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Evaluation and validation of an LC method for the analysis of carotenoids in vegetables and fruit

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In many epidemiologic studies, an increased intake of fruits and vegetables was associated with a reduced risk of various cancers. Research on possible anticarcinogenic compounds is often focussed on carotenoids. We elaborated a method for the determination of lutein, zeaxanthin, α-carotene, β-carotene, lycopene and β-cryptoxanthin in fruit and vegetables. After extraction, carotenoids were dissolved and a fraction was injected onto the LC-system and chromatographed isocratically on a Vydac polymer C_{18} column. The mobile phase was a mixture of methanol and THF (95/5 v/v). Carotenoids were detected at 450 nm using a diode array detector. The separation between the individual carotenoids was satisfactory and the interference of other compounds small. Low recoveries (60%) for lycopene were found due to presence of hastalloy frits in LC system. Problems were solved by changing them for PAT (Peek Alloyed with Teflon) frits. Values for repeatability relative standard deviation (RSD_r) of carotenoids in carrot, spinach, tomato, corn and tangerine ranged from 1.9–4.9%. The mean standard recoveries for carotenoids in tomato and tangerine ranged from 93–107%. With this procedure, carotenoids may be determined in fruits and vegetables under routine conditions, yielding reliable and reproducible results. © 1997 Elsevier Science Ltd

INTRODUCTION

Consumption of more vegetables and fruits is associated with a reduced risk of cancer (Steinmetz *et al.* (I), 1991). In research the possible anticarcinogenic effects of fruits and vegetables are not only ascribed to carotenoids like β-carotene but also to others like lutein and lycopene. β-Carotene is retrieved in most orange vegetables and fruits and in dark green leafy vegetables. Lutein, a major oxygenated carotenoid is present in greens such as kale and spinach whereas lycopene is found in tomatoes. Lutein and lycopene are considered to be strong or stronger than β-carotene as risk-predictors in lung cancer (Steinmetz *et al.* (II), 1991), and detected at the highest levels in human plasma.

It is anticipated that the food industry may use this information in their advertising to promote their products. Therefore, we decided to develop and validate a LC method for the determination of lutein, zeaxanthin, α-carotene, β-carotene, lycopene and β-cryptoxanthin in fruits and vegetables.

The extraction procedure of the described method is based on modifications of methods published by Hart and Scott (1995) and Khachik *et al.*, (1992). The extracts were analyzed by LC as described by Epler *et al.*, (1992) and Craft *et al.*, (1992). The results obtained with this method are reported in this paper.

MATERIALS AND METHODS

Reagents

All reagents were of analytical purity: lutein, α-carotene, β-carotene and lycopene were purchased from Sigma (Sigma Chemical Co., St.Louis, MO, USA). Zeaxanthin, β-cryptoxanthin were bought from Roth (Roth, Karlsruhe, Germany).

Ethyl-β-apo-8'-carotenoate was acquired from Fluka (Fluka Chemie AG, Buchs, Switzerland). All other reagents like potassium hydroxide, ethanol absolute, methanol, tetrahydrofuran, hexane, acetone, petroleumether (40–65°C), ditert-butyl-methylphenol (BHT), sodium chloride and magnesium carbonate were purchased from Merck, (E. Merck, Darmstadt, Germany). All solvents in this study contained 0.1% (w/v) BHT.

All sample and standard preparations were performed under subdued light. Contact with air was avoided as much as possible and most experiments were carried out under nitrogen atmosphere.

Approximately 1 mg of each carotenoid was weighed into a 100 ml volumetric flask and dissolved in a specific solvent as mentioned in Table 1. These solutions were kept under nitrogen at −20°C for 4 months. one ml of each stock solution was diluted to 10.0 ml with each specific solvent as mentioned in Table 1. Absorbance difference (A–A_0) of each diluted stock solution was determined at suitable wavelengths (λ_{max}), using settings given in Table 1. A is the absorbance of the standard solution and A_0 is the absorbance of the blank. Concentration of each stock solution was calculated using specific absorption coefficients ($A^{1\%}_{1\,cm}$) (Bauernfeind, 1981) and (Hart & Scott, 1995) as expressed in Table 1.

