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Determination of β-Carotene Content and Vitamin A Activity of Vegetables by High-Performance Liquid Chromatography and Spectrophotometry

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ABSTRACT

A fast and sensitive high-performance liquid chromatographic (HPLC) method for the determination of the provitamin A, β-carotene, in vegetables is described. After alkaline saponification and organic extraction β-carotene is separated from other carotenoids on a reversed-phase HPLC column and determined by measurement of its absorbance at 445 nm. The total amount of carotenoids is determined by measurement of the absorbance at 445 nm of the extract prior to HPLC.

Results are presented of the analysis of β-carotene and total carotenoids in some vegetables bought at a local market in Dar es Salaam. The percentage β-carotene, of the total carotenoid content, showed a great variation. The vitamin A activity of the vegetables was calculated from the analytical results.

INTRODUCTION

Carotenoids, several of which have vitamin A activity, occur invariably in photosynthetic tissues and are responsible for the colour of many vegetables and fruits. Humans and animals cannot synthesize these

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compounds but are able to deposit dietary carotenoids as absorbed or with slight modification of their structure. The intestinal cleavage of these compounds to form vitamin A-active retinoids represents a major contribution to nutrition (Simpson, 1983).

In many developing countries vitamin A deficiency occurs among children during the weaning period, which constitutes a public health problem (Mrisho, 1981; Marks, 1975) since the resulting xerophthalmia may cause blindness. In those parts of the world carotenoids of vegetable products, especially of dark green leafy vegetables, are the main dietary source of vitamin A activity since the retinol-containing dairy and other animal products are not available or are too expensive (Simpson, 1983).

Most vegetables contain a number of different carotenoids, notably β-carotene, which has the highest vitamin A activity (Bauernfeind, 1972) and is the most widespread, although its concentration varies considerably. At present the number of known naturally occurring carotenoids exceeds 500 whereas the number of vitamin A active compounds is only a fraction of that. It is generally recognized that, in terms of biological activity, 1 μg of retinol is equivalent to 6 μg of β-carotene or 12 μg of mixed carotenoids (WHO, 1967; Food and Nutrition Board, 1980).

In order to establish the vitamin A activity of foodstuffs, a method based on separation with a gravity fed column was accepted by the Association of Official Analytical Chemists (AOAC, 1980). In this method, the carotenes as a group are separated from oxygenated carotenoids. However, this method does not separate β-carotene from the less active α- and γ-carotene as these compounds have a polarity similar to that of β-carotene. This possible overestimation of the vitamin A activity necessitates reappraisal of the figures for the vitamin A activities in food tables (WHO, 1982).

In this connection, we have developed a fast and reliable method for the determination of β-carotene in vegetables using high-performance liquid chromatography (HPLC). After alkaline saponification and organic extraction, β-carotene was separated on a reversed-phase HPLC column and detected by measurement of its absorbance at 445 nm. During sample treatment antioxidants are used to prevent oxidative degradation of carotenoids. The total amount of carotenoids was determined by measuring the absorbance of the extract at 445 nm prior to HPLC. In this way the total vitamin A activity of vegetables could be calculated more accurately.

MATERIALS AND METHODS

Apparatus

HPLC was performed using a system incorporating a Gilson Model 302 constant flow pump (Meyvis, Bergen op Zoom, The Netherlands), a Rheodyne injection valve type 7010 and a Perkin-Elmer variable wavelength detector type LC 75. A Knauer stainless steel column (250 × 4·6 mm inside diameter) was home-packed with ODS-Hypersil 3 μm (Shandon Southern Products, Astmoor, Great Britain, catalogue no. 580 × 18) by the balanced-density slurry technique using a Haskel pump type DSTV 150 (Doedijns, Rijswijk, The Netherlands). Elution profiles were displayed on a Kipp BD 8 recorder (Kipp Analytica, Delft, The Netherlands).

