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ANALYTICAL AND NUTRITIONAL ASPECTS OF CAROTENOIDS IN VEGETABLES AND FRUITS: SOME PRELIMINARY RESULTS

Tee E Siong
Division of Human Nutrition,
Institute for Medical Resarch,
50588 Kuala Lumpur

Lim Chin Lam
Food Technology Programme,
School of Industrial Technology,
Universiti Sains Malaysia,
11800 Pulau Pinang

ABSTRACT

The β-carotene contents of vegetables and fruits were determined using the AOAC open-column (magnesia and Hyflo Super Cel mixture) chromatographic method and compared with a newly developed reversed-phase HPLC method, in which carotenoids were separated isocratically on an octadecylsilane (C_{18}) column using a ternary mixture of acetonitrile, methanol and ethyl acetate. Preliminary results obtained for 20 vegetables and 10 fruits showed that the AOAC method gave falsely-elevated results for samples containing α-carotene, as well as those with very low β-carotene concentrations. On the other hand, the HPLC method successfully separated and quantitated the major carotenoids present, namely lutein, cryptoxanthin, lycopene, γ– and α-carotenes in addition to β-carotene. The carotenoid composition of most of the green vegetables was rather consistent, comprising only of lutein and β-carotene. In contrast, there was no clear pattern of carotenoids present in the other vegetables and fruits, where several other carotenoids were detected in varying proportions. Vitamin A activity, expressed as μg retinol equivalent (RE), and calculated on the basis of all provitamin A carotenoids (cryptoxanthin, γ–, α– and β-carotenes) detected, showed that most of the green leafy vegetables, including several *sayur kampung,* had high RE. Several green non-leafy and other vegetables were found to have low and medium RE. None of the fruits studied may be said to have high vitamin A activity. RE calculated on the basis of results from the AOAC method was found to be erroneously low for samples with significant proportions of provitamin A carotenoids other than β-carotene, and falsely elevated for those with α-carotene. Total carotenoid concentrations can be estimated by taking absorbance reading of sample extracts directly in a spectrophotometer or by the HPLC method. The study clearly shows that the HPLC method would give a more complete picture of the carotenoid composition as well as a more accurate quantitation of the provitamin A activity of the vegetables and fruits. The nutritional significance of the findings are obvious since these foods are important sources of vitamin A for the majority of the communities in the country.

We, nutrition scientists must realise the limitation in our nutrition training, and that there are barriers between scientists and the people. Breaking down these barriers requires inputs from other disciplines for a better understanding of people's perception about foods, effects of inappropriate food practices on health and other related issues. Next, attempts to change all identifiable improper practices 'all in one go' are unrealistic. Specific behavioural objectives which have the potential for change should be carefully identified and worked through well-designed communication strategies and plans. Well-managed programmes are another requirement for successful modification of undesirable behaviours.

Undesirable food habits and sedentary lifestyles among affluent populations during the past decade, I believe, have led to some chronic degenerative diseases such as atherosclerosis, hypertension, coronary heart diseases and some types of cancer. The Thai national health statistics have shown increasing morbidity and mortality rates for these degenerative diseases. I believe this trend exists in Malaysia and other ASEAN countries as well.

Therefore, exchange of information on practical approaches for behaviour modification will be beneficial towards solving problems of over-nutrition. We should be alert to these emerging problems and try to find a strategy to prevent or minimise these problems. Experiences from one country should be shared with others particularly in the ASEAN region. There should be no barriers among nutritionists, health personnel and other scientists from different countries since we all share a common goal viz. ensuring good health and good nutrition for the people. Experiences from the Food Habits Project among various centres in Thailand as well as among countries in the ASEAN region showed the benefits of collaboration in solving our common problems. I would strongly advocate that such collaboration be further strengthened and this can be facilitated if each party shares their expertise and genuinely work together with others.

Nutrition research, in countries like ours, is an urgent need and not a luxurious activity. Effective use of limited resources can be devised, provided that the problems to be researched are correctly identified. It is our responsibility to initiate research to find solutions for the problems in our countries before the problems become more widespread.

There is also a great need to create an atmosphere which favours inter-disciplines in the application of nutrition knowledge in combating malnutrition. An interdisciplinary approach is feasible provided that related scientists recognise the needs and try to find effective ways to integrate them. Our experience in community level interventions and behavioural modification has demonstrated this point. What lies ahead is for us to work out more of these linkages or further strengthen the existing ones. I would like to call for a harmonising of our efforts for the mutual benefit of people in our countries and perhaps the whole ASEAN region.

INTRODUCTION

Vitamin A deficiency remains one of the major public health nutritional problems in many developing countries, and is an important cause of preventable blindness. The group of compounds pertinent in this deficiency problem that afflicts particularly young children, is carotenoids since the major source of vitamin A in the diet of most communities in the region is carotenoids. Although an extensive literature on these compounds has been built up, carotenoids are still being actively studied all over the world, since many gaps in knowledge exist and new frontiers are being pursued[1].

A basic tool in carotenoid research and development activities is knowledge of the content and composition of this group of pigments, which are widely distributed in nature and found without exception in photosynthetic tissues. In recent years, there has been particular emphasis on understanding the types and concentrations of various carotenoids in foods. This is of importance firstly in relation to the provitamin A activity of the carotenoids. It is thought that previously reported values of vitamin A activity in food composition tables may have been on the high side since methodologies used were not sufficiently discriminative and thus had included carotenoids that do not possess vitamin A activity[2-5]. Some of these carotenoids may occur in higher concentrations than β-carotene, the most potent precursor of vitamin A. Secondly, carotenoids, including those without vitamin A activity, are now thought to play important roles beyond their classical functions in nutrition and vision. With their highly conjugated double bonds, carotenoids may act as free radical traps or antioxidants, and therefore play an important protective role in cancer development[6-9].

The author has embarked on a systematic study to deyelop improved methodologies for the separation and quantitation of retinol and several carotenoids in foods and biological specimens, especially serum. A systematic review of the literature on methods for carotenoid analysis was first carried out[10]. Initially, several studies into the physicochemical characteristics of retinol and several carotenoids in various solvents and laboratory conditions were carried out to assist in the choice of analytical conditions. A combination of uv-vis spectroscopy and HPLC was used in these studies. A HPLC system for the analysis of these compounds was next developed, studying particularly the solvent system, peak detection and quantitation, and sample preparation. The objective was to develop a simple system, workable for routine determination of retinol and several carotenoids in a wide variety of foods of both plant and animal origin.

The HPLC method developed was applied to a variety of foods. Each food sample was also simultaneously determined for vitamin A and carotenoids by the open-column chromatographic procedure of the AOAC[11], with the aim of determining if the difference in β-carotene values thus far reported using the AOAC method were significantly different from the more specific HPLC method. If differences eixst, it is hoped that the study will indicate which type of vegetables show the greatest differences. Such differences have been mentioned by various investigators, but there has not been a detailed comparison and for a wide variety of foods as the present study.

This preliminary report presents results of some of the fruits and vegetables studied. Reports on other food samples, including retinol and carotene content of foods of animal origin, will be presented in other communications.

MATERIALS AND METHODS

Solvents and Carotenoid Standards

Solvents used for sample preparation and pretreatment and for open-column chromatography procedures, were all analytical-grade reagents. Solvents for high-pressure liquid chromatography were of HPLC grade. All solvents for use as the mobile phase in HPLC were filtered through a 0.45 μm regenerated cellulose membrane filter and degassed using an ultra-sonic bath.

α– and β-carotenes and lycopene standards were purchased from Sigma Chemical Company. γ-Carotene, β-apo-carotenal, cryptoxanthin, zeaxanthin, and lutein were gifts from F. Hoffmann La-Roche, Switzerland. Structures of all carotenoid standards used in this study are given in Figure 1 and their uv-vis absorption spectra in Figure 2. The eight carotenoids used have varying structures, including the acyclic conjugated polyenelycopene; carotenoids with *psi, beta* and *epsilon* end-groups; oxygenated carotenoids; and an apocarotenoid. Stock solutions of these carotenoids were prepared in hexane (except that lutein and zeaxanthin were prepared in ethanol and β-apo-carotenal in petroleum ether) in concentrations of 100 μg per ml and stored in amber bottles below –20°C. Working solutions of 1 μg per ml of the standards were prepared daily. The appropriate extinction coefficients published in the literature[12] were used to calculate the exact concentration of each of the carotenoids. The preparation of all standard carotenoids were carried out with no unnecessary delay, in a room with subdued light and all windows tinted with a light-protective film. All sample treatment and analytical procedures were also carried out in this laboratory.

