Asia Pacific J Clin Nutr (2002) 11(2): 92–97

Original Article

Simultaneous analysis of retinol, β-carotene and tocopherol levels in serum of Vietnamese populations with different incomes

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In this study, we clarified the status of the fat-soluble vitamins retinol and tocopherol, as well as β-carotene, as antioxidants in the prevention of cardiovascular disease in middle-aged Vietnamese populations with different incomes. In order to measure simultaneously the serum concentrations of retinol, β-carotene and tocopherol, we carried out high-performance liquid chromatography analysis with three separate detectors. The analytical method was modified, omitting the saponification process, and used a multi-evaporating system with dry ice. This allowed the analysis to proceed more rapidly, use a small amount of serum (40 μL) and be free of hexane contamination to the environment. The analyses reflected an adequate status of vitamin A (serum retinol = 20 μg/dL), but inadequate status of β-carotene and vitamin E (serum β-carotene < 40 μg/dL; serum tocopherol < 600 μg/dL) in all three Vietnamese populations. As large numbers of Vietnamese subjects were observed with very low serum concentrations of β-carotene and tocopherol, higher consumptions of green and yellow vegetables, fruits, vegetable oils and other foods rich in vitamin E are recommended for these Vietnamese populations.

Key words: β-carotene, Can Gio district, fat-soluble vitamin, Ho Chi Minh City, retinol, tocopherol, Vietnam.

Introduction

Dietary patterns in Vietnam, which are characterised by a low fat intake,[1] are associated with a low consumption of fat-soluble vitamins. On the practical side, vitamin A deficiency and its clinical manifestations, including xerophthalmia and blindness, have been identified as a significant public health problem in Vietnamese children.[2] However, past studies in Vietnam have focused only on the evaluation of vitamin A status in children and pregnant subjects. In addition, another nutritional problem in Vietnam is the recent trend towards increasing fat intake[1] and cardiovascular disease (CVD) morbidity in urban areas.[3] Therefore, our purpose in this study was to investigate the status of two kinds of fat-soluble vitamins, retinol and tocopherol, as well as β-carotene, as antioxidants in the prevention of CVD in middle-aged populations with different incomes. The results of this study will provide the basis for dietary vitamin recommendations for Vietnamese populations. Serum concentrations of retinol, β-carotene and tocopherol were measured simultaneously by high-performance liquid chromatography (HPLC) using a simple modified procedure devised in our laboratory. This analytical method allowed us to use a small amount of serum, a minimum of time and was not associated with the unhealthy environmental influences of hexane.

Materials and methods

Subjects

A total of 296 healthy people (111 men and 185 women) from three districts in South Vietnam with different incomes were selected randomly for this study. The high-income district (Ben Thanh, $n = 100$) is located in the centre of Ho Chi Minh City in which the majority of people are engaged in business. The medium-income district (Nha Be, $n = 98$) is a suburb of Ho Chi Minh City inhabited mainly by labourers. The low-income district (Can Gio, $n = 98$) is a seaside community 60 km east of Ho Chi Minh City, where most people are farmers or fishermen. In order to investigate the fat-soluble antioxidant vitamin status of these populations, the study was limited to people in the age range associated with a high risk of CVD (40–59 years). The average age was 47 years for both men and women in these populations (Table 1).

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Accepted 10 October 2001

Anthropometric measurement

The subjects had their height measured while in bare feet and weight measured wearing light clothing. Body mass index (BMI) was calculated as the ratio of weight (kg) to height squared (m^2). As shown in Table 1, the average BMI indices were in the range of 19.5–22.5, showing normal anthropometry values for these populations.

Dietary intake

Dietary intake was recalled in the previous 24 h and recorded for three consecutive days by our staff members. Twenty-four-hour dietary intake was calculated based on the Table of Food Composition of Vietnam.[4]

Biochemical analysis

Blood samples were taken in the morning after an overnight fast, and were then centrifuged. The serum was stored at –80°C until use. This study was approved by the Medical Ethics Committee of the Nutrition Center of Ho Minh City.

Materials

All-*trans* retinol was purchased from Sigma (Germany). β-Carotene and tocopherol (vitamin E homologues) were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents were of analytical grade.