Individual working solutions were prepared by evaporating 5 ml standard stock solution to dryness under nitrogen and dissolving the residue in 10.0 ml of methanol/THF (75:25 (v/v)). The purity of the individual carotenoid standard solutions were determined by injecting 25 μl standard working solution in the LC-system. Purity was calculated as a percentage by dividing the individual peak area for each carotenoid by the total peak area of the chromatogram. Concentrations measured from absorbance readings were corrected for purity. For quantification of carotenoids in samples, a mixed standard solution was prepared. Working solutions were prepared fresh on day of use.

Table 1. Specific absorption coefficients ($A_{1cm}^{1\%}$), solvents and maximum wavelengths (λ_{max}) for carotenoids.

Carotenoid	Solvent	λ_{max}, nm	$A^{1\%}_{1\,cm}$
Lutein	Ethanol	445	2550
Zeaxanthin	Acetone	452	2340
β-Cryptoxanthin	Petroleum ether	452	2386
Ethyl-β-apo-8'-carotenoate	Petroleum ether	445	2500
α-Carotene	Hexane	444	2800
β-Carotene	Hexane	450	2560
Lycopene	Hexane	472	3450

Apparatus

The LC-system was equipped with a high pressure pump, 25 μl injection loop, autosampler, column oven adjustable at 20°C, a programmable photo diode array detector with a data management system from Waters, (Waters, Milford, MA, USA).

The LC-column was of stainless steel, 250* 4.6 mm id, packed with Vydac 201 TP, 5 μm particle size (Vydac, Hesperia, CA, USA). Supplied 'biocompatible' Hastalloy frit material was replaced for PAT (Peek Alloyed with Teflon) column frits (Alltech Associates, Inc., Deerfield, IL, USA). The guard column was a synthetic column, 10*4.6 mm id, packed with Vydac 201 TP C18, 10 μm particle size, available from Alltech (Alltech Associates, Inc., Deerfield, IL, USA).

Samples

Carrot, spinach, tomatoes, corn (canned) and tangerines were bought at a local supermarket.

Tangerines were peeled, corn (canned) was drained, other samples were washed. All samples were mixed in a food processor (stems of spinach were included) and freeze-dried. Samples were stored for three–six months at −20°C until analysis.

Extraction and saponification

Approximately 0.50–1.00 g freeze dried material was weighed into a 200 ml centrifuge tube. 0.2 g $MgCO_3$ and a suitable amount of internal standard (ethyl-β-apo-8'-carotenoate), approximately equal to the mean level of carotenoids in the weighed sample portion, were added. Carotenoids in fruits and vegetables were extracted with portions of methanol/THF (1:1 v/v) of 0°C until colourless. The first portion size was 100 ml and subsequent portions were 50 ml. After 1 min of homogenizing using an ultra-turrax, samples were centrifuged for 5 min at 2000 *g* and at a temperature of

