul and Southgate, 1978; Goodwin, 1980; Bauernfeind, 1981; Britton, 1983). It is not known whether the different components among matrices influence the extraction and quantitation of carotenoids. Analyses of carotenoid levels frequently employ HPLC because of its ability to distinguish between similar geometrical structures of carotenoid (Bushway and Wilson, 1982; Dietz *et al.*, 1988; Mejia *et al.*, 1988). Its rapidity and the small amount of sample required also make it suitable for routine analysis of samples. Extraction of analytes from sample matrix is an important step prior the HPLC analysis. A literature review has shown that procedures for extraction of analytes from foods involved various types of solvent, solvent combinations and procedures (Table 1) and the relative efficacies of the existing methods have not yet been evaluated.

This study aims to find a suitable extraction method for a wide range of sample matrices for the analysis of carotenoids. Evaluations for the method were conducted by (1) comparison of extraction yields of the analytes obtained using different reported methodologies, (2) modification of the selected methodology for the most effective conditions, (3) validation of the established method to indicate its accuracy and repeatability, (4) investigation of the possible application of the established method to various kinds of fruit and vegetable matrices.

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MATERIALS AND METHODS

Chemicals

Lycopene, α-carotene (type V) and β-carotene (type IV) were obtained from Sigma Chemical Company (St. Louis, USA), and β-apo-8′-carotenal was purchased from Fluka Company (Basel, Switzerland).

HPLC grade solvents including acetonitrile and methanol were obtained from BDH Ltd (Poole, UK) while chloroform and hexane were obtained from Mallinckrodt Australia Pty Ltd, (Victoria). High-purity oxygen-free nitrogen gas was obtained from Commonwealth Industrial Gasses (Australia).

Table 1. Summary of extraction methods used for fruits and vegetables

Carotenoids	Food	Solvent[a]	References
α-, β-carotene and cryptoxanthin	orange juice	petroleum ether: isopropanol (1:3), diethyl ether[b]	Reeder and Park, 1975
α-, β-, ζ-carotene, α-, β-cryptoxanthin	orange juice	dichloroethane: methanol (1:1), hexane, diethyl ether[b]	Stewart, 1977
α-, β-carotene, lycopene	tomato	acetone, petroleum ether[b]	Zakaria *et al.*, 1979
β-carotene	fresh plant material	acetone: hexane (2:3), acetone, hexane or ethanol: hexane (4:3), ethanol, hexane	Association of Official Analytical Chemists, 1984, Section 43.015
α- and β-carotene	fruit and vegetable	tetrahydrofuran (for high carotene content) tetrahydrofuran, petroleum ether (for low carotene content)	Bushway and Wilson, 1982
α- and β-carotene	fruit and vegetable	tetrahydrofuran	Bushway, 1985
α- and β-carotene	fruit and vegetables	acetone:petroleum ether (1:1)	Hsieh and Karel, 1983
carotenoids	citrus	acetone, methanol:acetone (1:1) acetone, diethyl ether[b]	Noga and Lenz, 1983

[a]Solvent ratios in the parentheses are by volume ratio.
[b]Sample extracts were saponified.

Chromatographic conditions

A liquid chromatograph was equipped with a Waters model 45 pump (Millipore-Waters Associates, USA), a Waters U6K injector, a Waters model 440 detector, and a Waters temperature controller. An Omni Scribe dual pen recorder (Huston Instrument, USA) was used to record a chromatographic profile at a chart speed of $0.25\,\text{cm}\,\text{min}^{-1}$ and 0.01 V full-scale (0.01 VFS). An integrator model 3392 A (Hewlet Packard, USA) was used to integrate the detector response into peak area in carotenoids analysis.

A NOVA PAK C_{18} reverse phase column (150×13.9 mm, particle size $5\,\mu$m) and Bondapak C_{18} guard column (CORASIL MICRON 37–50) were obtained from Millipore-Waters Associates, USA.

