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Simultaneous determination of retinol, carotenoids and tocopherols in human serum by high pressure liquid chromatography

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ABSTRACT

The determination of serum vitamins having antioxidant properties has gained in importance in recent years. This is mainly due to the observation that an inverse correlation exists between blood levels of these vitamins, including retinol, carotenoids and tocopherol, and diet-related chronic diseases such as coronary heart disease and cancers. This laboratory has been carrying out a series of studies into the nutritional and analytical aspects of retinol and carotenoids. A simple reversed-phase HPLC method has been developed in an effort to improve methodologies for the separation and quantitation of carotenoids and retinol in foods and biological specimens, especially blood serum. As an extension to these studies, trials were carried out to determine the feasibility of analysing tocopherols using the same chromatographic procedure. With the addition of another detector wavelength, the same procedure detected and quantitated 3 major tocopherols simultaneously with retinol and five carotenoids. Within-day and between-day precision of the procedure was satisfactory. Trials carried out were able to improve recovery of the vitamins. Experiments conducted also showed that the addition of ascorbic acid to the extracting ethanol was beneficial for the analytical procedure. The presence of peroxide in ethyl acetate used in the chromatography mobile phase caused drastic destruction to the vitamins analysed. The addition of ascorbic acid during sample preparation was able to inhibit this destruction. The method was used for the analysis of sera from 65 apparently healthy Malaysians with a mean age of 52.8 years (range 24-76 years). Mean retinol concentration of the group was 69.8 ± 18.8 μg/dl. The mean β-carotene concentration of the subjects studied was 33.8 ± 24.3 μg/dl, while the mean total carotenoid concentration was 180.2 ± 3.0 μg/dl. The most abundant carotenoid in the serum samples studied was lutein, comprising about one-third of all carotenoids quantitated. The concentrations of δ- and γ-tocopherols in the serum samples studied were too low to be identified with certainty and quantitated accurately. The mean α-tocopherol level was 1840 ± 528 μg/dl. For retinol, α-tocopherol and most of the carotenoids determined, there was no statistically significant difference in the mean levels between male and female subjects as well as among the three different ethnic groups. Results obtained in this study were very similar to those previously reported by this laboratory. It is hoped that more data on the serum concentrations of these vitamins can become available for various population groups, including during various disease conditions.

INTRODUCTION

The determination of the serum vitamins having antioxidant properties has gained in importance in recent years. An inverse correlation between blood levels of these vitamins, including retinol, carotenoids and tocopherol, and diet-related chronic diseases such as coronary heart disease and

cancers have been observed. Many experimental, clinical and epidemiological studies into the possible protective effects of dietary antioxidants in these diseases have been carried out (Tee, 1992).

This laboratory has been carrying out a series of studies into the nutritional and analytical aspects of retinol and carotenoids. A simple reversed-phase HPLC method has been developed in an effort to improve methodologies for the separation and quantitation of carotenoids and retinol in foods and biological specimens, especially blood serum. The method has been used successfully for the analysis of major carotenoids in a variety of fruits and vegetables (Tee & Lim, 1991), legumes, tubers and roots (Tee, Goh & Khor, 1995), the simultaneous determination of retinol and several carotenoids in foods of animal origin and processed foods (Tee & Lim, 1992), and human sera (Tee, Lim & Chong, 1994). A study of the biological utilisation of carotenoids in two local vegetables in rats was also conducted (Tee, Lim & Chong, 1995).

As an extension to these studies, trials were carried out to determine the feasibility of analysing tocopherols using the same chromatographic procedure. This paper reports the modifications that were made to enable the simultaneous determination of retinol, several carotenoids and tocopherols using the HPLC method developed, several tests carried out to determine its suitability for use and finally the application of the method to a small sample of human serum.

MATERIALS AND METHODS

Solvents and standards

Analytical-grade solvents were used for sample preparation. Petroleum ether (b.p. 40-60°C) used for sample preparation was re-distilled before use as impurities in the solvent were found to interfere with the retinol peak in the HPLC chromatogram. Solvents for liquid chromatography were of HPLC grade. All solvents for use as the mobile phase in HPLC were filtered through a 0.45-μm nylon membrane filter and degassed using an ultra-sonic bath.

Retinol, α- and β-carotene, lycopene, and α-, δ-, and γ-tocopherol standards were purchased from Sigma Chemical Company. Cryptoxanthin and lutein were gifts from F. Hoffmann La-Roche, Switzerland. Stock solutions of retinol, tocopherols and lutein were prepared in ethanol and of the other carotenoids in hexane, in concentrations of 100 μg per ml (2-5 mg/ml for tocopherols) and stored in amber bottles below -20ºC. Working solutions of appropriate concentrations of the standards were prepared for use daily. The absorptivities (extinction coefficients) used to calculate the exact concentration of each of the compounds are given in Table 1. Absorbance readings and absorption spectra of all standard solutions were monitored daily.

The preparation of all standard solutions were carried out rapidly, in a room with subdued light and with all windows tinted with a light-protective film. All sample treatment and analytical procedures were also carried out in this room.