Standard preparation

For quantification analysis, external standards of all-*trans* retinol, β-carotene and tocopherol were used. Solutions of these standards were prepared in ethanol and hexane, and butylated hydroxy toluene (BHT) was added (0.001%) to maintain the stability of the standards. The concentration for each working standard was 0.1 μg/mL. The standard solution was a mixture of all-*trans* retinol, β-carotene and tocopherol homologues. It was stored at 0–4°C in the dark and the stability was checked frequently by HPLC.

Analysis of retinol, β-carotene and tocopherol in serum

Figure 1 shows the analytical steps performed in our laboratory. For the analysis of each sample, 40 μL of serum were put in test tubes in duplicate. The solution was mixed with a vortex for 10 s. Extraction with hexane was carried out using a 20 mL glass funnel. In this protocol, a saponification process was not performed prior to the extraction. At the evaporation process, approximately 10 mL of the hexane phase obtained after two extractions was put in a 10 mL flask, followed by evaporation under a stream of nitrogen in a 40°C water bath. We used a simple, home-made hexane-evaporating system consisting of channels connected to a series of T-type joints, allowing the simultaneous evaporation

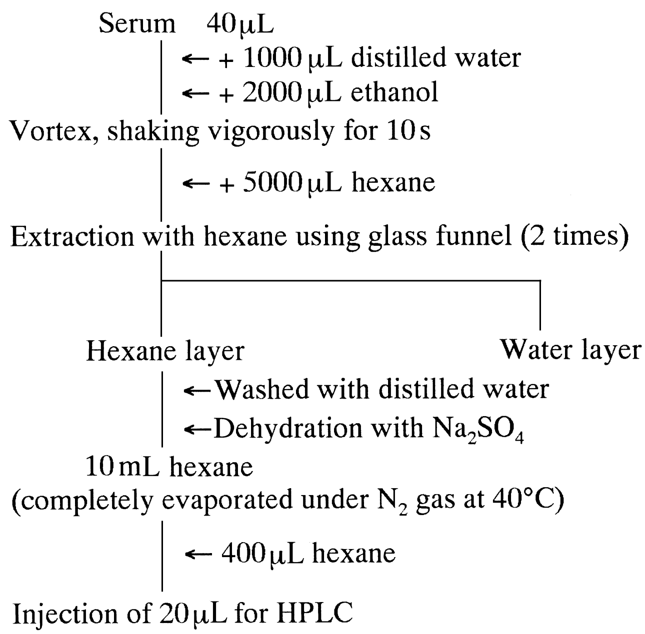

Figure 1. Simultaneous analysis of retinol, β-carotene and tocopherol in serum using high-performance liquid chromatography.

Table 1. Characteristics of the subjects in the three areas and their dietary intakes[9] compared with Vietnamese RDA[8]

Variables	Vietnamese RDA	Men Urban (n = 32)	Men Suburban (n = 40)	Men Rural (n = 39)	Women Urban (n = 68)	Women Suburban (n = 58)	Women Rural (n = 59)
Age		47 ± 5	46 ± 5	46 ± 5	47 ± 5	47 ± 6	47 ± 6
BMI		22.5 ± 2.6	21.2 ± 3.3	19.5 ± 2.3	22.4 ± 3.5	21.4 ± 3.7	19.9 ± 2.9
Protein†	12–14	15.8	15.0	15.0	16.3	14.5	14.3
Fat†	18–20	20.5	15.3	10.0	20.2	18.0	8.3
Carbohydrate†	66–70	63.7	69.7	75.0	63.5	67.5	77.4
Animal sources‡							
Meat	50	166	118	58	139	92	32
Fish	70	80	129	151	83	77	90
Egg	20	19	12	4	12	3	3
Milk	20	40	63	43	23	24	15
Vegetable sources‡							
Vegetables	300	323	289	171	291	208	156
Fruit	80	81	80	46	139	93	68

†Percentage of nutrient related to total energy; ‡Twenty-four-hour dietary intake (g/capita/day). BMI, body mass index; RDA, recommended dietary allowance.

of five samples. The recovery of evaporated hexane was carried out with dry ice, as shown in Fig. 2. With this equipment, most of the evaporated hexane was collected on dry ice.

HPLC analytical conditions

An HPLC LC-10AD (Shimadzu, Kyoto, Japan), was equipped with three detectors (two UV-VIS and one fluorescence). The conditions were as follows: column, NH2-1251-N 4.6∅ × 250 mm; mobile phase, hexane : 2-propanol (97:3); flow rate, 1 mL/min; and detection, UV 325 nm for retinol (Shimadzu SPD-10 A detector), VIS 450 nm for β-carotene (Shimadzu SPD-6AV detector), fluorescence 297 nm excitation and 327 nm emission for tocopherol (Shimadzu RF-10 A detector). The three detectors were used simultaneously with the eluent moving consecutively from the HPLC column to the VIS, fluorescence and UV detectors.