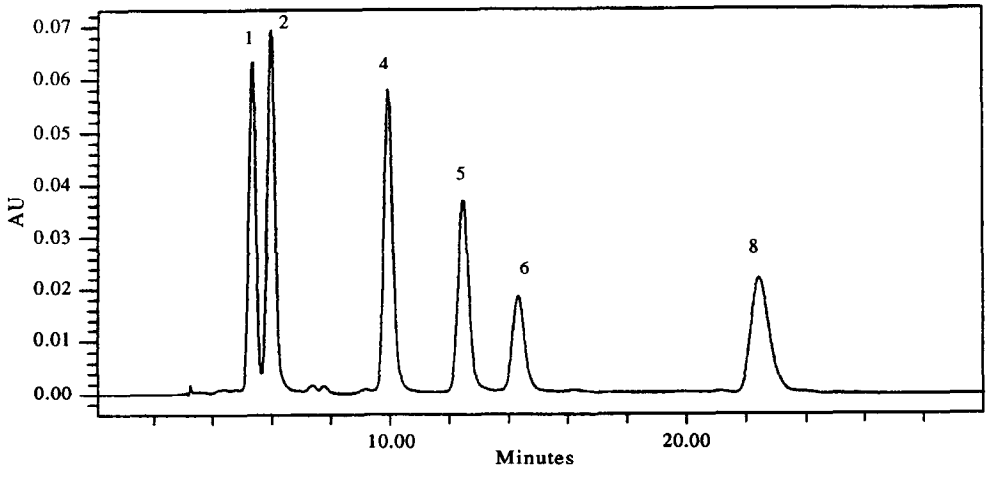

Fig. 1. LC-separation of a mixed standard solution containing approximately 4 μg of each carotenoid ml^{-1}. 1 = lutein, 2 = zeaxanthin, 4 = ethyl-β-apo-8'-carotenoate, 5 = trans-α-carotene, 6 = trans-β-carotene, 8 = trans-lycopene.

8°C. Supernatants were collected in 500 ml stoppered conical flask. [Note: in case β-cryptoxanthin had to be determined, the extract was saponified at room temperature for 2 h with an equal volume of KOH in methanol 10% (w/v).] After addition of 50 ml NaCl 10% (w/v), unsaponified as well as saponified solutions were extracted with 50 ml portions of petroleumether. Extractions were carried out until petroleumetherphase was colourless. From the saponified samples, combined petroleumether portions were washed with 100 ml portions of water until reaction of washes to phenolphthalein was neutral. Organic layers were evaporated to dryness. The residue was dissolved by ultrasonic agitation in methanol/THF (75:25 (v/v).

Determination

Aliquot portions (25 μl each) were injected onto the column of the LC-system and eluted with a mixture of methanol and tetrahydrofuran 95:5 (v/v) with a speed of 1.0 ml min^{-1}. Carotenoids were detected at 450 nm. Separated peaks were recorded and peak areas determined. Carotenoid concentrations in samples were quantified on basis of a mixed standard solution. Carotenoid concentrations were adjusted for recovery of the internal standard (ethyl-β-apo-8′-carotenoate). Carotenoids in the saponified mixture were calculated against a standard solution, which also was saponified.

RESULTS AND DISCUSSION

The described method is suitable for the determination of lutein, zeaxanthin, β-cryptoxanthin, α- and β-carotene and lycopene in fruits and vegetables. The separation between the individual carotenoids on the analytical system was satisfactory. The interference of other components was small. Figure 1 illustrates the separation between lutein, zeaxanthin, ethyl-β-apo-8′-carotenoate, α- ans β-carotene and lycopene.

Under the described LC-circumstances, cis-α-carotene, cis-β-carotene and cis-lycopene were separated from trans-isomers. As previously described by Craft *et al.* (1992), the main cis-isomers of α-carotene,