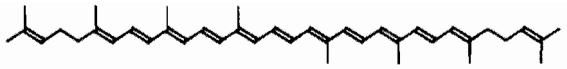
Sample Preparation and Pretreatment

Commonly consumed vegetables and fruits were purchased from markets and stalls. Edible portions of the foods were size-reduced in a blender and 2-10 g immediately weighed for analysis.

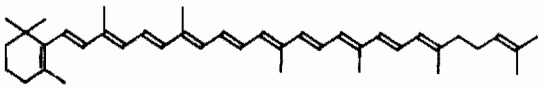
Sample pretreatment procedures were essentially those of the AOAC[11], except for the introduction of a saponification step. Preliminary studies carried out by the authors have shown that saponification was able to remove other pigments (mainly chlorophyll) from the food samples, which would otherwise interfere in the chromatography process, especially in the HPLC method. At the same time, carotene content was not affected.

To duplicate portions of the test sample were added a volume of 100% (w/v) potassium hydroxide equal to the weight of the food sample used, and 40 ml of ethanol. The mixture was saponified on an electric heating mantle for 30 minutes. The saponified mixture was cooled and extracted with 25–ml portions of hexane until the extract was colourless. The hexane extracts were pooled, washed till free of alkali, dried over sodium sulphate, and reduced to a small volume by heating over a water-bath with the aid of a stream of oxygen-free nitrogen. The resulting solution was made up immediately to a suitable volume (e.g. 25 ml) with hexane, referred to hereafter as the "test solution"

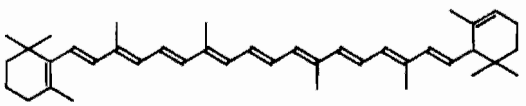
The total carotenoid concentration of the test solution was determined as described below. The solution was next subjected to two chromatographic and subsequent quantitation procedures: (1) by open-column chromatography using magnesia and Hyflo Super



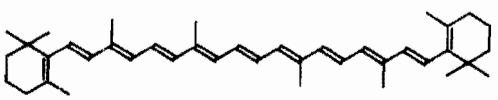
I· Lycopene (ψ,ψ –Carotene)



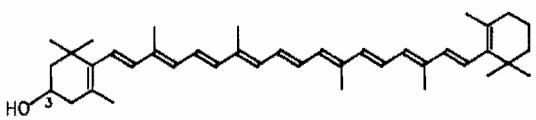
II· γ –Carotene (β,ψ –Carotene)



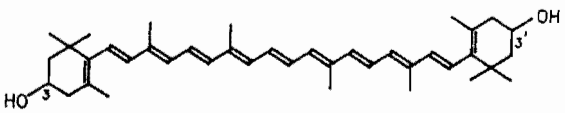
III· α –Carotene (β, ε –Carotene)



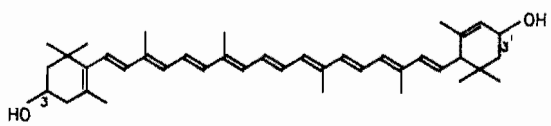
IV· β –Carotene (β,β –Carotene)



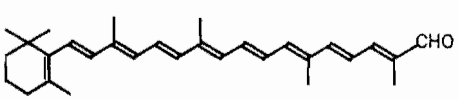
V· β–Cryptoxanthin, Cryptoxanthin, 3–Hydroxy– β –Carotene
(β,β –Caroten–3–ol)



VI· Zeaxanthin (β,β –Carotene–3,3'–diol)



VII· Lutein, "Xanthophyll", 3,3'–Dihydroxy– α –carotene
(β,ε –Carotene–3,3'–diol)



VIII· β –apo–8'–Carotenal (8'–apo– β –Caroten–8'–al)

Figure 1. Structures of Carotenoid Standards
(Systematic names of carotenoids given in parentheses)

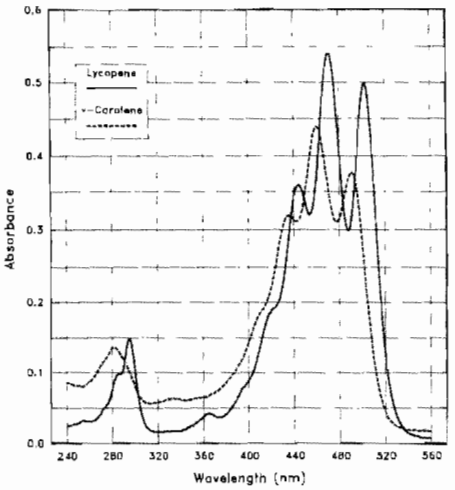


Figure 2a. UV-Vis Absorption Spectra of Lycopene and γ-Carotene in Hexane (2 µg/ml)

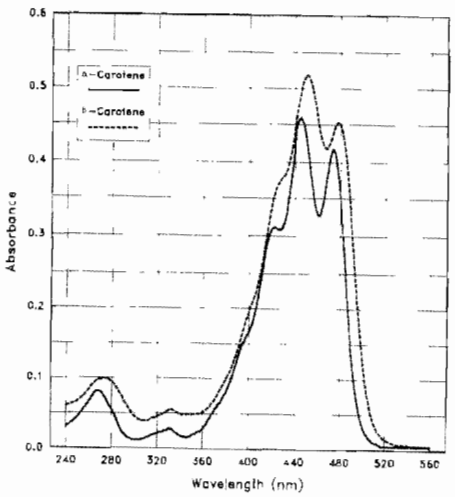


Figure 2b. UV-Vis Absorption Spectra of α- and β-Carotene in Hexane (2 µg/ml)

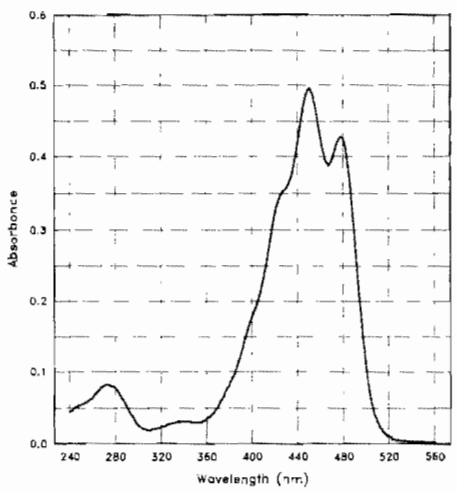


Figure 2c. UV-Vis Absorption Spectrum of Cryptoxanthin in Hexane (2 µg/ml)

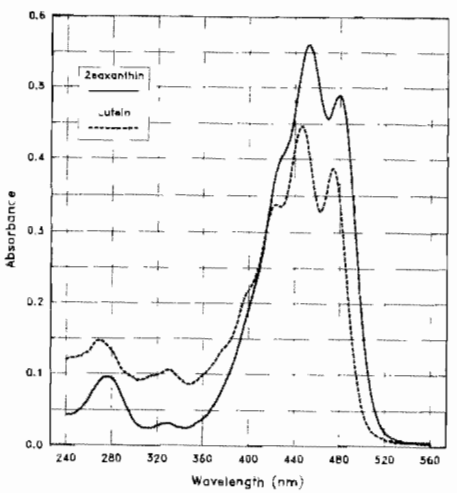


Figure 2d. UV-Vis Absorption Spectra of Zeaxanthin and Lutein in Ethanol (4 µg/ml)

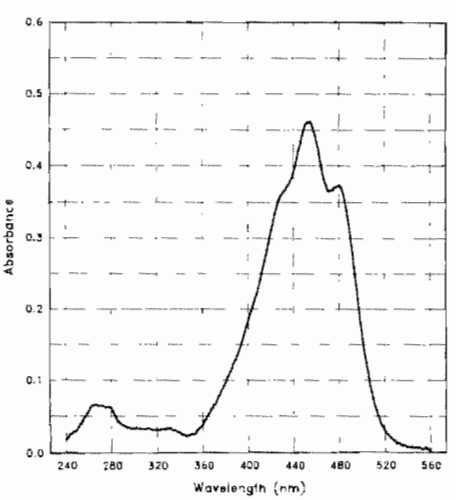


Figure 2e. UV-Vis Absorption Spectrum of β-apo-Carotenal in Petroleum Ether (2 µg/ml)

Cel mixture and quantitation using absorbance reading at 450 nm (AOAC method); and (2) high-pressure liquid chromatography and detection and quantitation at 436 nm (HPLC method). The analytical procedures are summarised in Figure 3.