Results

In Table 1, the 24-h dietary intakes of the three populations are shown.

HPLC analysis of retinol, β-carotene and tocopherol

Chromatograms obtained from analysis of the standard solution and serum are shown in Fig. 3. The three detectors (VIS, fluorescence and UV) were necessary to give separation of the peaks. The retention time was 4 min for β-carotene, 5 min for tocopherol and 10 min for retinol. In normal human serum, α-tocopherol is the major vitamin E form. However, γ- and δ-tocopherols are normally present in small amounts. β-Tocopherol is only present in trace quantities and is not normally detectable.

Measurements of retinol, β-carotene and tocopherol in serum

Serum concentrations of retinol, β-carotene, α- and γ-tocopherols are shown in Table 2. Results are presented for men only, women only and the total group (both men and women).

As shown in Table 2, none of the subjects had serum retinol values below 20 µg/dL, which indicates inadequate retinol status.[5] However, the high- and medium-income populations had significantly higher serum retinol concentrations than the low-income population, and this was the same for both sexes. Furthermore, in all three districts, men had higher serum retinol concentrations than women.

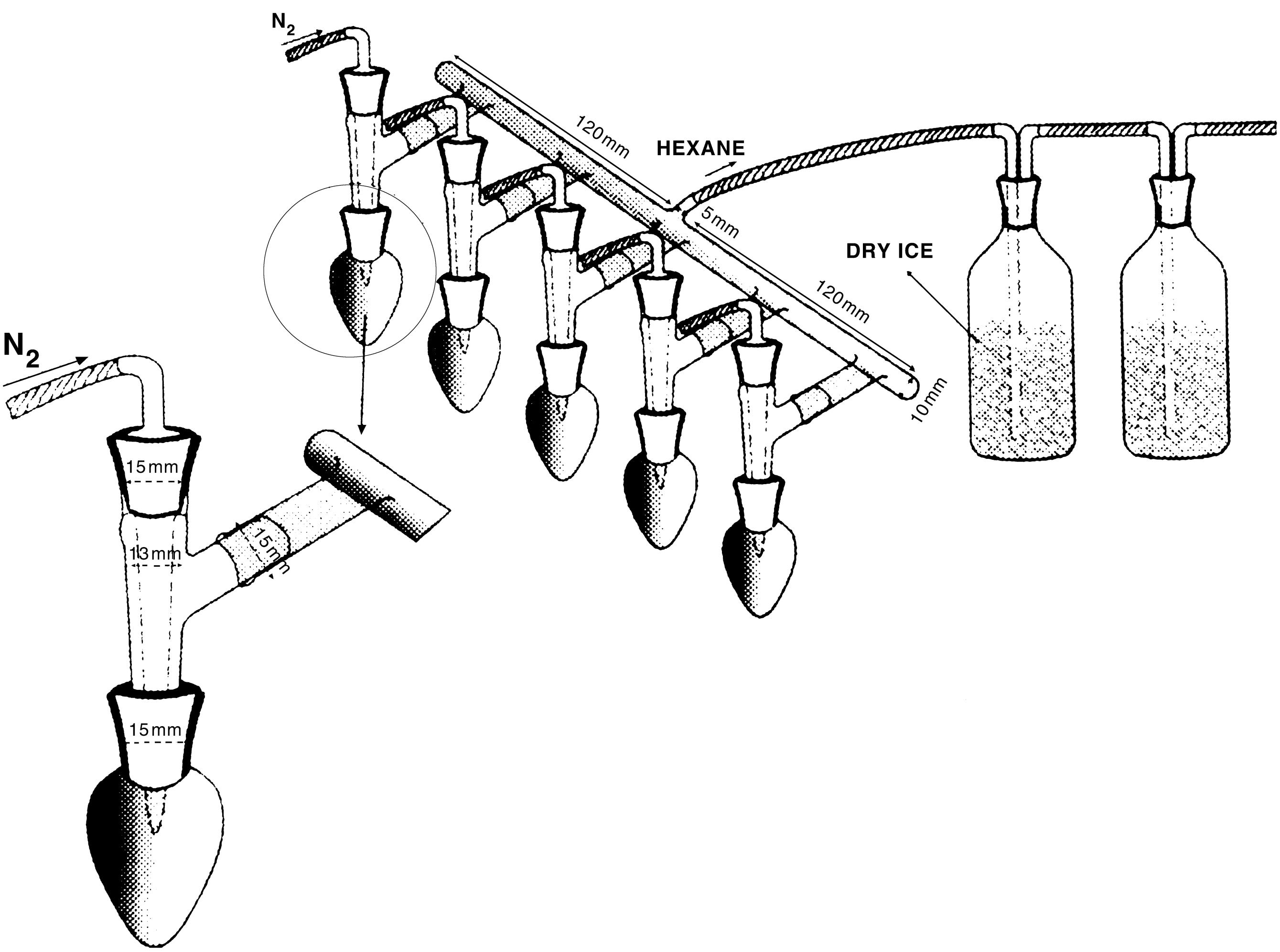

Figure 2. Hexane-evaporating system and recovery of evaporated hexane with dry ice.

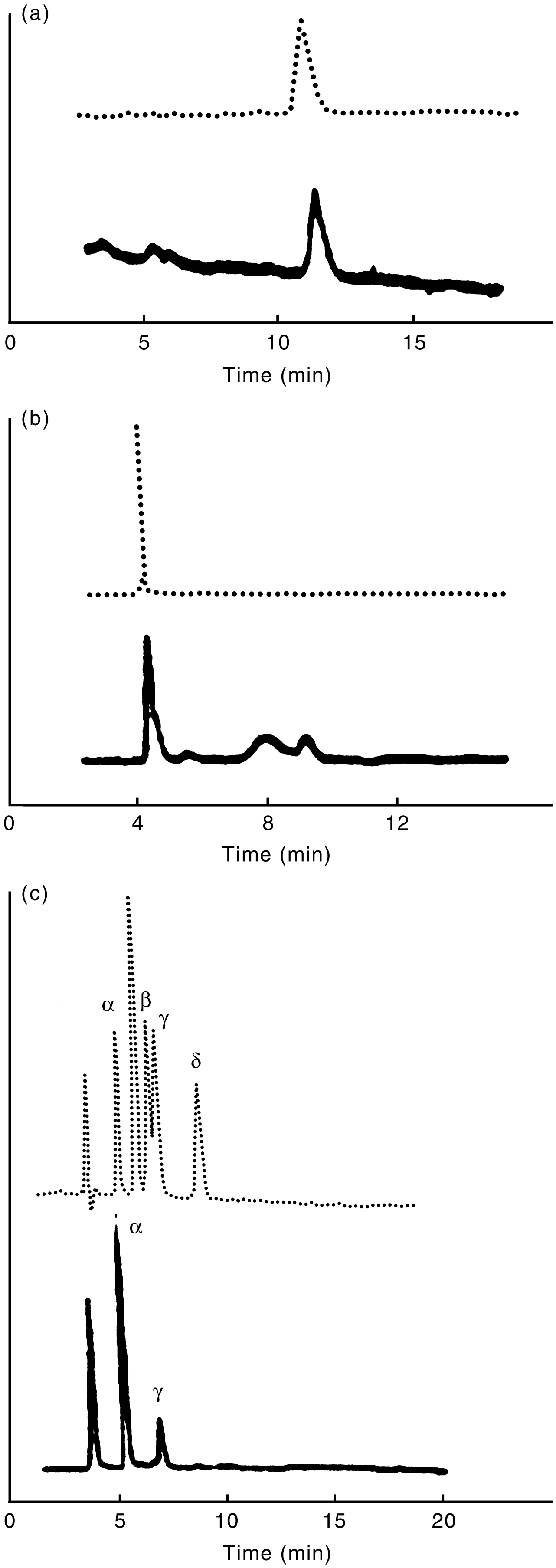

Figure 3. High-performance liquid chromatography chromatograms of (a) retinol standard (···) and serum (—) detected at 325 nm, (b) β-carotene standard (···) and serum (—) detected at 450 nm, (c) tocopherol standard (···) and serum (—) detected by fluorescence at 297 nm excitation and 327 nm emission.