Apparatus and other instruments

A spectrophotometer (Double-Beam Spectrophotometer, Model 220) from Hitachi Ltd (Tokyo, Japan) was routinely used for measuring the absorption of the working standard solutions.

A food processor (Breville Cyclonic Super Wizz, Hong Kong), an Omni-Mixer (Du Pont Company, CT, USA) and a rotatory evaporator (Büchi Rotavapor-RE, Buchi Laboratory-Techniques Ltd, Flawil, Switzerland) were required.

Laboratory conditions

Since the study involved handling light-sensitive compounds, all steps of the experimental procedures were performed in subdued lighting, with a 25 W red globe, (Phillips Ltd, Australia) providing the only illumination.

Preparation of standard carotenoid solutions

All of the solutions were prepared under red light and kept under a nitrogen atmosphere at $-20°$C. Working standards of each compound were prepared daily to the desired concentration from the stock solution, and checked daily by absorption at maximum wavelength. In addition, all solvents used in the preparation of stock solutions or working solutions were saturated with nitrogen before use.

β-Apo-8′-carotenal
A working solution of β-apo-8′-carotenal was prepared daily by diluting a stock β-apo-8′-carotenal solution (approximately 1 g/100 ml in chloroform) with hexane to obtain 0.40 ± 0.04 AUFS at 450 nm which was obtained by scanning its spectrum.

Lycopene
A concentrated solution of lycopene was prepaired daily (1 mg/10 ml) in chloroform and further diluted with hexane to obtain a working standard with an absorbance of 0.40 ± 0.04 at 472 nm. The concentration of working standard lycopene in μg/100 ml was calculated from its extinction coefficient ($E^{1\%}_{1\,\text{cm}}$ 3450 in hexane at 472 nm, Arroyave *et al.*, 1982).

α-Carotene
A working solution of α-carotene was prepared daily by diluting a stock solution of α-carotene (approximately

5 mg/50 ml in hexane) with hexane to obtain 0.40 ± 0.04 AUFS at 446 nm. The concentration of working solution α-carotene in μg/100 ml was calculated from its extinction coefficient ($E^{1\%}_{1\,cm}$ 2725 in hexane at 446 nm, Zechmeister and Polgar, 1943, as specified by Sigma Chemical Company).

β-Carotene

A working solution of β-carotene was prepared daily by diluting a stock solution of β-carotene (approximately 5 mg/50 ml in hexane) with hexane to obtain 0.40 ± 0.04 AUFS at 452 nm. The concentration of working standard β-carotene in μg/100 ml was calculated from its extinction coefficient ($E^{1\%}_{1\,cm}$ 2590 in hexane at 452 nm, Zechmeister and Polgar, 1943, as specified by Sigma Chemical Company).

Sample preparation

Canned tomato juice was selected as a representative sample of fruits and vegetables food groups in this study. This is because of the stability of the carotenoids during storage, the homogeneity and similarity of matrix to that of blended fruits and vegetables (Bauernfeind, 1981; Wolf, 1985; Sheft *et al.*, 1949; Farrow *et al.*, 1973). Canned tomato juice of Berrivale Orchards Ltd, Australia purchased from a local supermarket was used.

For application of the established method to various kinds of matrix, carrot and spinach were chosen as representative samples of root and leafy vegetables, with red and green colour. One kilogram each of carrot and spinach was obtained from a local fruit and vegetable shop. The purchased carrot was then cut into small pieces and sampled after cleaning and peeling. Carrot slices (250 g) were blended finely in a food processor. Purchased spinach was treated similarly. All processes were performed under red light.

Peak identification

Identification of carotenoids was based on retention times and comparison with a pure standard as well as co-chromatography with added standards of lycopene, α-carotene and β-carotene.

Comparative study of extraction procedures

Aliquots (2 g) of tomato juice from the same can were weighed and extracted as described by the six methods listed in Table 2. After extraction, the solvents were evaporated to dryness under a stream of nitrogen and the residues transferred to a 10 ml volumetric flask containing dried β-apo-8′-carotenal (internal standard) by dissolving with portions of 10 ml hexane and the extracts were then immediately chromatographed.