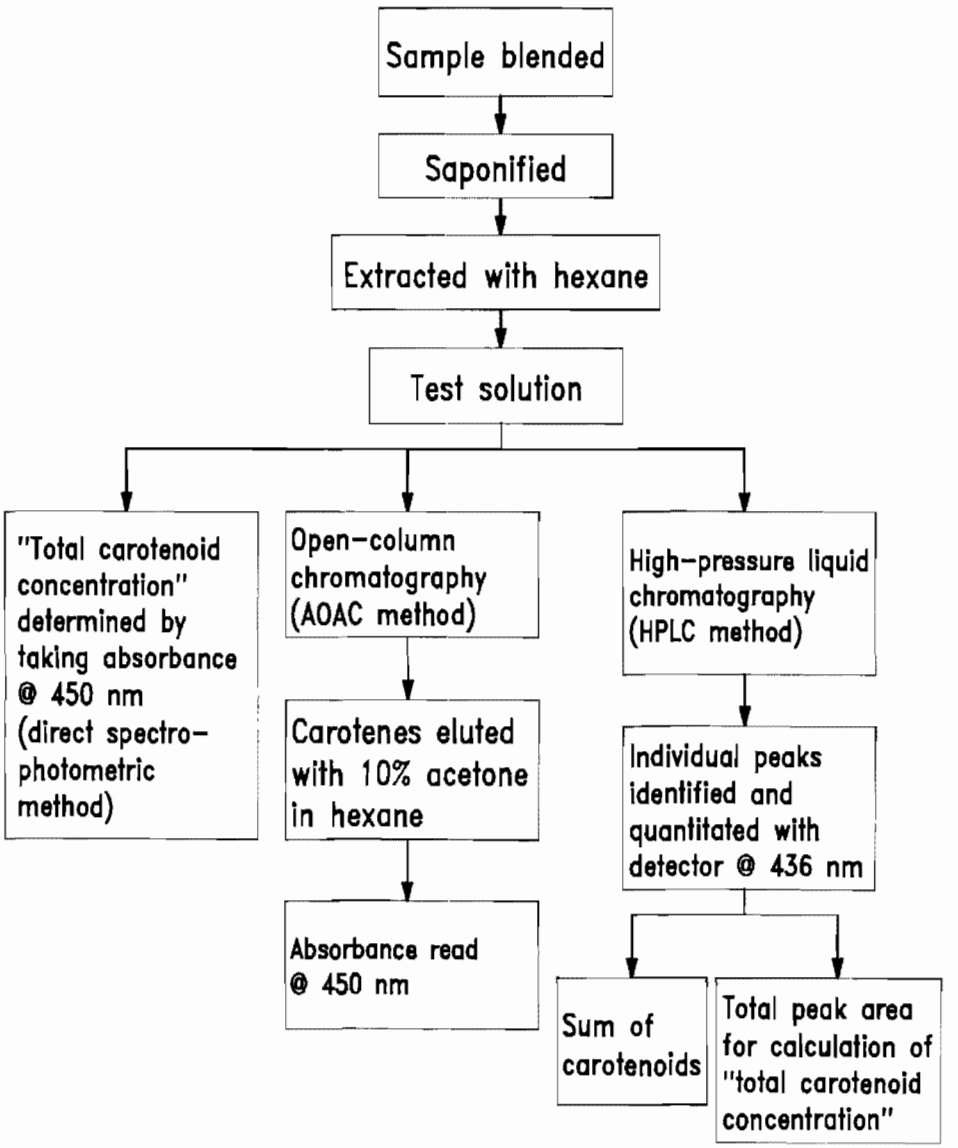


Figure 3. Summary of Analytical Procedures

Determination of Total Carotenoid Concentration

The test solution was read directly in a spectrophotometer at 450 nm and a β-carotene standard curve was used to calculate the total carotenoid content. The results obtained are referred to as having been obtained by the "direct spectrophotometric method".

The total carotenoid concentration was also determined by the high-pressure liquid chromatographic (HPLC) method described below.

Open-column Chromatography (AOAC method)

A suitable volume (e.g. 10 ml) of the test solution was pipetted into a glass column prepacked with a mixture of activated magnesia (Sea Sorb 43) (Fisher Scientific Co. or Sigma Chemical Co.) and diatomaceous earth (Hyflo Super Cel) (Fisher Scientific Co.), in the ratio of 1:1, for chromatography using the AOAC method[11]. β-Carotene was eluted from the column with approximately 80 ml of 10% (v/v) acetone in hexane. The eluate was evaporated on a water-bath with the aid of a stream of nitrogen and made up to a suitable volume (e.g. 10 ml) with hexane. The absorbance of the solution was read in a spectrophotometer at 450 nm and concentration of β-carotene calculated using a calibration curve prepared with the β-carotene standard.

High-pressure Liquid Chromatography (HPLC method)

HPLC conditions

A Waters high-pressure liquid chromatograph equipped with a 440 fixed-wavelength detector was used. A 436-nm wavelength kit was fitted onto the detector and an attenuation of 0.05 absorbance units full scale (AUFS) was set. Another detector with a 313-nm wavelength kit and connected in series to the first, was found to be useful in assisting monitoring of carotenoids, particularly the *cis-isomers of c*arotenes. A Waters 6000A solvent delivery system was used to deliver the mobile phase (acetonitrile-methanol-ethyl acetate; 88:10:2,v/v) at a rate of 2.0 ml/min. A stainless steel 30 cm X 3.9 mm I.D. 10 μm μBondapak C_{18} column was used for the chromatographic separation. This was preceded by a Waters Guard-PAK precolumn module housing a disposable Guard-PAK precolumn insert packed with the same material as that in the analytical column. Sample injection volumes, dispensed using a Rheodyne 7125 injector, were usually 50 to 100 μl. Peak areas were quantitated with a Waters 730 Data Module.

Chromatography of carotenoids

Hexane in the test solution was first evaporated off on a water-bath with the aid of nitrogen gas. The residue was immediately redissolved in a suitable volume of the mobile phase. After passing through a 0.45 μm regenerated cellulose membrane filter, suitable volumes were injected into the chromatograph. Identification and quantitation of the carotenoids were carried out by comparing with reference carotenoids similarly chromatographed. Some food samples were found to contain a few carotenoids which could not be identified. The concentrations of these carotenoids were estimated as β-carotene. The concentration of individual carotenoids were summed to give "sum of carotenoids". Total peak area from the chromatograms were used to determine total carotenoid content, calculated suing a β-carotene standard curve.

To assist in the identification of carotenoids in the samples, the pigments were eluted from the magnesia column (by the AOAC method) using a stepwise increase in the proportion of acetone in hexane. The absorption spectrum of each eluate was obtained and an aliquot injected into the HPLC to determine its purity and retention time. These data were compared with those given by authentic carotenoid standards.

RESULTS AND DISCUSSION

This preliminary report presents results for 20 vegetables and 10 fruits, listed in Table 1. The English names of the foods are first listed, followed by the names in Bahasa Malaysia (or other local names) and scientific names. Where the English names are not available, the local names of the foods are given.