Table 2 also shows that women in the high-income population had significantly higher serum β-carotene and α-tocopherol concentrations than those in the other two populations. However, in terms of β-carotene, 90–100% of these Vietnamese subjects had serum β-carotene values indicative of inadequate β-carotene status (< 20 μg/dL) or marginal β-carotene status (20–39 μg/dL).[5] In all three districts, more men than women were found to be in the deficient range (< 20 μg/dL). As for α-tocopherol values, 10–15% of subjects were deficient (< 200 μg/dL) and in 50–70% were in the marginal range (200–600 μg/dL), in all three populations.[5] There were significant differences among the three districts in γ-tocopherol values, with the highest values found in the high-income group, followed by the medium-income population and finally, the low-income population.

Discussion

Analytical method

Although there are reports describing the use of a saponification process before the extraction of retinol or tocopherol from serum,[6–8] this process is very troublesome and time consuming. In our study, we confirmed that with a small amount of serum (40 μL), the saponification process is not necessary and that yields of retinol, β-carotene and tocopherol are better than those obtained using the saponification process (data not shown). In this analysis, the same values were obtained when two different serum volumes, i.e., 40 μL and 80 μL, were analysed in duplicate. This means that a 40 μL quantity of serum is sufficient to obtain a satisfactory result. In addition, extraction without the saponification process and evaporation with a multi-evaporating system make for a more rapid analysis procedure. Therefore, application of this analytical method should allow one sample to be analysed in 50 min and five samples in 2 h. In order to eliminate errors associated with the use of an external standard, BHT was added to the standard solution to prevent oxidation of the standards. Because BHT is detected at 280 nm, it does not interfere with the standards or the samples. The stability of the working standard was checked frequently just before serum analysis under the same HPLC conditions. The use of dry ice allowed recovery of the evaporated hexane while preventing environmental pollution without using an expensive ventilation device (Fig. 2).

Vitamin status in the three populations

Although the low-income population had significantly lower serum vitamin A levels than the high- and medium-income populations, the vitamin A status of healthy 40–59-year-old subjects in all three populations was apparently adequate because the prevalence of acceptable retinol concentrations

Table 2. Mean serum concentrations of retinol, β-carotene and tocopherol

Variables	Total			Men			Women		
	High income (n = 100)	Medium income (n = 98)	Low income (n = 98)	High income (n = 32)	Medium income (n = 40)	Low income (n = 39)	High income (n = 68)	Medium income (n = 58)	Low income (n = 59)
Retinol† (µg/dL)	73 ± 25[a]	71 ± 28[a]	59 ± 23[b]	81 ± 26[d]	83 ± 30[d]	69 ± 25[e]	69 ± 24[f]	62 ± 22[f]	52 ± 18[g]
% Acceptable (≥ 20 µg/dL)	100	100	100	100	100	100	100	100	100
β-Carotene† (µg/dL)	19 ± 13[a]	12 ± 8[b]	13 ± 10[b]	14 ± 8	10 ± 7	10 ± 8	21 ± 14[f]	15 ± 8[g]	14 ± 11[g]
% Deficient (< 20 µg/dL)	60	81	79	71	92	98	54	73	73
% Marginal (20–39 µg/dL)	32	19	19	25	8	2	35	27	24
% Acceptable (≥ 40 µg/dL)	8	0	2	4	0	0	11	0	3
α-Tocopherol† (µg/dL)	680 ± 500[a]	540 ± 380[b]	460 ± 300[b]	670 ± 540	560 ± 320	520 ± 390	690 ± 500[a]	540 ± 410[b]	420 ± 210[b]
% Deficient (< 200 µg/dL)	10	10	16	15	11	19	7	11	15
% Marginal (200–600 µg/dL)	46	54	64	42	51	50	47	55	73
% Acceptable (≥ 600 µg/dL)	44	36	20	43	38	31	46	34	12
γ-Tocopherol† (µg/dL)	48 ± 33[a]	32 ± 20[b]	19 ± 14[c]	42 ± 34[d]	30 ± 22[d]	16 ± 13[e]	51 ± 32[f]	33 ± 19[g]	21 ± 14[h]

†Mean ± SD. Differences between [a] and [b], [b] and [c], [a] and [c]; [d] and [e]; [f] and [g]; [g] and [h]; [f] and [h]: statistically significant at $P < 0.05$.