Samples and sample preparation

Reproducibility studies

Left-over human sera sent to the laboratory for determination of serum lipid profile were pooled for a series of reproducibility studies. Sera that were stored for more than 5 days in the refrigerator were not used.

Aliquots of the pooled sera were processed as follows for the reproducibility study. Into glass-stoppered 15-ml centrifuge tubes were added 0.5 ml of serum and an equal volume of ethanol and the contents mixed in a vortex. After the addition of 2 ml of petroleum ether (b.p. 40-60ºC), the tubes were stoppered and shaken vigorously for 2 min. The tubes were centrifuged for 3 min or left to stand for 0.5 hour and 1.5 ml of the top petroleum ether layer pipetted out into graduated centrifuge tubes. The solvent in the tubes were evaporated to a volume of about 0.2 ml in a beaker of water placed over a hot-water bath. The water in the beaker should initially be cold to prevent spurting of the solvent in the tube. Care was taken not to evaporate the solvent totally dry as this can result in losses in carotenoids. Mobile phase was added immediately to the tubes to a volume of 1.5 ml and the tubes stoppered. The solutions were filtered with a 0.45 μm membrane filter before injecting into the HPLC.

Table 1. Extinction coefficient of vitamin standards

Standard	Solvent (wavelength maxima, nm)	Extinction Coefficient	Reference
Retinol	ethanol (325)	1570	Sigma Chemical Co.
α-Carotene	hexane (445)	2544	Sigma Chemical Co.
β-Carotene	hexane (450)	2500	Sigma Chemical Co.
Lutein	ethanol (445)	2550	De Ritter & Purcell (1981)
β-Cryptoxanthin	hexane (450)	2386	De Ritter & Purcell (1981)
Lycopene	benzene (485)	3370	De Ritter & Purcell (1981)
α-Tocopherol	ethanol (292)	75.8	Schudel, Mayer & Isler (1972)
δ-Tocopherol	ethanol (298)	87.3	Schudel, Mayer & Isler (1972)
γ-Tocopherol	ethanol (298)	91.4	Schudel, Mayer & Isler (1972)

For within-day reproducibility study, each pool serum was analysed 5 times on the same day. Thirty pool sera were so analysed. Ten pool sera were used for the between-day reproducibility study, each pool being analysed for three consecutive days. On each day, five replicate analyses were carried out.

Recovery studies

For each recovery run, two aliquots of 0.5 ml each of the serum sample were prepared. To the recovery tube was added 20 μl each of retinol, β-carotene and α-tocopherol standard containing 0.17, 0.19 and 7.57 μg respectively of the vitamins. To both tubes were added an equal volume of ethanol and processed as described above for reproducibility studies.

Effect of addition of vitamin C to samples

A number of left-over human sera were also used for a series of investigations into the effect of

addition of ascorbic acid. For each serum sample, two aliquots of 0.5 ml each were prepared. To the first aliquot was added an equal Volume of 95% ethanol containing 0.2% ascorbic acid. For the second aliquot, an equal volume of ethanol with no ascorbic acid was added. Both aliquots were then treated as described above for reproducibility studies.

Effect of peroxide on retinol, tocopherol and carotenoids

The mobile phase for these experiments were specially prepared using ethyl acetate which contained peroxide. To determine the presence of peroxide in the solvent, the potassium iodide (Kl) test was used (Nirenberg, 1985). The procedure consisted of observing colour change 5 min after adding the following and mixing on a vortex: 2.5 ml of water, 250 mg of crystalline KI, 0.5 ml of concentrated hydrochloric acid, and 1.5 ml of the test solvent. The presence of a brownish color indicated the presence of peroxides, the intensity of which depended on the amount of the impurities present. If desired, absorbance of the colour could be measured at 510 nm in a spectrophotometer using a blank solution prepared as above, but with pure ethanol as the organic solvent.

Serum samples used for the experiments were prepared as described in the study on the effect of ascorbic acid. Each serum sample used for the experiment was prepared in duplicate so that one aliquot contained ascorbic acid while the other did not contain added ascorbic acid.

Retinol, carotenoid and tocopherol concentrations of selected subjects

Blood samples sent to the laboratory for determination of serum lipid profile were selected based on the following criteria: (a) samples were from apparently healthy subjects who requested for routine medical examination; (b) samples were collected on the same day; and (c) sufficient volume was available. Blood samples meeting these criteria were allowed to clot and the sera separated by centrifugation. Aliquots of the sera from these samples were immediately taken and stored in the dark at -20ºC. The samples were analysed within one week of collection.

Serum samples were processed for analysis using the procedure described above. A modification to the procedure was the use of ethanol containing ascorbic acid (0.2% w/v) for the precipitation of serum protein. A total of 65 “normal” serum samples were analysed in duplicate, using a volume of 0.5 ml of serum for each analysis.