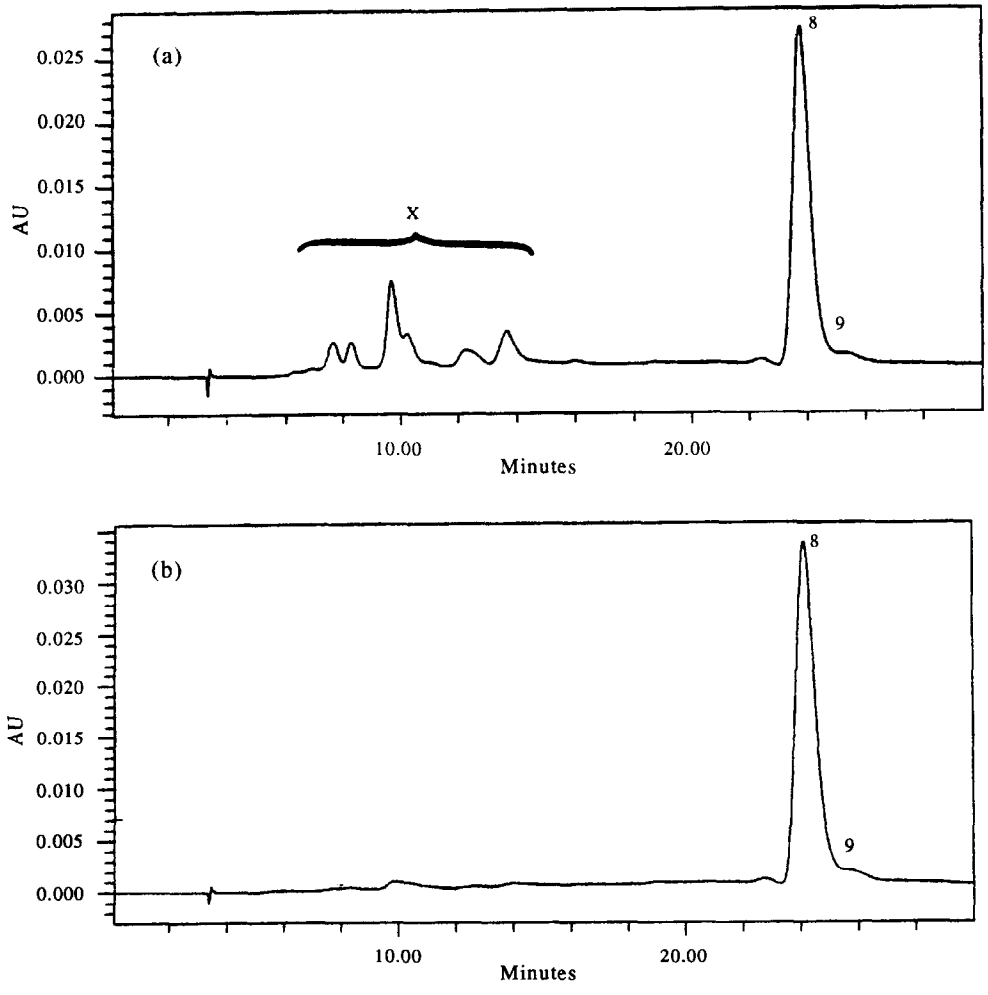

Fig. 2. Degradation of lycopene standard (appr. 10 μg ml^{-1}) due to hastalloy frit material (Chromatogram (a)). Same Lycopene standard on same LC column with PAT frit material (Chromatogram (b)). 8 = trans-lycopene, 9 = cis-lycopene, X = degradation products of lycopene.

β-carotene and lycopene elute immediately after the respective trans isomers. Most cis isomers were conveniently recognised using their second absorption maximum at 340 nm (O'Neil *et al.*, 1991) and/or their hypsochromic shifts of about 4 nm.

Methanol was chosen as mobile phase because of higher recoveries in case of methanol based solvents (Epler *et al.*, 1992). A polymeric C_{18} phase was selected to obtain a good separation between lutein and zeaxanthin.

Scott (1992), Epler *et al.* (1992) and Craft *et al.* (1992) described possible losses of carotenoids on stainless steel column frit materials. Epler and Craft propagated the use of 'biocompatible' hastalloy frit material. Since Vydac colums were equiped with this type of frit material, we tested our method with this material. Figure 2 shows a chromatogram of a standard lycopene. A considerable loss of approximately 40% of lycopene was observed when hastalloy frit material was used. As a result lycopene amounts in samples were quantified 0.6 of the real amount. Problems were solved by changing to PAT (Peek Alloyed with Teflon) frits yielding hardly any lycopene degradation products. Lycopene degradation products could be unnoticed among other carotenoid peaks in complex chromatograms of samples or mixed standard solutions. It was concluded from absorption maxima (410, 435 and 460 nm) in diode array spectra that these degradation products might be lycopene-epoxides. Khachik *et al.* (1992), ascribed these absorption maxima to the presence of di-epoxylycopene.

Stabilities of the stock solutions were checked on each day of use over four months by absorbance readings at the maximum absorption wavelengths. Concentrations were calculated by using specific absorption coefficients in Table 1. From this study, it was concluded that in case stock solutions were not stored at −20°C under nitrogen or they were older than 4 months, concentrations should be determined on the day of use.