Spectrophotometry was performed using a Vitatron MPS spectrophotometer equipped with a tungsten light source and a 445 nm interference filter (Meyvis, The Netherlands).

Reagents

Sodium ascorbate and sodium sulfide were obtained from E. Merck AG, Darmstadt, FRG. A 12% (w/v) sodium sulfide solution was prepared by dissolving 120 g of sodium sulfide in 200 ml of distilled water, whereafter 700 ml of glycerol (s.g. 1·23) was added. Diisopropylether, also obtained from Merck, was stabilized by the addition of 10 mg of butylated hydroxytoluene per liter. The HPLC mobile phase consisted of a mixture of methanol:acetonitrile:chloroform:water = 200:250:90:11 (v/v) and was flushed with a stream of helium gas for 10 min before use. The standard β-carotene was obtained from Merck (catalogue No. 2236), α-carotene from Sigma (catalogue No. C 0251), lycopene from Sigma (catalogue No. L 9879) and canthaxanthin (*trans*) from Fluka (catalogue No. 21385). γ-Carotene and β-apo-8′-carotenal (*trans*) were gifts from Hoffmann-La Roche. A β-carotene stock standard solution was obtained by dissolving approximately 20 mg of β-carotene in 10 ml of methylene chloride in a 100 ml brown-glass flask followed by dilution with *n*-hexane to 100 ml. This solution was stored in the dark at −20 °C and was stable for at least one month. A working standard solution was prepared daily by dilution of an aliquot of the stock standard solution with mobile phase to a concentration of approximately 1 μg per milliliter.

Standardization

The concentration of β-carotene in the stock standard solution was determined using spectrophotometry. An aliquot of 1 ml of this solution was diluted with cyclohexane to 200 ml, whereafter the concentration of β-carotene was determined by measuring the absorbance of the diluted solution at 457 nm in a Varian Cary 219 spectrophotometer, using $E = 2505$ at 457 nm for a solution of 1 % (w/v) β-carotene in cyclohexane (Hejno, 1964).

Sample treatment

The vegetables investigated were bought at a local market in Dar es Salaam, Tanzania, on different occasions and stored in plastic bags sealed under nitrogen in the dark at −20 °C for a period not exceeding one month.

A portion of 5–20 g of vegetables was brought into a 250 ml brown-glass saponification flask. After adding 10 ml of a freshly prepared aqueous solution of 10 % (w/v) sodium ascorbate, 5 ml of sodium sulfide solution and 50 ml of a freshly prepared 2 mol/liter ethanolic KOH solution, the resulting mixture was refluxed on a boiling waterbath for 30 min. The flask was then cooled to room temperature and, after the addition of 100 ml of diisopropylether, the contents were mixed by shaking. After separation of the two layers, the upper layer was transferred to a 250 ml separating funnel containing 100 ml of a 5 % (w/v) KOH solution. After shaking, the lower aqueous layer was discarded and the layer of diisopropylether was washed further with consecutive 100 ml portions of water until no longer alkaline to phenolphthalein paper. Usually, three portions sufficed. The diisopropylether, containing the carotenoids, was dried with strips of blue-ribbon filter paper. Finally, an aliquot was evaporated in a vacuum rotary evaporator under nitrogen. The residue was dissolved in HPLC mobile phase to a concentration in the range 1–20 μg β-carotene per milliliter.

Spectrophotometry

The total concentration of carotenoids was determined by measuring the absorbance at 445 nm of the final extract in a Vitatron spectrophotometer with the working standard solution as the reference.

High-performance liquid chromatography

HPLC analysis of β-carotene was carried out by injecting 20 μl of the extract on to the HPLC column. The column was eluted isocratically with the mobile phase at a flow rate of 1·5 ml/min. The effluent was monitored with the detector set at a wavelength of 445 nm, being the absorption maximum of β-carotene in the mobile phase. The recorder was set at 10 mV full scale. Duration of the chromatographic run was about 15 min per sample. The concentration of β-carotene in the original sample was calculated from peak heights with the working standard solution as the reference. In routine analysis, this solution was run before each series of five samples.