High-pressure liquid chromatography procedure

HPLC conditions

A Gilson liquid chromatograph equipped with a 305 pump was used. A Gilson 116 UV dual wavelength detector set at 325 nm (0.01 AUFS) and 295 nm (0.005 AUFS) was connected in series to a Waters 440 fixed-wavelength detector at 436 nm (0.005 AUFS) to enable the simultaneous detection of retinol, tocopherols and carotenoids, respectively. A stainless steel 30 cm x 3.9 mm (i.d.) 10-mm μBondapak C_{18} column was used for the chromatographic separation. This column was preceded by a pre-column module housing a disposable pre-column insert packed with the same material as that in the analytical column. The mobile phase used consisted

of a ternary mixture of acetonitrile, methanol and ethyl acetate (88:10:2, v/v/v), delivered at a rate of 2 ml/min. The ethyl acetate used were tested and found to be negative for peroxide before use. The sample injection volume, dispensed with a Gilson 234 autoinjector, was usually 200 µl. Data were analysed and stored using a Gilson 715 HPLC system controller software, operating in a IBM-AT compatible microcomputer.

Chromatography of retinal, carotenoids and tocopherols

Serum samples were prepared as described above. After filtering through a 0.45-µm membrane filter, aliquots were injected into the HPLC for simultaneous detection and quantitation of retinol, carotenoids and tocopherols.

Peak areas of retinol, α- and β-carotenes, lutein, cryptoxanthin, lycopene and the tocopherols were quantitated and their concentrations calculated using reference standards of these compounds, similarly chromatographed. Total peak area obtained at 436 nm for each sample was used for calculating total carotenoid concentration in the sample. "Sum of carotenoids" is an arithmetic summation of the five carotenoids mentioned above.

Statistical analyses

Since the number of cases analysed were small, non-parametric methods for not normally distributed observations were used for statistical analyses. The Wilcoxon matched-pairs signed-ranks test was used for determining significant differences in mean retinol, tocopherol and carotenoid levels between samples with and without the addition of ascorbic acid. For determining, differences in vitamin levels between male and female subjects the Mann-Witney U test for two independent samples was used. The Kruskal-Wallis one-way analysis of variance was used to examine differences in the levels of these vitamins among the three ethnic groups. All statistical tests were carried out using the SPSS for Windows statistical package (version 5.0). For all tests carried out, a P value of <0.05 was taken as statistically significant.

RESULTS AND DISCUSSION

Simultaneous detection and quantitation of retinol, tocopherols and carotenoids

The HPLC method described was developed in this laboratory for the simultaneous determination of retinol and carotenoids in serum (Tee, Lim & Chong, 1994). This study has shown that the same HPLC system is also able to separate tocopherols from retinol and its precursors. The detector setup was as previously used, namely connecting a Gilson UV 116 detector in series to a Waters 440 fixed-wavelength detector with a 436-nm filter. The former detector was set to simultaneous detect at 2 wavelengths, that is, 325 nm for retinol and 295 nm for tocopherols. The second detector was for detecting carotenoids. The controller software was able to separately quantitate the areas of peaks detected by the two detectors, although one of them was a third-party detector. The software also enabled re-analysis of data, using the most appropriate integration parameters for the peaks in each chromatogram.

A chromatogram of retinol, carotenoid and tocopherol standards separated using the configurations described above is shown in Figure 1, while Figure 2 shows a chromatogram of a serum sample analysed using the method described. As can be seen from the chromatogram of the standards, all the five carotenoids were fully separated, except for α- and β-carotenes. The three forms of tocopherols were also well separated. All the five carotenoids were detected and quantitated in the serum samples studied. For the tocopherols, the concentrations of delta and gamma forms were too low to be identified with certainty and quantitated accurately. The rest of this paper will therefore only report on α-tocopherol concentration.

Reproducibility studies

Within-day variation studies carried out by daily repeated analyses of 30 pool sera gave mean coefficient of variation (CV) of less than 10 for all the vitamins, except for lycopene, α- and β-carotene. Larger variations were observed for the latter 3 compounds (Table 2). Reproducibility of results obtained for between-day variation studies was also good, with mean CV values of less than 10 for all compounds (Table 3).