Validation of the established method

Recovery

In order to determine recovery of added analytes under an established extraction procedure, standard calibration curves of the pure carotenoids were prepared on the same day as the analysis as follows. Known quantities of lycopene, α-carotene and β-carotene standard solutions were added to a 10 ml volumetric flask and the mixture was dried under a stream of nitrogen, redissolved in 2 ml chloroform and made up to the mark with HPLC mobile phase. The 20 μl of resulting solution was injected for HPLC analysis. After chromatography of these solutions, calibration curves were obtained by plotting the peak area ratios of lycopene, α-carotene and β-carotene to β-apo-8′-carotenal versus the concentrations of added carotenoids.

Aliquots of 2.0 g canned tomato juice, with and without the addition of known quantities of the pure carotenoids, were treated with hexane containing β-apo-8′-carotenal (the internal standard) and further extracted using the established procedure described (Scheme 1). After chromatography, the concentrations of endogenous carotenoids and endogenous plus added carotenoids in each sample were calculated using standard calibration curves. Subtraction of the endogenous carotenoids gives recovered values of added carotenoids. Percentage recoveries of added carotenoids were then calculated.

Table 2. Extraction methods for comparative study using canned tomato juice

Method 1	Association of Official Analytical Chemists, 1984, Section 43.015	2–5 g sample extracted 5 min with 100 ml acetone: hexane (4:6)
Method 2	Association of Official Analytical Chemists, 1984, Section 43.015	2–5 g sample extracted 5 min with 140 ml ethanol: hexane (4:3)
Method 3	Folch *et al.*, 1957	2 g sample extracted with 34 ml chloroform:methanol (2:1)
Method 4	Chen *et al.*, 1981	2 g sample extracted with 34 ml dichlomethane:methanol (2:1)
Method 5	Hara and Radin, 1978	2 g sample extracted with 36 ml hexane:isopropanol (3:2)
Method 6	Hsieh and Karel, 1983	2–5 g sample extracted with acetone:petroleum ether (50:50)

Note: Solvent ratios in the parentheses are by volume ratio.

Precision

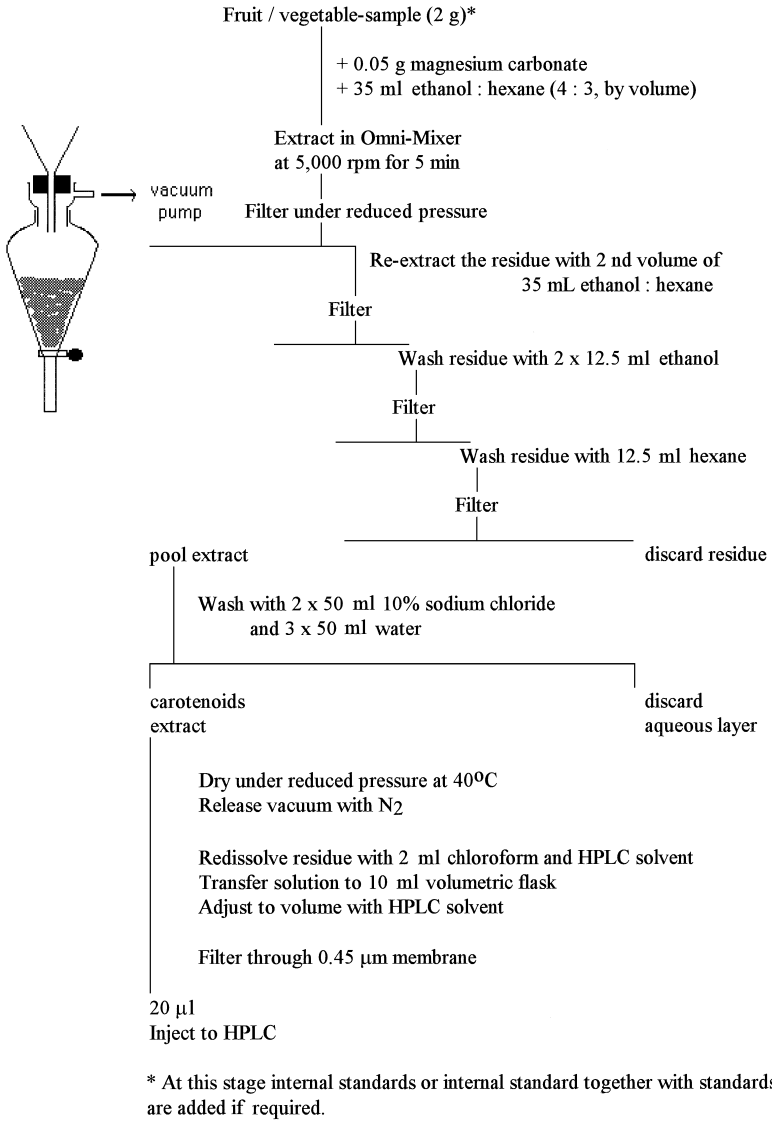
To demonstrate the reproducibility of the established procedure, endogenous carotenoids in an aliquot of 2.0 g canned tomato juice were determined in duplicate (all samples were from the same production lot) for nine consecutive days. Prior to extraction each sample was treated with hexane containing β-apo-8′-carotenal and the extraction procedure followed as in Scheme 1. After chromatography, peak area ratios of lycopene and β-carotene to the internal standard, β-apo-8′-carotenal were converted into concentrations (μg/10 ml) by means of standard addition curves and further converted to μg/g sample.