Recovery experiments

Recovery experiments were carried out in duplicate by the addition of a certain volume of the stock standard solution of β-carotene to the saponification flask. The amount of β-carotene added was comparable to the amount expected to be present in the sample. After evaporation of the solvent in a gentle stream of nitrogen, the sample was added and saponified as described above.

Calculation of the vitamin A activity

The vitamin A activity of the vegetables examined was calculated as retinol equivalents according to the formula given by the WHO (1982), i.e.:

Retinol equivalents (μg/g) = 1/6 [β-carotene] (μg/g) + 1/12 [remaining carotenoids] (μg/g)

The amount of remaining carotenoids was calculated as the difference between total carotenoids and β-carotene.

RESULTS

Characteristics of the analytical method

Typical elution profiles of extracts of pumpkin and dried potato leaves and of the working standard solution, spiked with α- and γ-carotene,

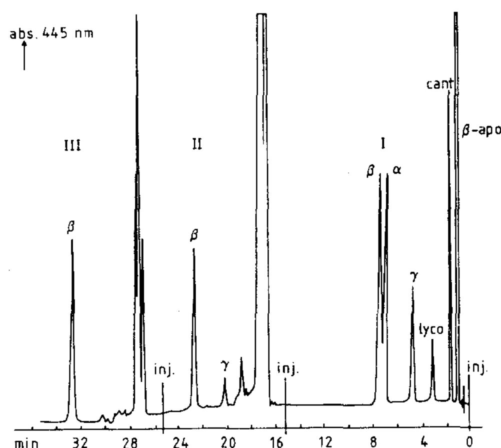

Fig. 1. Typical elution profiles of the working standard solution (I) spiked with α- and γ-carotene, canthaxanthin, β-apo-8′-carotenal and lycopene, and of extracts of pumpkin (II) and dried potato leaves (III).

canthaxanthin, β-apo-8′-carotenal and lycopene, are shown in Fig. 1. In blank experiments only a solvent was recorded. The HPLC procedure enables a good separation of α-, β- and γ-carotene from other carotenoids. The latter, having a higher polarity, show shorter retention times on the reversed phase column.

The partition of α-, β- and γ-carotene between the stationary and the

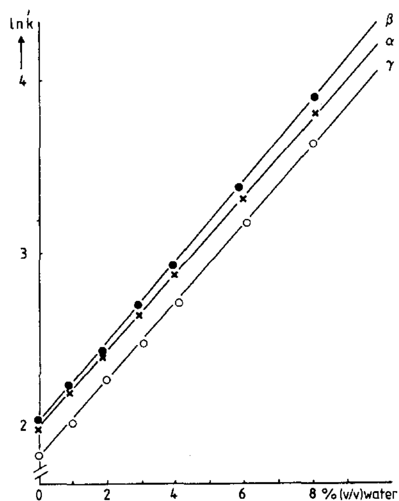

Fig. 2. The relationship of the capacity factors (k') of α-, β- and γ-carotene with the water content of the HPLC mobile phase.

mobile phase can be influenced by varying the water content of the mobile phase. The solubility of these compounds in the mobile phase decreases with increasing water content, which results in longer retention times.

Figure 2 shows the relationship between the capacity factors (k') of α-, β- and γ-carotene and the water content of the mobile phase. In the range of the water content applied (0, 1, 2, 3 and 4%, v/v), β-carotene was separated from γ-carotene while α- and β-carotene were sufficiently separated using water contents of the mobile phase from 2% (this method) up to 4%. This means that, if the β-carotene peak of a certain sample is interfered with by sample components, the chromatographic separation can be enhanced by changing the water content of the mobile phase.