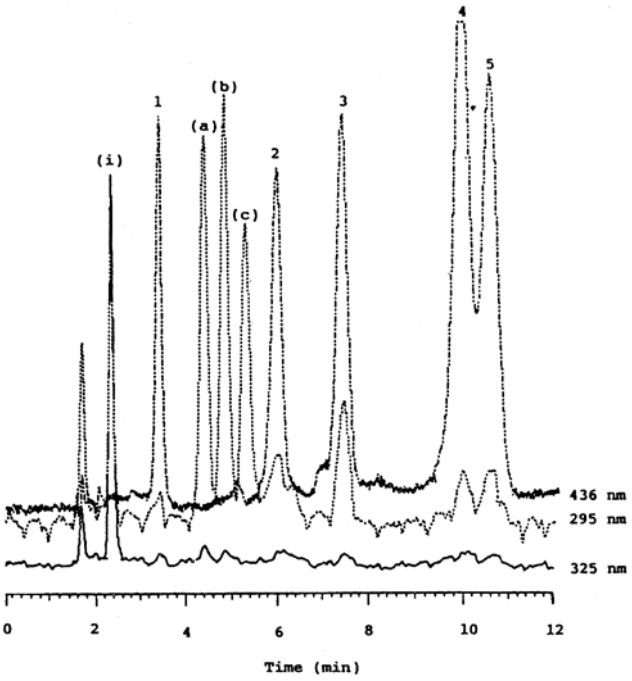

Figure 1. HPLC chromatogram of a mixture of retinol, tocopherol and carotenoid standards. Retinol and tocopherols were detected using Gilson UV-116 dual wavelength detection at 325 nm and 295 nm respectively. Carotenoids were detected using Water's 440 fixed wavelength detector at 436 nm which was connected in series to the UV detector. Other chromatography conditions are as given in the text. A volume of 50 µl of the standard mixture was injected. (i) = retinol; (a) = δ-tocopherol; (b) = γ-tocopherol; (c) = α-tocopherol; 1 = lutein; 2 = ß-cryptoxanthin; 3 = lycopene; 4 = α-carotene; 5 = ß-carotene.

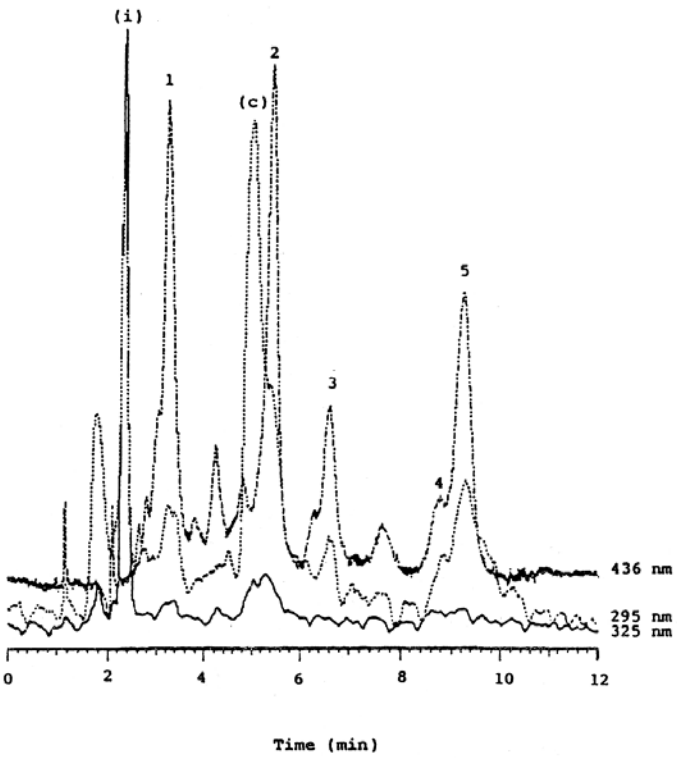

Figure 2. HPLC chromatogram of a sample of human serum. Chromatography conditions are as described for Figure 1. 0.5 ml serum was used for the analysis, and 100 µl sample extract was Injected. (i) = retinol; c = α-tocopherol; 1 = lutein; 2 = ß-cryptoxanthin; 3 = lycopene; 4 = α-carotene; 5 = ß-carotene. Peaks (a) (δ-tocopherol) and (b) (γ-tocopherol) in the serum samples were too low to be identified with certainty and quantitated accurately.

Recovery studies

In the first set of studies carried out, mean recovery values obtained upon addition of retinol and α-tocopherol were over 90% and CV of not over 14% (Table 4). However, unsatisfactory results were obtained for β-carotene, with low recovery and high CV values. The loss of carotene probably occurred during the heating of the petroleum ether extract to almost dryness in a boiling waterbath. It was noticed that turbidity occurred in most of the tubes on standing, before the addition of mobile phase. This turbidity, probably due to water condensation when the tubes were cooled, appeared to have a drastic effect on β-carotene but not to retinol and α-tocopherol.

To determine if the results could be improved, a second set of experiments were carried out, with more precautions being taken during the heating step. The extracts were heated to about 0.2 ml, followed immediately by the addition of mobile phase, and then stoppered tightly with glass stoppers. It was noticed that no turbidity occurred in most of the tubes except for two cases.

Recovery values for β-carotene improved to a mean of 82.7% and the CV was reduced to 26% (Table 4).

Effect of addition of vitamin C to samples

A loss of retinol has been reported to occur during the analysis of stored, frozen sera, probably during the extraction step. The addition of ascorbic acid to ethanol has been reported to be able to prevent this loss (Driskell, Bashor & Neese, 1985). Since some of the vitamins determined in this study are highly labile, experiments were thus carried out to determine if the addition of ascorbic acid could reduce loss of these compounds.