Standard addition curves of each carotenoid were constructed by plotting the peak area ratios of each carotenoid to internal standard versus the known concentration of added analyte. These values were obtained from the analyses of canned tomato juice with and without the addition of pure carotenoids in conjunction with the use of an internal standard.

The endogenous values obtained were then calculated for % coefficient of variation within the day and between run.

Investigation of a possible application of the established method to various matrices

For the investigation, canned tomato juice, carrot and spinach were used as representative matrices. Prior to extraction, known quantities of α- and β-carotene together with β-apo-8′-carotenal were added to 2.0 g samples of canned tomato juice, carrot and spinach. The addition of these carotenoids was made at concentration levels which covered the general range expected in carrot and spinach that were 12.4, 24.8, 49.6 and 99.2 μg/10 ml for α-carotene; and 25.5, 50.9, 101 and



Scheme 1. Summary of the extraction method for the analysis of carotenoids in fruits and vegetables.

201 μg/10 ml for β-carotene, respectively. Samples, without the addition of carotenoids, were also treated with β-apo-8′-carotenal and were then extracted and percentage recoveries of added compound were calculated.

RESULTS AND DISCUSSION

Comparative study of extraction procedures

The mean peak area ratios of lycopene and β-carotene relative to β-apo-8′-carotenal obtained using the six extraction methods are shown in Fig. 1. Table 3 shows the changes in carotenoid contents of the extract solution with time. Mean peak area ratios of lycopene and