Table 1: Names of Vegetables and Fruits Studied

	English name	Local name	Scientific name
	Green, leafy vegetables		
1.	*Cekur manis*	*Cekur manis*	*Sauropus androgynus*
2.	*Cemperai*	*Cemperai*	Champereia griffithii
3.	Chinese cabbage	*Pak-coy*	*Brassica chinensis*
4.	Drumstick leaves	*Daun kelor*	*Moringa oleifera*
5.	Fern shoots	*Pucuk paku*	*Diplazium esculentum*
6.	Papaya shoots	*Daun betik*	*Carica papaya*
7.	*Pegaga gajah*	*Pegaga gajah*	*Hydrocotyl javanica*
8.	*Ranti*	*Ranti*	*Solanum nigrum*
9.	Spinach	*Bayam putih*	*Amaranthus viridis*
10.	Swam cabbage	*Kangkung*	*Ipomoea aquatica*
11.	*Tanki*	*Tanki*	*Neptunia oleracea*
12.	Tapioca shoots	*Pucuk ubi kayu*	*Manihot utilissima*
	Green, non-leafy vegetables		
13.	French bean	*Kacang buncis*	*Phaseolus vulgaris*
14.	Long bean, dark green	*Kacang panjang*	*Vigna sinensis*
15.	Paprika/Bell pepper	*Lada hijau besar*	*Capsicum annuum*
16.	Snake gourd	*Ketola ular*	*Tricosanthes anguina*
	Other vegetables		
17.	Carrot	*Lobak merah*	*Caucus carota*
18.	Chilli, red	*Lada merah*	*Capsicum annuum*
19.	Pumpkin	*Labu merah*	*Cucurbita maxima*
20.	Tomato	*Tomato*	*Lycopersicum esculentum*
	Fruits		
1.	Banana	*Pisang emas*	*Musa sapientum*
2.	Banana	*Pisang tanduk*	*Musa sapientum*
3.	*Buah kundang*	*Buah kundang*	*Bouea macrophylla*
4.	Mango (Black-gold)	*Mangga*	*Mangifera indica*
5.	Musk lime	*Limau kesturi*	*Citrus microcarpa*
6.	Orange	*Limau manis*	*Citrus nobilis*
7.	Papaya	*Betik*	*Carica papaya*
8.	Papaya exotica	*Betik eksotika*	*Carica papaya*
9.	Tree tomato	*Tomato pokok*	*Cyphomandra betacea*
10.	Watermelon, red	*Tembikai*	*Citrullus vulgaris*

Total Carotenoid Content

The total carotenoid concentration of each vegetable or fruit was determined by two methods: (1) taking the absorbance reading of the hexane extract at 450 nm previous to any chromatographic separation ("direct spectrophotometric method"); and (2) calculating the concentration from total peak area of HPLC chromatograms (HPLC method). There are two major limitations of total carotenoid concentrations determined by either method. Detection at a single wavelength (450 nm for the "direct spectrophotometric method" and 436 nm for the HPLC method) is not adequate since absorption maxima for the various carotenoids differ considerably. The calculation based on β-carotene as the reference standard is also an approximation since the different carotenoids have different extinction coefficients.

Table 2 shows total carotenoid concentrations in the vegetables and fruits obtained by the "direct spectrophotometric" and HPLC methods. Figure 4 shows the ratios of results obtained by the former method to those by the HPLC method. A ratio of unity indicates similar results were given by the two methods. For most of the vegetables and fruits (27 out of 30 samples studied), the ratios obtained were between 0.6 and 1.0, i.e. varying within b20% from 0.8. The results indicate that the HPLC method tended to give slightly higher total carotenoid concentrations. In spite of the limitations mentioned above, the data obtained could provide useful estimations on total carotenoid concentrations. In spite of the limitations mentioned above, the data obtained could provide useful estimations on total carotenoid concentrations.

Table 2: Total Carotenoid Content[1] of Vegetables and Fruits Determined by Direct Spectrophotometric and HPLC Methods

	Name of Vegetable/Fruit	Direct Spectrophotometric method	HPLC method
	Green, leafy vegetables		
1.	*Cekur manis*	31822	39910
2.	*Cemperai*	11833	17310
3.	Chinese cabbage	2703	3728
4.	Drumstick leaves	12986	14565
5.	Fern shoots	1944	2278
6.	Papaya shoots	2120	2517
7.	*Pegaga gajah*	4281	4832
8.	*Ranti*	8330	9462
9.	Spinach	5481	7058
10.	Swamp cabbage	2083	2078
11.	*Tanki*	14788	16708
12.	Tapioca shoots	5738	6975
	Green, non-leafy vegetables		
13.	French bean	525	784
14.	Long bean (dark green)	776	1070
15.	Paprika/Bell pepper	300	453
16.	Snake gourd	302	338

	Other vegetables		
17.	Carrot	9468	9997
18.	Chilli, red	5678	6077
19.	Pumpkin	1830	2212
20.	Tomato	1276	1224
	Fruits		
1.	*Pisang emas*	99	119
2.	*Pisang tanduk*	239	332
3.	*Buah kundang*	1029	1353
4.	Mango (Black-gold)	540	615
5.	Musk lime	406	419
6.	Orange	334	515
7.	Papaya	2489	3664
8.	Papaya exotica	3107	4057
9.	Tree tomato	1604	1583
10.	Watermelon (red)	4748	4365

[1] Mean of duplicate analyses; expressed as μg per 100 g of edible portion of sample

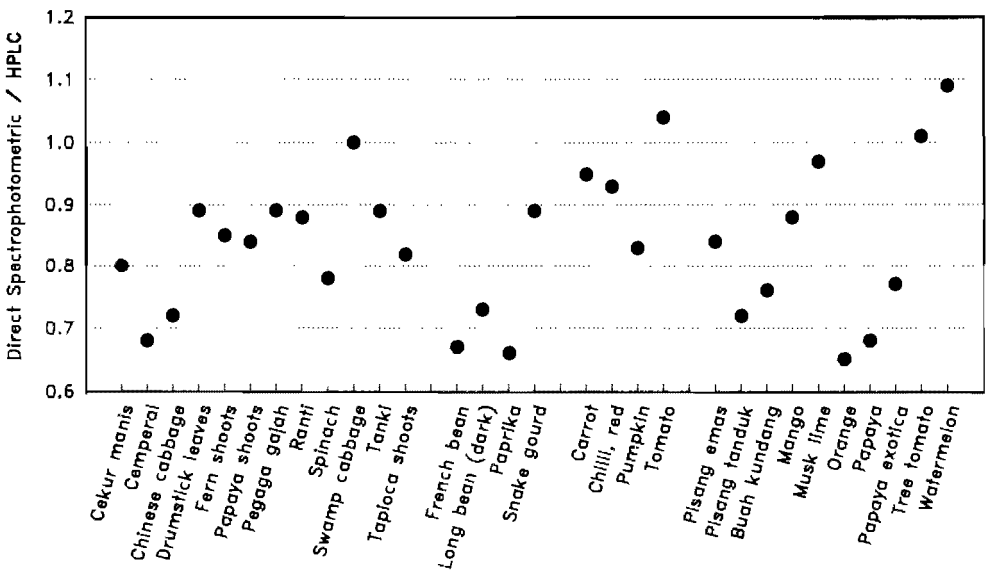


Fig. 4. Ratio of Total Carotenoid Concentrations of Vegetables and Fruits Determined by the Direct Spectrophotometric and HPLC Methods

Carotenoid Composition

Only the HPLC method was able to give the carotenoid composition of the vegetables and fruits studied. The HPLC conditions employed gave satisfactory separation for lutein (retention time, RT = 3.6 min), cryptoxanthin (RT = 6.0 min), lycopene (RT = 7.5 min), γ-carotene (RT = 9.0 min), α-carotene (RT = 10.2 min), and β-carotene (RT = 10.8 min). Zeaxanthin, structurally very similar to lutein (Figure 1) was minimally separated from the latter. β-Apo-8'-carotenal, with the carbon skeleton shortened to 30, was also

minimally separated from lutein. However, all three carotenoids had slightly different absorption spectra (Figure 2). When vegetable extracts were fractionated on the magnesia column using step-wise increase of acetone in hexane as the eluant, the fraction eluted from the column with a RT of 3.3 minutes in the HPLC chromatogram was found to have an absorption spectrum similar to that of lutein α- and β-carotenes, differing only in the position of the double-bond in one of the two end groups (Figure 1), were not completely separated. However, there was no difficulty in accurate identification and quantitation of these two pigments.

A chromatogram of a mixture of these carotenoids is given in Figure 5. It can be seen that elution order of the carotenoids on the reversed-phase C_{18} column was as expected, i.e. the more polar compounds were eluted earlier. As can be seen from the chromatogram, the oxygenated carotenoids or xanthophylis were eluted early. Lutein and zeaxanthin, the dihydroxy pigments were eluted first, followed by the hydroxy carotenoid cryptoxanthin, and then the straight-chain carotenoid lycopene. The non-polar carotenoid hydrocarbons, γ—, α— and β-carotenes were eluted last from the column.