Results of 19 experiments carried out on the effect of addition of ascorbic acid to serum samples are shown in Tables 5a and 5b. The Wilcoxon signed-ranks test showed that, with the exception of β-carotene, there was no significant difference in the mean values of the compounds studied with or without the addition of ascorbic acid. Mean β-carotene value with the addition of ascorbic acid was found to be significantly higher than without the addition of the vitamin (P=0.022). Although mean levels of α-tocopherol and lutein without the addition of ascorbic acid were not significantly lower, the peaks of these vitamins in the HPLC chromatogram showed signs of deterioration. It was thus felt that the addition of vitamin C would be beneficial to the analytical procedure, and was done for the analysis of "normal" serum samples reported below.

Table 2. Coefficient of variation obtained from within-day variation study

	Retinol	α-tocopherol	lutein	ß-cryptoxanthin	lycopene	α-carotene	ß-caroene	Total carotenoids
Mean	3.9	4.9	4.7	5.6	10.1	15.7	11.0	5.5
SD	2.0	2.9	2.4	3.1	4.3	4.8	5.9	2.7
Minimum	0.9	1.0	0.4	0.7	2.3	5.7	1.7	1.4
Maximum	8.8	11.3	8.8	16.3	22.3	23.8	28.9	12.0

Values in table were obtained from the analysis of 30 pool sera. Each pool was analysed in one day with 5 replicate analyses per pool

Table 3. Coefficient of variation obtained from between-day variation study

	Retinol	α-tocopherol	lutein	ß-cryptoxanthin	lycopene	α-carotene	ß-caroene	Total carotenoids
Mean	2.6	5.1	3.4	6.6	6.5	10.6	6.7	4.0
SD	1.0	2.2	1.2	4.4	3.6	3.3	4.2	2.0
Minimum	1.5	1.4	1.4	1.9	1.9	4.9	0.8	1.4
Maximum	4.4	10.0	5.5	17.0	11.1	15.4	12.6	7.6

Values were obtained from the analysis of 10 pool sera. Each pool was analysed in one day with 5 repeated analyses on each day

Table 4. Recovery values for retinol, ß-carotene and α-tocopherol

	Retinol	ß-carotene	α-tocopherol
First set			
Number of analysis	12.0	12.0	12.0
Mean	94.2	56.0	92.5
SD	13.6	24.8	12.2
Minimum	76.2	20.1	79.3
Maximum	116.5	97.6	122.2
CV	14.4	44.3	13.1
Second set			
Number of analysis	13.0	13.0	13.0
Mean	95.3	82.7	95.7
SD	12.5	21.6	11.6
Minimum	75.4	36.8	78.3
Maximum	119.7	106.6	122.0
CV	13.1	26.1	12.1

The addition of ascorbic acid does not interfere with the HPLC chromatogram since it is not extracted by petroleum ether.

Effect of peroxide on retinol, tocopherol and carotenoids

Ethyl ether, isopropyl ether, dioxane, tetrahydrofuran and many other ethers tend to absorb and react with oxygen from the air to form unstable peroxides which may detonate with extreme violence when they become concentrated by evaporation or distillation, or when disturbed by unusual heat, shock or friction (Steere, 1971). In addition, peroxides in such solvents may bring about oxidation of carotenoids in solution.

The series of nine experiments carried out were sufficient to clearly show that the presence of peroxide in the ethyl acetate used in the preparation of the mobile phase caused considerable destruction to most of the compounds studied. The addition of ascorbic acid during sample preparation was able to inhibit this destruction. Except for retinol and lutein, the mean level of the vitamins were significantly lower without the addition of ascorbic acid (Tables 6a & b). The most drastic effects were seen for tocopherol and most of the carotenoids, where the losses were greater than 50%. Even though ethyl acetate only makes up 2% of the mobile phase, the presence of minute amounts of the peroxide has been shown to be able to cause serious damages. It is thus important to check all ethyl acetate used for the presence of peroxide, even if it is HPLC grade.

Table 5a. The effect of addition of ascorbic acid (AA) to serum sample

	Retinol		α-tocopherol		lutein		β-cryptoxanthin	
	without AA	with AA	without AA	with AA	without AA	with AA	without AA	with AA
Number	19		19		19		19	
Mean (µg/dl)	73.0	71.2	1596	1663	37.2	39.4	37.6	37.4
SD (µg/dl)	24.9	24.1	453	463	10.2	9.4	13.2	12.5
P (two-tail)*	0.108		0.184		0.084		0.904	

*mean values with and without ascorbic acid addition compared using Wilcoxon Matched-Pairs Signed-Ranks Test

Table 5b. The effect of addition of ascorbic acid (AA) to serum sample

	lycopene		α-carotene		β-carotene		Total carotenoids	
	without AA	with AA	without AA	with AA	without AA	with AA	without AA	with AA
Number	19		19		19		19	
Mean (μg/dl)	17.9	17.8	8.4	8.9	29.6	33.7	181.3	189.9
SD (μg/dl)	4.4	5.6	3.6	4.2	16.1	18.0	30.4	44.6
P (two-tail)*	0.809		0.469		0.022		0.064	

*mean values with and without ascorbic acid addition compared using Wilcoxon Matched-Pairs Signed-Ranks Test

Table 6a. The effect of addition of ascorbic acid (AA) on mobile phase containing peroxide