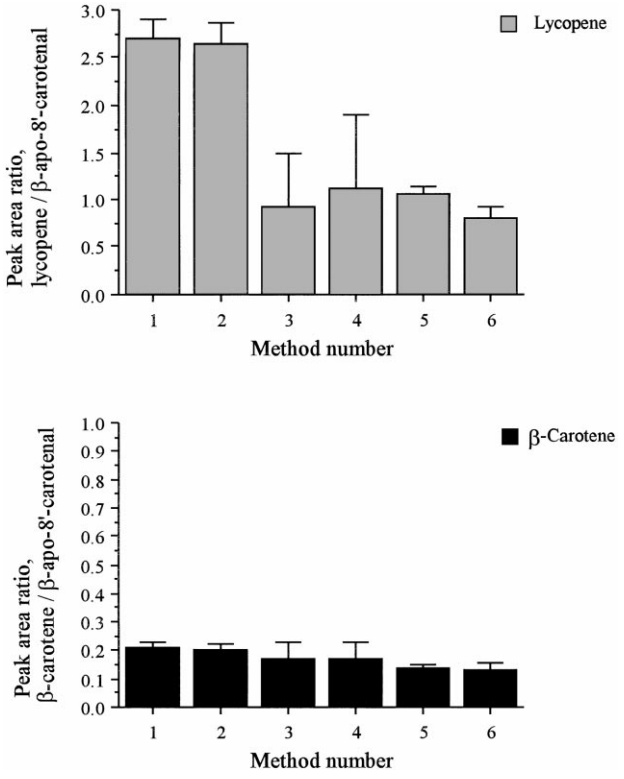


Fig. 1. Peak area ratios of lycopene and β-carotene relative to internal standard (β-apo-8′-carotenal) extracted from canned tomato juice using six extraction methods (Method 1, Association of Official Analytical Chemists (AOAC), 1984, Section 43.015 using acetone: hexane; Method 2, AOAC 1984, Section 43.015 using ethanol: hexane; Method 3, Folch *et al.*, 1957; Method 4, Chen *et al.*, 1981; Method 5, Hara and Radin, 1978; Method 6, Hsieh and Karel, 1983). The values presented are the mean of six replicate determinations with one injection using two x 2 g samples from three cans of tomato juice. One-way ANOVA and Duncan's test at 95 percent confidence limit: Lycopene—Methods 1 and 2 are not significantly different. Methods 1 and 2 are each significantly different from methods 3, 4, 5 and 6. β-Carotene—Methods 1 and 2 are not significantly different and either method is not significantly different from methods 3 or 4. Methods 3 and 4 are not significantly different. Methods 1 and 2 both are significantly different from methods 5 and 6.

Table 3. The change in lycopene and β-carotene content of the extracts with time[a]

Method	Time (min)[b] 0	60	180
Lycopene			
1	2.70 ± 0.20	2.66 ± 0.17	2.66 ± 0.22
2	2.65 ± 0.22	2.65 ± 0.20	2.56 ± 0.23
3	0.92 ± 0.57[c]	0.60 ± 0.29	0.49 ± 0.24[c]
4	1.11 ± 0.80[d]	0.54 ± 0.26	0.40 ± 0.23[d]
5	1.05 ± 0.08	1.04 ± 0.11	1.04 ± 0.10
6	0.80 ± 0.12	0.79 ± 0.12	0.80 ± 0.13
β-Carotene			
1	0.21 ± 0.02	0.20 ± 0.02	0.20 ± 0.02
2	0.20 ± 0.02	0.19 ± 0.02	0.19 ± 0.02
3	0.17 ± 0.06[e]	0.12 ± 0.02	0.12 ± 0.02[e]
4	0.17 ± 0.06[f]	0.15 ± 0.04	0.12 ± 0.02[f]
5	0.14 ± 0.01	0.14 ± 0.02	0.14 ± 0.01
6	0.13 ± 0.03	0.12 ± 0.02	0.13 ± 0.03

[a]Lycopene and β-carotene content presented are the mean peak area ratios of lycopene and β-carotene relative to β-apo-8′-carotenal from six replicate determinations with one injection using two × 2 g samples from three cans of tomato juice.
[b]Represents time intervals of HPLC analysis following the sample preparation.
[c] Means are significantly different ($P < 0.05$), using paired t-test.
[d] Means are significantly different ($P = 0.05$), using paired t-test.
[e] Means are significantly different ($P < 0.05$), using paired t-test.
[f] Means are significantly different ($P < 0.05$), using paired t-test.