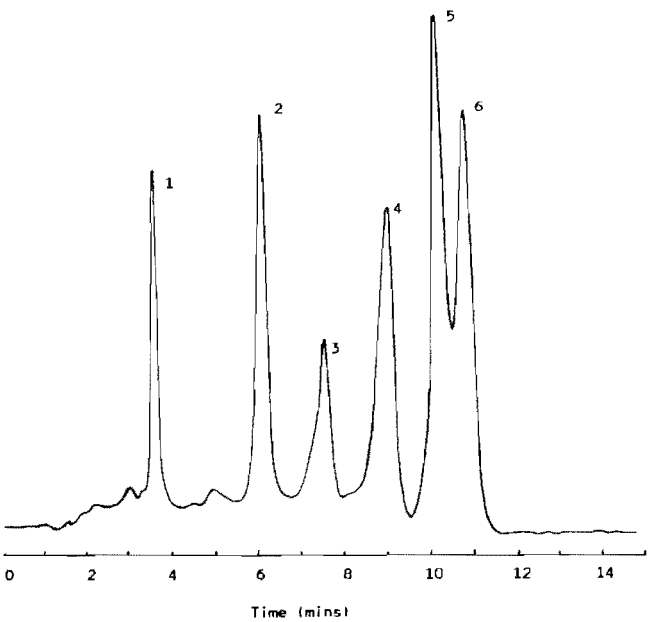


Figure 5. HPLC Chromatogram of Carotenoid Standards. Detector @ 436 nm, 0.02 AUFS. Other chromatography conditions as given in text. Concentrations of lutein, cryptoxanthin and lycopene were 0.5 µg/ml, and of α-, β- and τ-carotenes were 1.0 µg/ml. 100 µl used for injection. 1 = Lutein; 2 = cryptoxanthin; 3 = lycopene; 4 = τ-carotene; 5 = α-carotene; 6 = β-carotene

The concentrations of the major carotenoids quantitated are given in Tables 3 and 4 for the vegetables and fruits respectively. The carotenoids are tabulated in the order of their elution from the HPLC column, except for "other carotenoids" not identified. Figures 6 and 7 give the composition of the carotenoids, expressed as the percentage of each carotenoid to the sum of all carotenoids.

For most of the green vegetables (leafy and non-leafy), the major carotenoids detected were rather consistent, and simple HPLC chromatograms were obtained (Table 3 and Figure 6). In most cases, only β-carotene and lutein were obtained. The former was found in all the vegetables studied, and was clearly the major carotenoid in most of the

vegetables. In 13 of the green vegetables studied, β-carotene made up over 40% of the sum of all carotenoids. For the remaining three samples, at least 20% of the carotenoids was β-carotene. Lutein was also detected in all vegetables in fairly high proportions. Except for two samples, lutein made up over 25% of the sum of all carotenoids in these vegetables. The other carotenoids were encountered infrequently. α-Carotene was found only in *cemperai,* whilst γ-carotene, lycopene and cryptoxanthin were not encountered. For three of the vegetables in these two groups, a small proportion (< 20%) of the carotenoids was contributed by a few unidentified carotenoids.

Table 3: Content[1] of Major Carotenoids in Vegetables

	Name of Vegetables	Lutein	Crypto-xanthin	Lycopene	gamma-Carotene	alpha-Carotene	beta-Carotene	Others[2]	Sum[3]
	Green, leaf vegetables								
1.	*Cekur manis*	29913	0	0	0	0	13351	3292	46556
2.	*Cemperai*	9871	0	0	0	3677	3218	0	16766
3.	Chinese cabbage	963	0	0	0	0	3022	0	3985
4.	Drumstick leaves	7128	0	0	0	0	7536	0	14663
5.	Fern shots	1002	0	0	0	0	1438	0	2440
6.	Papaya shoots	821	0	0	0	0	1829	0	2650
7.	*Pegaga gajah*	1305	0	0	0	0	3840	0	5145
8.	*Ranti*	2888	0	0	0	0	7048	0	9936
9.	Spinach	4175	0	0	0	0	3177	0	7352
10.	Swamp cabbage	335	0	0	0	0	1895	0	2229
11.	*Tanki*	6236	0	0	0	0	11395	0	17631
12.	Tapioca shoots	1676	0	0	0	0	5720	0	7396
	Green, Non-leafy vegetables								
13.	French bean	460	0	0	0	0	236	154	849
14.	Long bean (dark green)	423	0	0	0	0	569	153	1144
15.	Paprika/Bell pepper	223	0	0	0	0	267	0	490
16.	Snake gourd	225	0	0	0	0	148	0	372
	Other vegetables								
17.	Carrot	0	0	0	0	0	6769	0	10179
81.	Chilli, red	941	7154	0	0	0	1663	1971	6328
19.	Pumpkin	941	0	0	0	756	578	0	2273
20.	Tomato	130	0	723	0	0	365	0	1128

[1] Mean of duplicate analyses; expressed as μg per 100 g of edible portion of sample
[2] Unidentified carotenoids
[3] Summation of all carotenoids tabulated.

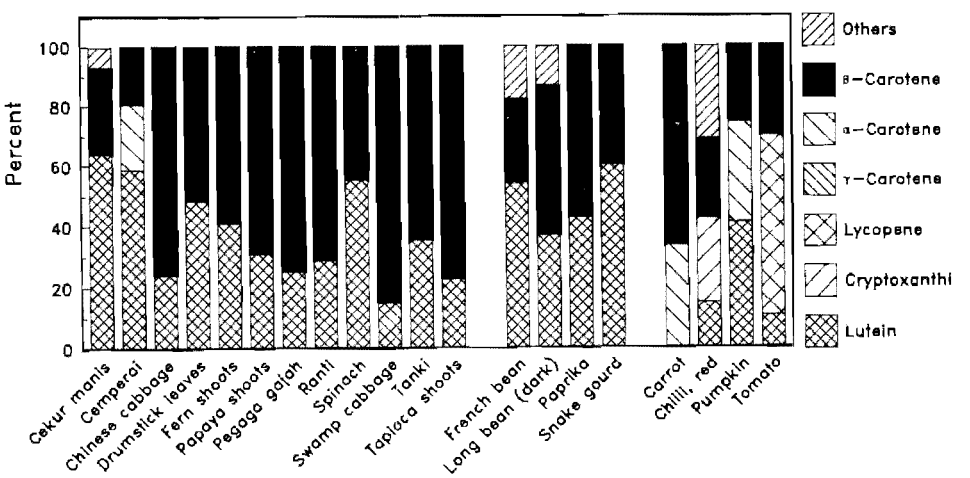


Figure 6. Carotenoid Composition of Vegetables

In contrast to the green vegetables, the carotenoid composition of the other vegetables was rather different (Table 3 and Figure 6). Although β-carotene and lutein were found in all these fruit and root vegetables (lutein was not detected in carrot), several other carotenoids were encountered. α-Carotene was detected in carrot and pumpkin, while cryptoxanthin was found in red chilli. Lycopene was detected only in tomato, and made up about 60% of the carotenoids quantitated.

The fruits also presented rather different carotenoid compositions from those obtained for the green vegetables (Table 4 and Figure 7). There was no clear pattern of carotenoids present in the samples studied. β-Carotene was detected in all the fruits, but its proportion varied considerably, ranging from 100% in mango to less than 10% for five other fruits. Lutein was found in five of the ten fruits studied, but in smaller proportions than in green vegetables. Cryptoxanthin was found in most of the fruits studied, and contributed to over 50% of the carotenoids in three of the fruits. As for the vegetables, α-carotene was infrequently encountered, having been detected only in the two banana species, contributing to about 50% of total carotenoids. Lycopene also occurred infrequently, being detected only in papaya (including the cultivar Exotica) and watermelon (red variety). In the last named, it constituted over 80% of all the carotenoids. γ-Carotene was ound in small proportions (<5%) in four of the fruits studied. Four of the fruits studied also had significant proportions of the unidentified carotenoids.