	Retinol		α-tocopherol		lutein		β-cryptoxanthin	
	without AA	with AA	without AA	with AA	without AA	with AA	without AA	with AA
Number	9	9	9	9	9	9	9	9
Mean (μg/dl)	55.8	55.2	624	1340	30.2	31.0	10.7	30.5
SD (μg/dl)	12.7	13.4	355	194	11.0	11.6	7.2	9.7
P (two-tail)*	0.6784		0.0077		0.3743		0.0077	

*mean values with and without ascorbic acid addition compared using Wilcoxon Matched-Pairs Signed-Ranks Test

Table 6b. The effect of ascorbic acid (AA) on mobile phase containing peroxide

	lycopene		α-carotene		β-carotene		Total carotenoids	
	without AA	with AA	without AA	with AA	without AA	with AA	without AA	with AA
Number	9	9	9	9	9	9	9	9
Mean (μg/dl)	2.4	13.2	1.1	5.2	3.6	21.0	64.3	131.1
SD (μg/dl)	2.4	5.7	1.2	2.7	2.7	11.3	27.3	44.1
P (two-tail)*	0.0077		0.0077		0.0077		0.0077	

*mean values with and without ascorbic acid addition compared using Wilcoxon Matched-Pairs Signed-Ranks Test

Serum retinol and carotenoid concentrations of selected subjects

Age and sex distribution of subjects

The mean age of the 65 subjects studied was 52.8 years (Table 7). Sex ratio of male to female subjects was 1.1:1. There were almost equal numbers of Chinese and Malay subjects, each constituting slightly less than 30% of the subjects studied. The remaining 45% were Indian subjects. Because of the manner of obtaining blood samples for study, there was little control over the selection of subjects for inclusion in the study so as to have a ratio of ethnic groups that

is more reflective of the racial composition in the country. Thus differences between ethnic groups observed should be taken as tentative.

Serum retinol level

The mean serum retinol concentration of 65 apparently healthy Malaysians with no reported vitamin deficiencies was 69.8 µg/dl, and the median was 66.3 µg/dl (Table 8). Serum retinol concentrations of most of the subjects were clustered between 40 and 90 µg/dl.

There was no statistically significant difference between the mean retinol concentration of male subjects (73.3 µg/dl) and that of the female subjects (65.9 µg/dl) (P= 0.1378) (Table 8). Mean retinol concentrations of the three ethnic groups were not significantly different (P= 0.7070) (Table 9).

Mean retinol level of the subjects in this study (69.8 ± 18.8 µg/dl) is very similar to that reported in another recent study by this laboratory, using the same method (74.2 ± 23.0 µg/dl) (Tee, Lim & Chong, 1994). These values are however much higher than retinol levels reported several years ago by this laboratory for a series of studies of villages in Peninsular Malaysia where mean serum retinol level of some 500 adults (18-45 years, sexes combined) was about 47 µg/dl (Chong *et al.*, 1984). The higher values can, in part, be explained by the differences in analytical methods; the earlier study had used the Carr-Price method for analysis.

Table 7. Age (years) of study subjects by sex and ethnic groups

		Sex		Ethnic		
	Combined	Male	Female	Chinese	Malays	Indian
Number	65	34	31	17.0	19.0	29.0
Mean	52.8	53.1	52.4	53.0	51.4	53.5
Median	52.0	53.0	52.0	52.0	54.0	52.0
SD	10.7	10.4	11.1	7.8	9.8	12.8
Minimum	24.0	36.0	24.0	40.0	36.0	24.0
Maximum	76.0	76.0	75.0	72.0	66.0	76.0

More importantly, the higher retinol levels could be because the subjects in the two recent series were relatively well-nourished urban executives. Because of the way the serum samples were obtained, it was not possible to determine the dietary intake of the subjects, including the possibility that the subjects could have been on vitamin supplements.

The International Vitamin A Consultative Group (IVACG) (Arroyave *et al.,* 1982) has recommended that 20 µg/dl may be used as a cut-off for acceptable serum levels of vitamin A, without age differentiation. The mean serum retinol concentration of the subjects studied was well above this cut-off level. None of the subjects had a serum level below 20 µg/dl.