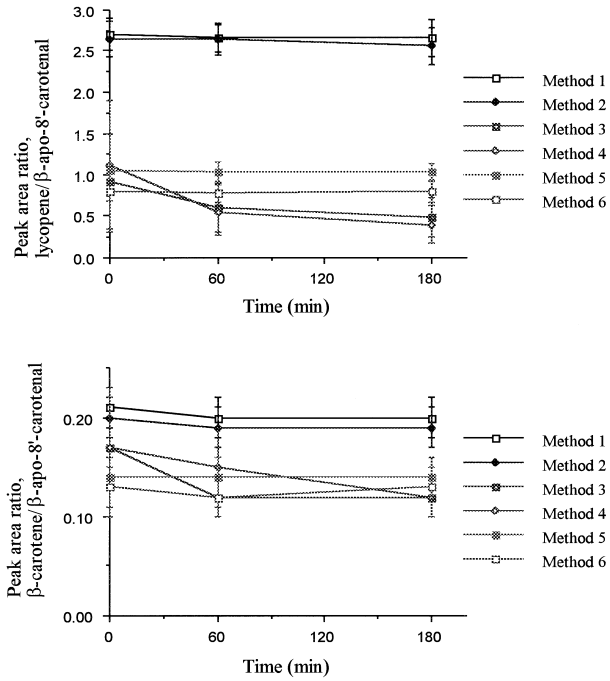


Fig. 2. Stability of lycopene and β-carotene content of the extracts with time. The values presented are the mean of peak area ratios of lycopene and β-carotene relative to internal standard (β-apo-8′-carotenal) of six replicate determinations with one injection using six extraction methods as in Figure 1. Time zero (0 min) represents basal time of HPLC analysis following sample preparation. Paired t-test: Lycopene—Methods 3 and 4 at 180 min are significantly different from those at 0 min ($P < 0.05$ and $P = 0.05$, respectively). β-Carotene—Methods 3 and 4 at 180 min are significantly different from those at 0 min ($P < 0.05$ for both).

Table 4. Weights of magnesium carbonate and volumes of extractant used in method 2

	Proportion, weight and volume			
Proportion relative to Association of Official Analytical Chemists, 1984, Section 43.015	1:1	1:2	1:3	1:4
Extraction step				
Sample (g)	2	2	2	2
Magnesium carbonate (g)	0.1	0.05	0.03	0.025
Ethanol (ml)	80	40	27	20
Hexane (ml)	60	30	20	15
Washing step				
Ethanol (ml)	2×25	2×12.5	2×8.3	2×6.25
Hexane (ml)	25	12.5	8.3	6.25
Water (ml)	5×100	5×50	5×33.3	5×25