Table 4: Content[1] 1 of Major Carotenoids in Fruits

Name of Fruit	Lutein	Crypto xanthin	Lycopene	gamma-Carotene	alpha-Carotene	beta-Carotene	Others[2]	Sum[3]
1. *Pisang emas*	27	0	0	0	62	40	0	128
2. *Pisang tanduk*	37	0	0	0	157	92	0	286
3. *Buah kundang*	457	155	0	52	0	301	514	1477
4. Mango (Black-gold)	0	0	0	0	0	615	0	615
5. Musk lime	65	446	0	0	0	12	0	522
6. Orange	30	332	0	0	0	25	218	605
7. Papaya	0	1483	2003	118	0	228	294	4125
8. Papaya exotica	0	615	2333	189	0	321	304	3760
9. Tree tomato	0	1236	0	0	0	599	0	1834
10. Watermelon (red)	0	457	5301	90	0	324	0	6171

[1]Mean of duplicate analyses; expressed as μg per 100 g of edible portion of sample
[2]Unidentified carotenoids
[3]Summation of all carotenoids tabulated

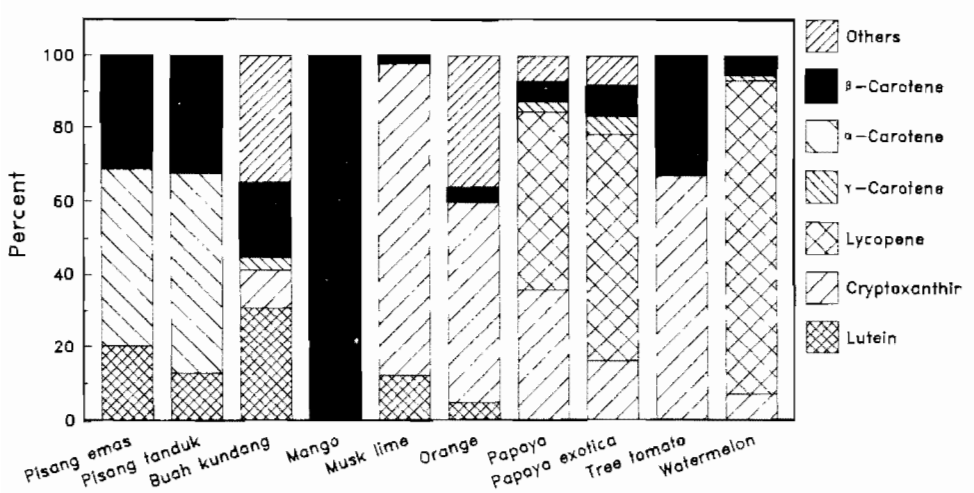


Figure 7. Carotenoid Composition of Fruits

β-Carotene Content

β-Carotene concentrations determined by the AOAC and HPLC methods are tabulated in Table 5. Figure 8 shows the ratio of the carotene concentration determined by the two methods.

Table 5: β-Carotene Content[1] of Vegetables and Fruits Determined by the AOAC and HPLC Methods

	Name of Vegetable/Fruit	AOAC method	HPLC method
	Green, leafy vegetables		
1.	*Cekur manis*	12363	13351
2.	*Cemperai*	6788	3218
3.	Chinese cabbage	2604	3022
4.	Drumstick leaves	7724	7536
5.	Fern shoots	1273	1438
6.	Papaya shoots	1709	1829
7.	*Pegaga gajah*	3266	3840
8.	*Ranti*	6760	7048
9.	Spinach	2947	3177
10.	Swamp cabbage	1729	1895
11.	*Tanki*	11459	11395
12.	Tapioca shoots	4607	5720
	Green, non-leafy vegetables		
13.	French bean	221	236
14.	Long bean (dark green)	520	569
15.	Paprika/Bell pepper	154	267
16.	Snake gourd	138	148
	Other vegetables		
17.	Carrot	9027	6769
18.	Chilli, red	1743	1663
19.	Pumpkin	1170	578
20.	Tomato	352	365
	Fruits		
1.	*Pisang emas*	82	40
2.	*Pisang tanduk*	219	92
3.	*Buah kundang*	303	301
4.	Mango (Black-gold)	495	615
5.	Musk lime	18	12
6.	Orange	40	25
7.	Papaya	208	228
8.	Papaya exotica	314	221
9.	Tree tomato	582	599
10.	Watermelon (red)	246	324

[1]Mean of duplicate analyses; expressed as μg per 100 g of edible portion of sample.

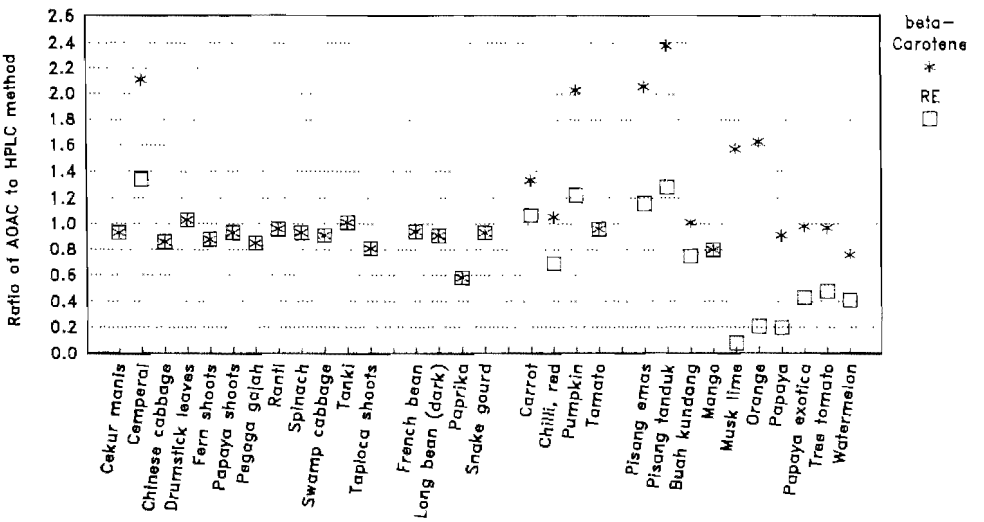


Figure 8. Ratio of beta-Carotene and Retinol Equivalent (RE) of Vegetables and Fruits Determined by the AOAC and HPLC Methods

For the green leafy vegetables, the ratios of β-carotene concentration determined by the AOAC and HPLC methods clustered between 0.8–1.2, i.e. varying within ±20% from unity. Only in one vegetable was the ratio outside this range; *cemperai* was found to have an exceptionally high ratio of 2.1. This high ratio was due to the presence of α-carotene in this vegetable which was eluted together with β-carotene from the magnesia column and erroneously estimated together with it in the AOAC method. For most of the other vegetables, the ratio tended to be less than 1.0, indicating that the HPLC method gave slightly higher results.

The ratios of β-carotene content determined by the AOAC and HPLC methods for the green non-leafy and other vegetables were slightly more varied, with most of the ratios between 0.6–1.4. For pumpkin, the ratio was exceptionally high at 2.0, due to the presence of α-carotene which was giving erroneously high result by the AOAC method.

For the fruits, there was considerable variation in the ratios of β-carotene concentration given by the two methods. For six of the fruits studied, the ratios were between 0.7 and 1.0. For the only two fruits with α-carotene, i.e. the two species of banana, the ratios were greater than 2.0. The reason for this over-estimation by the AOAC method has been explained above. Two other fruits with ratios of about 1.6 were musk lime and orange. These fruits were found to have low levels of β-carotene (less than 100 μg per 100 g edible portion), which made up only a small proportion of all the carotenoids detected. The relatively insensitive and non-specific nature of the AOAC method, especially for foods with low β-carotene, could be the reason for the over-estimation by this method.

Results obtained from the HPLC method showed that highest concentrations of β-carotene were found in green leafy vegetables, particularly *cekor manis* and *tanki* Table 5). Both *sayur kampung* had a β-carotene content of over 11,000 μg per 100 g of edible portion. Seven other green leafy vegetables were found to have a β-carotene content of

between 3,000 – 10,000 μg per 100 g of vegetable. With the exception of carrot (β-carotene about 7,000 μg per 100 g), none of the green non-leafy and other vegetables was found to have high β-carotene content. All the fruits studied were not rich in β-carotene, with concentrations of less than 1000 μg per 100 g of sample. The sample of mango studied was interesting in that all the carotenoids was β-carotene, and the concentration was the highest of all the fruits (about 600 μg per 100 g).