Table 8. Serum retinol, tocopherol and carotenoid level (μg/dl) of subjects

	Retinol	α-tocopherol	Carotenoids						
			lutein	β-cryptoxanthin	lycopene	α-carotene	β-carotene	Sum of carotenoids	Total carotenoids
Combined (n=65)									
Mean	69.8	1840	43.1	31.9	18.5	8.5	33.8	135.7	180.2
Median	66.3	1820	41.5	30.4	16.6	7.3	26.3	124.5	165.7
SD	18.8	528	14.2	13.6	12.2	6.3	24.3	49.8	63.0
Minimum	40.4	177	20.1	8.5	3.3	0.0	5.5	43.9	59.5
Maximum[1]	139.0	3290	83.9	70.1	68.8	35.0	132.5	266.4	338.0
Female (n=31)									
Mean	65.9	1900	39.2	32.3	19.6	7.6	36.1	134.8	177.8
Median	64.4	1900	37.0	30.9	16.9	7.3	35.2	126.2	167.4
SD	17.0	467	12.4	12.5	12.4	4.8	25.6	49.2	61.2
Minimum	41.9	1070	20.1	9.2	4.7	0.0	5.5	43.9	59.5
Maximum	117.4	3080	70.3	66.7	62.6	24.4	132.5	255.2	333.1
Male (n=34)									
Mean	73.3	1780	46.6	31.5	17.5	9.2	31.6	136.6	182.3
Median	67.7	1660	45.6	27.3	15.1	7.1	24.5	117.9	160.7
SD	19.8	579	14.9	14.8	12.2	7.4	23.2	51.0	65.4
Minimum	40.4	177	22.7	8.5	3.3	0.0	6.1	76.3	107.8
Maximum	139.0	3290	83.9	70.1	68.8	35.0	108.9	266.4	338.0
P (two-tail)*	0.1378	0.3056	0.0431	0.5457	0.3579	0.6316	0.3119	0.8029	0.8644

*mean values between male and female subjects compared using Mann-Whitney U Test

Table 9. Serum retinol, tocopherol and carotenoid level (μg/dl) of subjects by ethnic groups

	Retinol	α-tocopherol	Carotenoids						
			lutein	β-cryptoxanthin	lycopene	α-carotene	β-carotene	Sum of carotenoids	Total carotenoids
Chinese (n=17)									
Mean	68.6	1830	40.7	38.0	15.9	8.0	38.0	140.7	186.6
Median	71.9	1690	36.6	37.6	16.4	6.9	26.0	133.6	183.6
SD	15.6	527	13.2	15.9	7.3	6.1	33.5	19.6	59.5
Minimum	40.4	1070	22.7	16.8	3.3	0.0	8.4	76.3	111.6
Maximum	93.6	2080	63.6	66.7	27.3	26.1	132.5	255.2	333.1
Malay (n=19)									
Mean	66.7	1660	41.0	29.1	21.5	9.8	39.2	140.6	183.2
Median	61.6	1630	40.9	26.7	18.8	7.7	35.2	137.1	180.2
SD	11.9	527	15.8	10.1	14.1	7.3	25.6	55.2	71.3
Minimum	53.5	177	20.1	10.8	4.7	2.9	5.5	43.9	59.5
Maximum	90.9	2610	83.9	59.9	62.6	35.0	108.9	266.4	338.0
Indian (n=29)									
Mean	72.5	1960	45.8	30.1	18.1	7.9	27.7	129.6	174.5
Median	68.1	1950	46.5	30.9	16.3	7.1	24.6	117.7	161.4
SD	23.7	513	13.6	13.6	13.2	5.7	14.7	47.3	60.9
Minimum	45.6	1120	23.7	8.5	5.8	0.0	6.1	54.7	74.9
Maximum	139.0	3290	70.3	70.1	68.8	24.4	59.4	254.8	332.5
P (two-tail)*	0.7070	0.2543	0.2678	0.1732	0.4705	0.5554	0.3810	0.6441	0.7720

*mean values among ethnic groups compared using Kruskal-Wallis 1-Way Anova

The U.S. Interdepartmental Committee on Nutrition for National Defense (ICNND, 1963) had recommended 20-49 μg/dl as an "acceptable" serum vitamin A level, and a level of >50 μg/dl as "high". Using these values, the serum vitamin A levels reported in these two recent studies, and even those of the rural villagers reported earlier, would be considered as "high". It would appear that these levels suggested by ICNND may need to be revised upwards. Using the mean and two standard deviation of the values obtained in these two recent studies, it may be suggested that the "acceptable" range for serum retinol be from 30-110 μg/dl. These values may be regarded as tentative, until data from larger population groups can become available. Using this cutoff, none of the subjects in this study and the study last year (Tee, Lim & Chong, 1994) were found to have a low retinol level (below 30 μg/dl). Three subjects (4.5%) in this study and 7 (7.0%) in the earlier study were found to have a "high" retinol level (>110 μg/dl). For comparison, it can be noted that the Centres for Disease Control (CDC), Atlanta has used a reference range of 25-115 μg/dl (CDC, 1993)

Serum carotenoid concentrations

Tables 8 and 9 tabulate the mean concentrations of the five major carotenoids according to sex and ethnic groups of the subjects respectively, namely lutein, cryptoxanthin, lycopene, α- and β-carotene. All the five carotenoids were detected in all the serum samples, except α-carotene which was not detected in 2 subjects. Together, the five carotenoids make up about 75% of the total carotenoid concentration. Concentrations of most of the carotenoids showed large variations.

The mean β-carotene concentration of the subjects studied was 33.8 ± 24.3 μg/dl (Table 8), with most of the individual values clustered around 10-50 μg/dl. Similar values were reported in the earlier study of this laboratory (29.2 ± 21.3 μg/dl) (Tee, Lim & Chong, 1994).