Purity of stock standard solutions as determined with HPLC ranged from 94–100%. The response of the LC system was linear over the range 0–5 μg ml^{-1} for each carotenoid except for lycopene which was linear over the range of 0–3.5 μg ml^{-1}. The smaller linearity range of lycopene was explained by the lesser solubility of this compound in the injection solvent. The composition of the injection solvent was a compromise between good solubility of carotenoids, compatibility with mobile phase and no deformation of peaks. As described by Khachik *et al.* (1988), interaction between carotenoids, injection solvent and mobile phase may cause multiple LC-peaks, leading to misinterpretations. Raising the THF-concentration of the injection solvent from 25 to 30% (v/v) resulted in this phenomenon. With the injection solvent used, no multiple peaks were observed, whereas the solubility of lycopene was restricted at 3.5 μg ml^{-1}. The detection limit for the individual carotenoids was approximately 0.1 μg ml^{-1} for standard solutions, which corresponds with carotenoid concentrations of 1 μg $100g^{-1}$ in vegetables or fruits.

Table 2. Analytical data and statistics for carotenoids in carrot, spinach, tomato, corn and tangerine. N = 10 analysis for each carotenoid. Mean content is expressed as mg $100g^{-1}$ wet weight.

	Mean (mg $100g^{-1}$)	Literature (mg $100g^{-1}$)	s_r (mg $100g^{-1}$)	RSD_r (%)	r (mg $100g^{-1}$)	Recovery (%)
Carrot						
Lutein	0.298		0.011	3.5	0.031	
α-Carotene	4.87	0.5–8.5	0.098	2.0	0.27	
β-Carot. (cis + trans)	13.0	1.8–14.7	0.248	1.9	0.7	
Cis-β-carot.	0.49		0.053	10.9	0.15	
Spinach						
Lutein	5.0	4.4–15.9	0.21	4.2	0.6	
β-Carot. (cis + trans)	0.9	3.0–6.7	0.22	24.1	0.6	
Cis-β-carot.	0.38		0.09	23.5	0.25	
Corn						
Lutein	0.199	0.5–2.3	0.004	2.1	0.011	
Zeaxanthin	0.315		0.006	1.9	0.017	
β-Carot. (cis + trans)	0.050	0.01–0.07	0.002	4.9	0.007	
Cis-β-carot.	0.021		0.002	10.5	0.006	
Tomato						
Lutein	0.050	0.1	0.002	4.1	0.006	96.0
Zeaxanthin						97.3
α-Carotene						93.3
Trans-β-carot.	0.377	0.11–0.66	0.015	4.0	0.042	96.9
Lycopene (trans + cis)	2.73	0.88–4.20	0.067	2.5	0.19	106.8
Lycopene (cis)	0.111		0.018	16.0	0.050	
Tangerine						
β-Cryptoxanthin	0.92	0.106	0.03	3.7	0.10	97.8

s_r is standard deviation, RSD_r is repeatability relative standard deviation, r is repeatability.

There were negligible differences between lutein, zeaxanthin, α-carotene, β-carotene and lycopene determinations in spinach and a mixture of corn, tomato and carrot, with and without saponification of the extracts. Values were within margins of repeatability conditions. From the above, observations it was concluded that, in particular, luteine and zeaxanthin are not present in their esterified configuration. The results for β-cryptoxanthin in tangerines were different. A low concentration of β-cryptoxanthin (0.06 mg $100g^{-1}$) was determined when a tangerine extract was not saponified. Results for β-cryptoxanthin in tangerine were comparable when extracts were saponified for 2, 3, 3.5 and 4 h, respectively.

The method was validated for different matrices like carrot, spinach, tomato, corn (canned) and tangerine (Table 2).

Recoveries were determined by spiking samples with various concentrations of different standards before extraction and saponification. Saponification was not included in the recovery study of tomato. Recovery data for β-cryptoxanthin in tangerine were determined after saponification of samples.