Vitamin A Activity (Retinol Equivalent)

Conventionally, the nutritional significance of carotenoids is related to the provitamin A activity of these compounds. Although over 500 carotenoids have been reported to occur naturally, only a few are known to possess provitamin A activity and occur in significant amounts in natural foods[13,14]. For vitamin A activity, a carotenoid must have at least one unsubstituted β-ionone ring with an attached polyene side of at least 11 carbon atoms. Consistent with these important structural requirements, the following carotenoids identified in this study have been known to possess provitamin A activity: β-carotene, α-carotene, γ-carotene, and cryptoxanthin. The vitamin A activity of β-carotene, expressed as μg retinol equivalent (RE) was calculated as RE = (μg β-carotene)/6[15]. The other three carotenoids mentioned, possessing only one unsubstituted β-ionone ring, may be expected to have about 50% of the biological activity of β-carotene. The formula used for these provitamin A carotenoids was therefore RE = (μg carotenoid)/12.

The RE values calculated from β-carotene determined by the HPLC method and those calculated based on all carotenoids with provitamin A activity by the same analytical method are given in Table 6 for the vegetables. The differences in RE between the two methods of calculation, expressed as a percentage of RE from all carotenoids, are also given in the Table. For the green leafy vegetables, with the exception of *cemperai,* since no other carotenoids with provitamin A activity were detected, there was no difference in RE calculated by the two methods. For *cemperai,* excluding α-carotene from the calculation would result in a 36% error in RE content for the vegetable. There was also no difference in RE calculations for green non-leafy vegetables. For the other vegetables, namely carrot, red chilli and pumpkin, differences in RE ranged from 20-40%.

Table 6: Retinol Equivalent (RE)[1] of Vegetables Determined by the AOAC and HPLC Methods

	Name of Vegetable	AOAC method[2]	HPLC method		
			β-carotene[3]	total[4]	% difference[5]
	Green, leafy vegetables				
1.	*Cekur manis*	2061	2225	2225	0
2.	*Cemperai*	1131	536	843	36.4
3.	Chinese cabbage	434	504	504	0
4.	Drumstick leaves	1287	1256	1256	0
5.	Fern shoots	212	240	240	0
6.	Papaya shoots	285	305	305	0
7.	*Pegaga gajah*	544	640	640	0
8.	*Ranti*	1127	1175	1175	0
9.	Spinach	491	530	530	0
10.	Swamp cabbage	288	316	316	0
11.	*Tanki*	1910	1899	1899	0
12.	Tapioca shoots	768	953	953	0

	Green, non-leafy vegetables				
13.	French bean	37	39	39	0
14.	Long bean (dark green)	87	95	39	0
15.	Paprika/Bell pepper	26	45	45	0
16.	Snake gourd	23	25	25	0
	Other vegetables				
17.	Carrot	1504	1128	1412	20.1
18.	Chilli, red	290	277	423	34.5
19.	Pumpkin	195	96	159	39.5
20.	Tomato	59	61	61	0

[1] Mean of duplicate analyses; expressed as μg per 100 g of edible portion of sample

[2] Calculated as RE = μ (μg carotene)/6

[3] Based on β-carotene only; RE = (μg β-carotene)/6

[4] Based on β-carotene and all other provitamin A carotenoids, i.e. α-carotene, γ-carotene, cryptoxanthin; RE = [(μg β-carotene)/6] + [(μg other carotenoids)/12]

[5] Calculated as: $\frac{\text{total RE} - \text{RE from } \beta\text{-carotene}}{\text{total RE}} \times 100$

Since other provitamin A carotenoids besides β-carotene were detected in all the fruits studied, except mango, the RE values calculated on the basis of all these carotenoids were higher than those calculated based on β-carotene only (Table 7). The vitamin A activity calculated on the basis of β-carotene alone would result in RE values which were 25 – 95% lower.

Table 7: Retinol Equivalent (RE)[1] of Fruits Determined by the AOAC and HPLC Methods.

	Name of Fruit	AOAC method[2]	HPLC method		
			c-carotene[3]	total[4]	% difference[5]
1.	*Pisang emas*	14	7	12	43.8
2.	*Pisang tanduk*	36	15	28	46.0
3.	*Buah kundang*	51	50	67	25.6
4.	Mango (Black-gold)	82	103	103	0
5.	Musk lime	3	2	39	95.1
6.	Orange	7	4	32	87.1
7.	Papaya	35	38	171	77.8
8.	Papaya exotica	52	54	120	55.6
9.	Tree tomato	97	100	203	50.8
10.	Watermelon (red)	41	54	99	45.8

[1] Mean of duplicate analyses; expressed as μg per 100 g of edible portion of sample

[2] Calculated as RE = (μg carotene)/6

[3] Based on β-carotene only; RE = (μg β-carotene)/6

[4] Based on β-carotene and all other provitamin A carotenoids, i.e. β-carotene, γ-carotene, cryptoxanthin; RE = [(μg β-carotene)/6] + [(μg other carotenoids)/12]

[5] Calculated as: $\frac{\text{total RE} - \text{RE from } \beta\text{-carotene}}{\text{total RE}} \times 100$

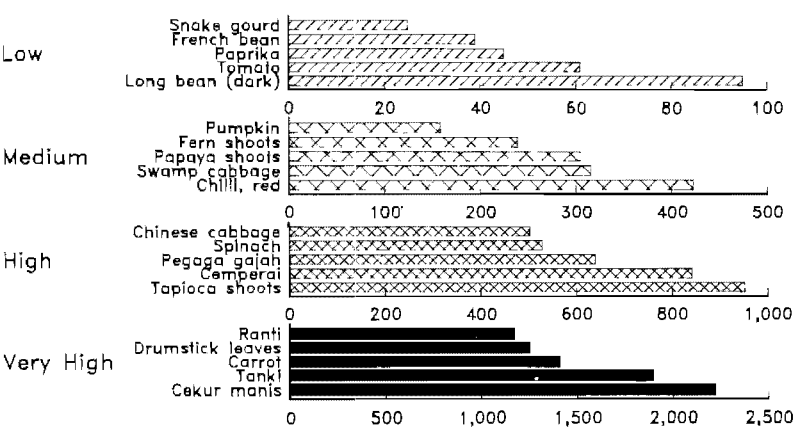
The RE values calculated from β-carotene determined by the AOAC method and all carotenoids with provitamin A activity by the HPLC methods are given in Table 6 for the vegetables. As can be expected, with the exception of *cemperai*, the ratios of RE calculated by the two methods were exactly the same as ratios obtained for β-carotene for the green vegetables (leafy and non-leafy) (Figure 8), because β-carotene was the only provitamin A carotenoid detected for these vegetables. Owing to the presence of β-carotene in *cemperai* the RE for this vegetable was falsely elevated when calculated from carotene determined by the AOAC method since α-carotene possesses only half the provitamin A activity of β-carotene.

For the other vegetables except tomato, the presence of other provitamin A carotenoids resulted in ratios of RE determined by the AOAC and HPLC methods that were different from ratios of β-carotene given by the two methods (Figure 8). RE obtained from the AOAC method (Table 6) for carrot and pumpkin would be falsely elevated due to the presence of α-carotene. The reason for this is as explained above for *cemperai*. On the other hand, RE for red chilli given by the AOAC method would be falsely low since the procedure was not able to quantitate cryptoxanthin.

Similarly, because of the presence of other provitamin A carotenoids in fruits (except for mango), the ratios for RE determined by the AOAC and HPLC methods were different from the β-carotene ratios. Ratios for RE were all lower than those for β-carotene, and were generall below 1.0 (Table 7 and Figure 8). These data show that the AOAC method was underestimating the vitamin A activity of the fruits, since the method was not able to quantitate the other provitamin A carotenoids.

To facilitate easy identification of vegetables which are good sources of vitamin A activity, these foods were grouped into four categories, namely low (<100 μg RE per 100 g edible portion), medium (100–499 μg RE), high (500–999 μg RE) and very high (> 1,000 μg RE). These are illustrated graphically in Figures 9. Vegetables with high and very high RE were all green leafy vegetables, with the exception of carrot, a root vegetable. Of particular interest are three kampung vegetables with over 1,000 μg RE, namely *cekur manis, tanki* and *ranti*. Several other local vegetables were in the high RE category. A few other green leafy vegetables and red chilli and pumpkin made up the group with medium RE. All the green non-leafy vegetables, as well as tomato were found to be poor sources of vitamin A.