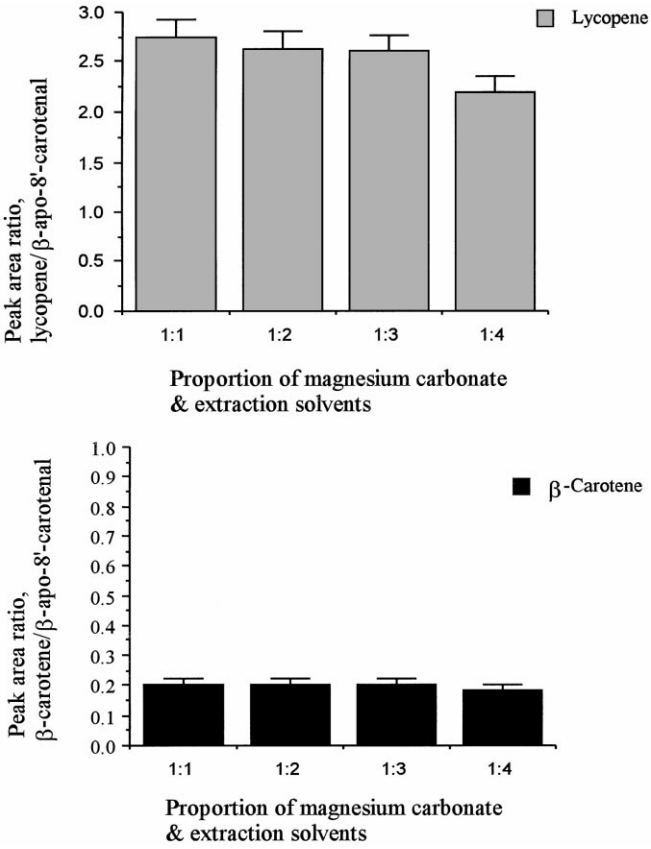


Fig. 3. The effect of varying the weights of magnesium carbonate and volumes of extraction solvents on the extraction yields of lycopene and β-carotene expressed as peak area ratios relative to the internal standard (β-apo-8′-carotenal). The values presented are the means of six replicate determinations with one injection using two $\times$ 2 g samples from three cans of tomato juice. One-way ANOVA and Duncan's test at 95% confidence limit: Lycopene—1:1, 1:2 and 1:3 proportions of weight to volume (Association of Official Analytical Chemists, 1984, Section 43.015) are not significantly different to each other but the 1:1 and 1:2 proportions are significantly different from 1:4. β-Carotene—no significant differences were found among the four proportions used.

Table 5. Per cent extraction of carotenes from canned tomato juice, carrot and spinach using 'single' and 'double' extraction

Samples	% Extraction of carotenes[a]			
	β-apo-8′ carotenal	lycopene	α-carotene	β-carotene
Canned tomato juice				
'single extraction'	100.0	100.0	—	100.0
	(0.0)[b]	(0.0)	—	(0.0)
'double extraction'	100.0	100.0	—	100.0
	(0.0)	(0.0)	—	(0.0)
Carrot				
'single extraction'	100.0	—	93.7	94.4
	(0.0)	—	(6.3)	(5.6)
'double extraction'	100.0	—	99.1	99.1
	(0.0)	—	(0.9)	(0.9)
Spinach				
'single extraction'	100.0	—	—	98.1
	(0.0)	—	—	(1.9)
'double extraction'	100.0	—	—	99.2
	(0.0)	—	—	(0.8)

[a]Per cent extraction of carotenes are presented as relative extracted peak areas of β-apo-8′-carotenal, lycopene, α-carotene and β-carotene to overall extracted peak areas of each compound from sample using 'single' and 'double' extraction plus that extracted from the corresponding residue using same extraction procedure of the modified AOAC method. The values presented are the mean of duplicate determinations with two injections using 2.0 g samples.
[b]Values in parentheses are per cent extraction of carotenes from the discarded residues.

β-carotene relative to β-apo-8′-carotenal were plotted at time intervals of 60 and 180 min following sample preparation as shown in Fig. 2. These results show that the method of AOAC is more efficient than the other tested methods. The AOAC method was thus selected for further evaluation. Reduction of the employed volume of solvents and weight of magnesium carbonate in the extraction process to 1/2, 1/3 and 1/4 of the recommended values (Table 4) was explored to economise the use of chemicals. Results in Fig. 3 show that the use of chemicals can be reduced to 1/2 of the recommended values without any significant difference in the extraction yield of lycopene and β-carotene in comparison with the official recommended values. Double extraction using 35 ml of ethanol and hexane mixture (4:3, by volume) gave better recovery of β-apo-8′-carotenal, lycopene, α-carotene and β-carotene from matrices of canned tomato juice, carrot and spinach than the single extraction procedure (Table 5). To facilitate separation of the hexane and aqueous phase during the washing stage, 10% sodium chloride solution was used rather than water for the first and second wash. Results of this set of studies provide a new established extraction procedure for plant matrix weights up to 2 g. The summary of the established extraction procedure is shown in the Scheme 1.

Table 6. Recovery of carotenes added to canned tomato juice[a]

Compound	Concentration			
	Initial (μg/10 ml)	Added (μg/10 ml)	Found (μg/10 ml) mean ± SD	Recovery (%)
Lycopene	89.7	68.0	151 ± 1.1	95.6
	89.7	136	216 ± 1.1	95.7
	89.7	232	308 ± 1.2	95.7
α-Carotene	0.0	12.4	12.7 ± 0.1	102
	0.0	24.8	25.2 ± 0.0	102
	0.0	49.6	50.4 ± 0.1	102
	0.0	99.2	101 ± 0.1	102
β-Carotene	5.5	3.9	8.7 ± 0.3	92.6
	5.5	7.9	12.5 ± 0.4	93.3
	5.5	15.7	19.9 ± 0.3	93.9
	5.5	25.5	30.9 ± 0.5	99.7
	5.5	50.9	56.2 ± 0.5	99.7
	5.5	101	106 ± 0.5	99.5
	5.5	201	205 ± 0.4	99.5

[a]The experiments were performed in duplicate. Two 2.0 g aliquots of canned tomato juice with and without addition of standards were extracted as described in Scheme 1.