Except for lutein, mean concentrations of all the five carotenoids listed in Table 8 were not significantly different for the female and male subjects. Mean lutein concentration of male subjects was found to be significantly higher than that of the female subjects (P=0.0431). No statistically significant differences in the mean concentrations of all carotenoids listed in Table 9 were observed for the serum samples of Chinese, Indian and Malay subjects.

The most abundant carotenoid in the serum samples studied was lutein, comprising about one-third of all carotenoids quantitated (Table 10). Cryptoxanthin and β-carotene were the next abundant carotenoids, each contributing about 25% of all carotenoids. Lycopene and α-carotene together made up the remaining 20%. Considerable variations in these proportions were observed for the individual subjects.

The mean total carotenoid concentration of the subjects studied was 180.2 ± 63.0 μg/dl (Table 8), very similar to that obtained in our previous study (196.0 ± 83.2) (Tee, Lim and Chong, 1994). There was no significant difference between female and male subjects (P=0.8644). Total carotenoid concentrations for the three ethnic groups were not significantly different (P=0.7720) (Table 9).

Blood concentrations of the carotenoids reflect the level of dietary intake of these pigments. As

has been pointed out earlier (Tee, Lim & Chong, 1994), the guidelines for interpretation of serum carotene levels suggested by ICNND (1963) are not applicable to the β-carotene concentrations reported here. The values given in the guidelines should be more correctly termed as total carotenoids, as the colorometric method used was not able to quantitate the individual carotenoids. None of the subjects was found to have a total carotenoid concentration of less than 40 μg/dl, the level considered to be "low" in the ICNND guidelines.

As has been done for serum retinol values, a similar revision of the cut-off levels for total carotenoids is proposed, taking into consideration the mean values obtained in this study and the earlier study of Tee, Lim and Chong (1994). It is suggested that the "acceptable" range for serum total carotenoids be from 50-330 μg/dl. A similar range for serum β-carotene is also proposed to be from 5-70 μg/dl. These values should be regarded as tentative, until data from larger population groups or more clinical data can become available. Using this cutoff, only one subject (0.6%) in the combined data from this study and the study last year (Tee, Lim & Chong, 1994) was found to have a low total carotenoid level (below 50 μg/dl). Twelve subjects (7.3%) in the two combined studies were found to have a "high" total carotenoid level (>330 μg/dl). One subject (0.6%) was found to have a low β-carotene level (<5 mg/dl) and 10 subjects (10%) had a high β-carotene level (>70 μg/dl). CDC (1993) has adopted a reference range of 2-80 μg/dl for β-carotene, while a range for total carotenoids has not been proposed.

Table 10. Percentage of individual carotenoids

	Proportion (%) of individual carotenoids				
	Lutein	Cryptoxanthin	Lycopene	α-carotene	β-carotene
Mean	33.7	23.7	13.4	6.0	23.2
Median	31.3	23.4	12.7	5.5	21.4
SD	10.7	6.3	6.2	3.2	9.7
Minimum	16.0	9.3	3.2	0.0	7.1
Maximum	65.4	45.8	33.2	16.2	57.8

Serum tocopherol concentrations

As has been mentioned above, the concentrations of δ- and γ-tocopherols in the serum samples studied were too low to be identified with certainty and quantitated accurately. The mean α-tocopherol level of the 65 subjects studied was 1840 ± 528 μg/dl, with a median of 1830 μg/dl. Based on the results obtained from this group of subjects, a tentative reference range of 800-2900 μg/dl is suggested. Only 1 subject (1.5%) in the group studied had a α-tocopherol level less than 800 μg/dl and two subjects (3.1%) had a level greater than 2900 μg/dl. The range used by CDC (1993) is 500-2650 μg/dl.

There was no statistically significant difference between mean α-tocopherol concentration of male subjects (1780 μg/dl) and that of the female subjects (1990 μg/dl) (P= 0.3056) (Table 8). Mean α-tocopherol concentrations of the three ethnic groups were also not significantly different (P = 0.2543) (Table 9).

CONCLUSIONS

The HPLC method developed in this laboratory for simultaneous determination of retinol and several major carotenoids has been used successfully for the analysis of fruits and vegetables, legumes and tubers, foods of animal origin and sera. Results obtained in this study have shown that with the addition of another detection wavelength, the method is also suitable for the detection and quantiation of the three major tocopherols. Simultaneous determination of three important groups of antioxidant vitamins become possible in this method. It is the intention that a single method that can be applicable to different matrices would obviate the need to continuously change instrument settings and conditions.

Several experiments carried out have provided further refinement to the method. These include precautions to be taken during preparation of the extract for chromatography, the importance of using pure solvents such as petroleum ether and ethyl acetate, and the use of ascorbic acid to inhibit destruction to the vitamins analysed. Further improvements to the method are being undertaken by this laboratory.

Data on the serum concentration of the antioxidant vitamins of the local population groups are still lacking. In view of the increasing interest in the protective role of these vitamins in certain chronic diseases such as cancer and coronary heart disease, the present HPLC procedure should facilitate more refined epidemiological and experimental studies into such a relationship. This laboratory is employing the developed method in several studies in this area.

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