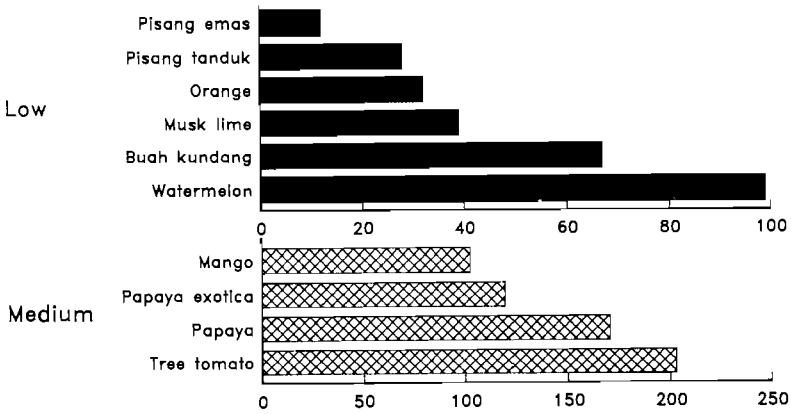
Figure 9. Classification of Vegetables According to Retinol Equivalent (RE)



Low = <100 μg RE/100 g e.p.
Medium = 100–499 μg RE/100 g e.p.
High = 500–999 μg RE/100 g e.p.
Very High = >1000 μg RE/100 g e.p.
All x-axis scales in μg RE/100 g e.p.

A similar grouping was made for the fruits studied (Figure 10). None of thefruits may be considered as having high or very high vitamin A activity. The two species of papaya, tree tomato and mango were found to have medium RE, while the other fruits were poor sources of vitamin A, particularly *pisang emas.*

Figure 10. Classification of Fruits According to Retinol Equivalent (RE)



Low = <100 µg RE/100 g e.p.
Medium = 100–499 µg RE/100 g e.p.

All x–axis scales in µg RE/100 g e.p.

CONCLUSION

A non-aqueous reversed-phase HPLC method for the determination of carotenoids in various vegetables and fruits has been developed. The method uses the basic configurations of an HPLC system, and would thus be useful for routine determinations. A ternary mixture of acetonitrile, methanol and ethyl acetate was used to separate the carotenoids isocratically in an octadecylsilane (C_{18}) column. A fixed wavelength detector at 436 nm was used to detect the carotenoids, the peaks being monitored and quantitated in an integrator. The method gave satisfactory separation and quantitation of lutein, cryptoxanthin, lycopene, γ–, α– and β-carotenes. The emphasis has been on major carotenoids that occur in sufficient amounts to contribute significantly to dietary intake.

This is the first report on the concentration of major carotenoids in relation to total carotenoids in a number of Malaysian vegetables and fruits. It is also the first report of a parallel study of carotenoid determination by the AOAC and the HPLC methods for a fairly large number of samples. Findings from the study have clearly shown that the HPLC method would give a more complete picture of the carotenoid composition as well as a more accurate quantitation of the provitamin A activity of the vegetables and fruits. Depending on the composition of the carotenoids present, the AOAC method could under- or over-estimate the β-carotene concentration and therefore the RE activity. The HPLC procedure reported could be useful for up-dating the vitamin A activity of plant materials in the current Malaysian Food Composition Table, thereby providing the correct identification of foods rich in provitamin A activity. The nutritional significance of the findings are clear since these foods are important sources of vitamin A for the majority of the communities in the country. Furthermore, there is currently a great deal of interest in carotenoids not possessing vitamin A activity as they may be associated with lower cancer risk.

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REFERENCES

1 Tee ES. Carotenoids and Retinoids in Human Nutrition. Lim CL, Chong YH (eds). Kuala Lumpur: Institute for Medical Research, 1988.

2 Zakaria M. Simpson K, Brown PR, Krstulovic A. Use of reversed-phase high-performance liquid chromatographic analysis for the determination of provitamin A carotenes in tomatoes. *J Chromatogr* 1979; 176:109-117.

3 Beecher GR, Khachik F. Evaluation of vitamin A and carotenoid data in food composition tables. *J Natn Cancer Inst* 1984; 73:1397-q404.

4 Underwood BA. Vitamin A in animal and human nutrition. In: Sporn MB, Roberts AB, Goodman DS (eds). The Retinoids. Voume 1. Oriando: Academic Press, 1984; 281-392.

5 Bureau JL, Bushway RJ. HPLC determination of carotenoids in fruits and vegetables in the United States. *J FD Sci* 1986; 51:128-130.

6 Peto R, Doll R, Buckley JD, Sporn MB. Can dietary beta-carotene materially reduce human cancer rates? *Nature* 1981; 290-201-208.

7 Oison JA. Carotenoids, vitamin A and cancer. *J* Nutr 1986; 116:1127-1130.

8 Ritenbaugh C. Carotenoids and cancer. *Nutr Today* 1987; January/February:14-19.

9 Temple NJ, Basu TK. Does beta-carotene prevent cancer? A critical appraisal. *Nutr Res* 1988; 8;685-701.

10 Tee ES. Review of Methods for Carotenoids and Retinoids Analysis. Kuala Lumpur: Institute for Medical Research, 1989 (unpublished).

11 Williams S (ed). Official Methods of Analysis of the AOAC. 14th Edn., Virginia: Association of Official Analytical Chemists, 1984; 834-835.

12 De Ritter, E. Absorption maxima and E (1%/1cm) values for carotenoids. In: Bauernfeind JC (ed). Carotenoids as Colorants and Vitamin A Precursors. New York: Academic Press, 1981; 883-923.

13 Bauernfeind JC. Carotenoid Vitamin A precursors and analogs in foods and feeds. *J agric Fd Chem* 1972; 20-456-473.

14 Goodwin TW. Metabolism, nutrition and function of carotenoids. *Annu Rev Nutr* 1986; 6:273-297

15 National Academy of Sciences. Fat-soluble vitamins: vitamin A. In: Recommended Dietary Allowances, 9th Edn., Washington, D.C.: National Academy of Sciences, 1980; 55-60.

Proc Nutr Soc Mal 1990; 5:28-32

EFFECT OF VITAMIN E FROM PALM OIL ON CHEMICALLY-INDUCED MAMMARY CARCINOGENESIS IN RATS

K. Nesaretnam
Palm Oil Research Institute of Malaysia,
Bangi, Selangor.

Khor Hun Teik
Department of Biochemistry,
University of Malaya, Kuala Lumpur.

Chong Yoon Hin
Palm Oil Research Institute of Malaysia,
Bangi, Selangor.

J. Ganesan
Division of Pathology,
Institute for Medical Research,
50588 Kuala Lumpur.

ABSTRACT

Female Sprague-Dawley rats at 50 days old were treated with a single dose of 5 mg 7,12-dimethylbenz(α)anthracene (DMBA) intragastrically. The rats were fed on semisynthetic diets containing 20% by weight of refined, bleached and deodorised palm oil (RBDPO), soybean oil (SBO), coconut oil (CNO) and vitamin E-free palm oil (EFPO) three days after DMBA treatment, for 20 weeks. The results showed that the rats fed on semisynthetic diets containing different fats did not differ significantly in the rate of growth during the experimental period. However, rats fed on RBDPO diet gave the lowest tumour incidence, the least number of mammary tumours and the smallest number of tumours per rat as compared to the other dietary groups. In contrast, rats fed on the diet containing EFPO had a higher tumour incidence, higher total number of tumours and larger number of tumours per rat that were comparable to that in rats fed on SBO diet. The results therefore suggest that the protective effect of palm oil may be due to vitamin E— tocopherols and tocotrienols, in the oil.

INTRODUCTION

Palm oil triglycerides are structurally different from those of other vegetable oils[1,2]. Palm oil is also rich in tocotrienols, the structural analogues of the more common vitamin E, tocopherols[3].

Animal studies have consistently shown that dietary fats play a promotional role in certain types of cancers[4]. Polyunsaturated oils are more effective than saturated fats in promoting tumour growth.