Results were examined for outliers by the Grubbs test at the $P = 0.05$ level of significance. One outlier was found in the repeatability analysis ($n = 10$) of lycopene in tomato. There was one outlier for lutein and α-carotene in the recovery analysis ($n = 10$). Repeatability relative standard deviations (RSD_r) for carotenoids in carrot, spinach, tomato, corn and tangerine varied from 1.9-4.9% (concentration range 0.05–13.0 mg $100g^{-1}$). These values for the accompanying levels are acceptable according to the IUPAC (1989) Harmonized Protocol (Pocklington, 1990), according to which the acceptable within-laboratory method performance (RSD_r) may range from one-half to two-thirds of the predicted RSD_R for the levels of interest. RSD_R is reproducibility relative standard deviation. For cis-β-carotene in carrot, trans- and cis-β-carotene in spinach and cis lycopene in tomato the RSD_r ranged from 10.5–24.1%. These compounds were detected around the detection limit.

Mean standard recoveries for carotenoids in tomato and tangerine ranged from 93–107%. Recovery of the internal standard ethyl-β-apo-8′-carotenoate during the whole procedure without saponification was 96% ($n = 4$, $s_r = 0.5\%$).

The values for carotenoids in carrot, spinach, corn, tomato and tangerine were compared with values in literature (Mangels *et al.*, 1993); (Hart & Scott, 1995). In Table 2, a comparison with a range of values, given by Mangels, was made because of variability of food carotenoid levels affected by variety, season, etc., from a review of 180 articles, in general a high confidence code for these carotenoids in these samples were given. Carotenoid levels were determined after saponification of samples. Generally, results were in good agreement, except for β-carotene in spinach. A possible cause might be the inclusive of stems, with less β-carotene, in the homogenation of the sample. β-Cryptoxanthin in tangerine is much higher compared with levels in literature, possibly due to incorrect analysis previously.

The described procedure determines lutein, zeaxanthin, α-carotene, β-carotene and lycopene in vegetables and fruit with satisfactory, reliable and reproducible results.

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REFERENCES

Bauernfeind, J. C. (1981). *Carotenoids as colorants and Vitamin A precursors*. Academic press, New York, USA, pp. 883–923.

Craft, N. E., Wise, S. A. & Soares, J. H.Jr. (1992). Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. *J. Chromatogr.*, **589**, 171–176.

Epler, K. S., Zeigler, R. G., Craft, N. E., Sander, L. C. & Wise, S. A. (1992). Evaluation of reversed-phase lquid chromatographic colums for recovery and selectivity of selected carotenoid. *J. Chromatogr.*, **595**, 89–101.

Hart, D. J. & Scott, K. J. (1995). 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. *Food Chem.*, **54**, 101–111.

Khachik, F., Beecher, G. R., Vanderslice, J. T. & Furrow, G. (1988). Liquid chromatographic artifacts and peak distortion: sample–solvent interactions in the separation of carotenoids. *Anal. Chem.*, **60**, 807–811.

Khachik, F., Mudlagiri, B. G., Beecher, G. R., Holden, J., Lusby, W. R., Tenorio, M. D. & Barrera, M. R. (1992). Effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables. *J. Agric. Food Chem.*, **40**, 390–398.

Mangels, A. R., Holden, J. M., Beecher, G. R., Forman, M. R. & Lanza, E. (1993). Carotenoid content of fruits and vegetables: an evaluation of analytical data. *J. Am. Diet. Assoc.*, **93**, 284–296.

O'Neil, C. A., Schwartz, S. J. & Catignani, G. L. (1991). Comparison of liquid chromatographic methods for determination of cis-trans isomers of β-carotene. *J. Assoc. Off. Anal. Chem.*, **74**, 36–42.

Pocklington, W. D. (1990). Harmonized protocols for the adoption of standardized analytical methods and for the presentation of their performance characteristics. *Pure Appl. Chem.*, **62**, 149–162.

Scott, K. J. (1992). Observations on some of the problems associated with the analysis of carotenoids in foods by HPLC. *Food Chem.*, **47**, 403–404.

Steinmetz, K. A. & Potter, J. D. (1991*a*). Vegetables, fruit and cancer. I. Epidemiology. *Cancer, Causes and Control*, **2**, 325–357.

Steinmetz, K. A. & Potter, J. D. (1991*b*). Vegetables, fruit and cancer. II. Mechanisms. *Cancer, Causes and Control*, **2**, 427–442.