Table 7. Within and between run variation[a]

Day	Lycopene	β-Carotene
1		
Mean[b]	65.1	3.4
CV, %	3.0	4.6
2		
Mean	68.0	3.7
CV, %	2.1	6.8
3		
Mean	68.2	3.8
CV, %	1.7	6.6
4		
Mean	73.1	3.9
CV, %	5.9	5.1
5		
Mean	71.3	3.9
CV, %	3.3	6.1
6		
Mean	65.4	3.4
CV, %	6.1	7.7
7		
Mean	69.2	3.5
CV, %	2.5	7.3
8		
Mean	66.9	3.5
CV, %	4.4	5.7
9		
Mean	68.8	3.5
CV, %	5.7	10.5
Overall (between run)		
Mean	68.6	3.6
CV, %	5.3	7.5

[a]Variability of a canned tomato juice (Lot II). The determination was done in duplicate on each of nine days. α-Carotene was not detected in this sample.
[b]Mean (and standard deviation), in μg/g.

Validation of the established method

The recoveries of lycopene, α-carotene and β-carotene added to canned tomato juice are shown in Table 6. They ranged from: 95.6 to 95.7% for lycopene (68.0–232 μg/10 ml added); 102% for α-carotene (12.4–99.2 μg/10 ml added); and 92.6 to 99.7% for β-carotene (3.9–201 μg/10 ml added). These data are similar to those of Hsieh and Karel (1983) who reported a recovery of added β-carotene from tomatoes at 91.0% and, from dried apricots, 99.0%.

Results of the precision study, shown in Table 7, both within and between runs, indicate that the assay procedure provided a successful determination of carotenoids within an acceptable precision range. The variation within a day gave coefficients of variation of 1.7–6.1% for lycopene and 4.6–10.5% for β-carotene. Overall precision or the precision between each of the 9 days of the experimental period showed a coefficient of variation of 5.3% for lycopene and 7.5% for β-carotene. Zakaria *et al.* (1979) reported values for mean ± SD (% CV) of lycopene and β-carotene in tomato juice of six replicate samples to be 9.78 ± 0.67 (6.99%) and 1.22 ± 0.03 (2.42%), respectively. Bushway and Wilson (1982) reported a similar coefficient of variation in various kinds of fruits and vegetables as follows: 1.10–14.16% for α-carotene and 1.26–8.60% for β-carotene.

Investigation of a possible application of the established method to various matrices

In this study, carrot and spinach were chosen as representatives of root, leafy, red and green vegetables with differences in both their carotenoid contents and in the type of matrix. Percentage recoveries of the analytes obtained were found to be unaffected by the composition of matrice over the explored range because the average % recoveries of added carotenoids from canned tomato juice, carrot and spinach were: 101, 99.8 and 101% for α-carotene (12.4, 24.8, 49.6 and 99.2 μg/10 ml of added α-carotene); and 98.1, 99.7 and 96.1% for β-carotene (25.5, 50.9, 101 and 201 μg/10 ml of added β-carotene). Results of this study thus show that it is possible to use the established method for the routine analysis of various fruits and vegetables.

CONCLUSION

Results of the studies showed that the established extractions with two solvents of low biological hazard, ethanol and hexane, can be sucessfully used for the analysis of carotenoids in fruits and vegetables with good recoveries and precision. The method is also applicable to various kinds of matrix, i.e. canned tomato juice, carrot and spinach.

ACKNOWLEDGEMENT

The authors are grateful to the International Development Program of Australian Universities and Colleges for financial support.

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