TABLE 1
Total Carotenoids, β-Carotene and Vitamin A Activity of Some Vegetables

Sample	*Total carotenoids* (μg/g)	*β-carotene* (μg/g)	*β-carotene* (Percent of total)	*Vitamin A activity*[a] (μg/g)
Dried potato leaves	531	78	15	51
Dried potato leaves	481	71	15	46
Dried potato leaves	296	30	10	27
Dried potato leaves	170	22	13	16
Cassava leaves, fresh	150	31	21	15
Cassava leaves	282	31	11	26
Amaranthus, fresh	254	45	18	25
Amaranthus, fresh	239	64	27	25
Amaranthus	208	7	3	18
Salad	100	22	22	10
Salad	57	17	30	6
Pea leaves	78	19	24	8
Pea leaves	12	1·1	9	1·1
Pumpkin leaves	36	6	17	3·5
Green peas	20	2·1	11	1·8
Green peas	18	1·5	8	1·6
Pigeon peas	4·3	0·4	9	0·4
Brassica	1·8	0·5	28	0·2
Brassica	1·3	0·3	23	0·1
Bit root as spice	0·4	<0·2	10	<0·1

[a] The vitamin A activity is expressed in μg retinol per gram. 1 μg of retinol is equivalent to 12 μg (total carotenoids minus β-carotene) or 6 μg of β-carotene (WHO, 1982).

TABLE 2
Recovery of β-Carotene Added to Some Vegetables

Vegetable	*β-carotene*		*Total carotenoids*	
	Sample (μg/g)	*Recovery*[a] (%)	*Sample* (μg/g)	*Recovery*[a] (%)
Salad	21	97	89	95
Salad	17	96	82	95
Spinach	9	100	61	99
Spinach	7	98	51	98

[a] The added amounts of β-carotene per gram of sample were 12 μg for the β-carotene determination and 50 μg for the determination of total carotenoids.

As far as has been investigated, the detector response was linear from concentrations corresponding to 0·05 up to 2000 μg of β-carotene per gram of sample. Assuming that the signal to noise ratio should be at least three, the detection limit of the method described in this paper corresponds to a concentration of 0·2 μg per gram of sample.

Some vegetables, bought at a local market in Dar es Salaam, Tanzania, were analysed for β-carotene and total carotenoids, whereafter the vitamin A activity was calculated. The results are given in Table 1.

The percentage of β-carotene, as well as the vitamin A activity, varied widely, depending on the type of vegetable.

Recoveries

As β-carotene is heat-labile in the presence of oxygen (Simpson *et al*, 1976; Simpson & Chichester, 1981), we determined its stability during sample treatment by measuring the recovery of β-carotene added to salad and spinach. The samples were analysed in duplicate for β-carotene as well as for total carotenoids with and without the addition of β-carotene. The results, given in Table 2, show that, for β-carotene as well as for total carotenoids, reproducible results were obtained and that the recovery of β-carotene added to vegetables was good.

DISCUSSION

The HPLC method described in this paper provides a fast and reliable method for the determination of β-carotene in vegetables. Reproduci-

bility and recovery were good. The linearity range and the sensitivity permit a determination of β-carotene in vegetables in a concentration as low as 0·2 μg per gram of sample. The heat-labile carotenoids were protected from oxidative degradation during sample treatment by the use of the antioxidants sodium ascorbate in the reflux procedure and butylated hydroxytoluene in the extraction procedure. Metal ions which may catalyse oxidation of carotenoids during refluxing were inactivated by the addition of sodium sulfide. In case of interference of the β-carotene peak by sample components, the chromatographic separation can be enhanced by changing the water content of the mobile phase.

The method of analysis for β-carotene by HPLC and for total carotenoids by spectrophotometry in the same extract allows the calculation of more reliable data for the vitamin A activity of foodstuffs. In this way, overestimation is less likely to occur.

As processing reduces the vitamin A activity of foodstuffs (Anon., 1972; Sood & Bhat, 1974), a study on the effect of traditional sun-drying on the total carotenoids and β-carotene contents of some commonly eaten Tanzanian vegetables is in progress.

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