was 100%, based on results of this study. However, the specific concern in these three populations is the very low β-carotene values in serum (< 40 µg/dL), apparent evidence that in 90–100% of Vietnamese in this study (Table 2), the intake of green and yellow vegetables and fruits rich in β-carotene is inadequate. However, based on the Vietnamese recommended dietary allowance (RDA)[9] in Table 1, the evaluation data from the dietary intake survey[10] conducted in these populations reflected an essentially adequate intake of vegetables and fruits in the high- and medium-income populations, while intake was not adequate in the low-income population. In addition, the fat-energy percentages of these populations were: 20% of the total energy consumed by the high-income population, 15–18% of the total energy consumed by the medium-income population and 8–10% of the total energy consumed by the low-income population (Table 1). It may be estimated that low-fat intake limits the absorption of β-carotene despite the intake of fruits and vegetables being sufficient in the high- and medium-income populations. The major concern is for the low-income district because this population had an inadequate intake of vegetables and fruits, less than half the Vietnamese RDA (Table 1), associated with a very low intake of fat (8–10% of total energy).

There have been reports on serum β-carotene levels in various countries.[11–13] In a rural population of East Pakistan, the distribution of serum β-carotene levels showed that 22.7% of men and 33.3% of women had serum β-carotene levels below 40 µg/dL,[11] while in this study, almost all of the men and women in all three Vietnamese populations had values lower than 40 µg/dL. In French populations of 40–50 and 50–65 years of age, serum β-carotene levels averaged 50 µg/dL,[12] 2.5–3 times higher than those in middle-aged Vietnamese populations. In US adults, reported serum β-carotene levels were 133 µg/dL for men and 158 µg/dL for women,[13] approximately 10 times higher than those of the Vietnamese populations. The normal physiological serum β-carotene concentration is usually 50–300 µg/dL.[14]

Higher serum retinol and lower β-carotene concentrations in men than in women were observed in all three Vietnamese populations studied. This may represent a fundamental difference in the food consumption patterns of men and women. Women consumed a higher amount of vitamin A from fruits or carotene sources (Table 1), whereas men consumed a higher amount of vitamin A from animal products including meat, fish, egg and milk (Table 1). Consequently, these fundamental differences in vitamin A intake between men and women should be re-examined and suitable recommendations aimed at health promotion for both sexes are needed.

Another interesting point in these Vietnamese income-based populations is the significant number of individuals with serum tocopherol levels below 600 µg/dL, despite the mean serum tocopherol values of these populations approaching normal physiological levels (500–1000 µg/dL). The mean serum tocopherol concentrations in the Vietnamese populations were also found to be relatively close to those of adults in Pakistan (i.e., 760 μg/dL for men and 730 μg/dL for women).[11] However, in that study, 34% of the Pakistani population had serum tocopherol values below 600 μg/dL, while in this study, 80% of low-income, 65% of medium-income and 55% of high-income Vietnamese subjects had low values. In the US adult population, men and women had mean serum tocopherol levels of 1060 μg/dL and 1030 μg/dL, respectively,[13] reaching the upper limit of the normal level. Thus, because large numbers of Vietnamese subjects had very low serum concentrations of β-carotene and tocopherol, it is important to promote habitual intake of foods rich in β-carotene and tocopherol, as well as to increase fat consumption in middle-aged Vietnamese populations. Consuming adequate quantities of green and yellow vegetables and fruits is necessary, especially for the low-income population. Vegetable oils are also good sources of vitamin E for these populations. Analysis of the vegetable oils consumed in Vietnam showed soybean, peanut and cooking oil (a mixture of soybean, peanut, palm and coconut oils) to contain considerable amounts of vitamin E.[15] Moreover, in addition to a higher intake of the vitamin E contained in vegetable oils, increasing the consumption of these oils would also facilitate the absorption of fat-soluble vitamins in Vietnamese subjects. Besides, other food sources rich in vitamin E, such as nuts and eggs, should also be recommended for low- and medium-income populations in this study. An appropriate dietary vitamin E requirement has been proposed, represented by a vitamin E : polyunsaturated fatty acid ratio in the range of 0.4–0.5.[16] Therefore, further studies are required to establish adequate, as well as optimal, recommendations for vitamin E intake in the Vietnamese populations studied herein.

Conclusion

In this report we have presented a modified method allowing the simultaneous analysis of serum retinol, β-carotene and tocopherol concentrations. The advantages of this method include its rapidity, the simplified technique applicable to a very small amount of serum and environmental conditions free of hexane contamination. Employing the described method, we clarified the antioxidant fat-soluble vitamin status of middle-aged Vietnamese subjects, both men and women, in three districts with different incomes. In all three populations, adequate intake of vitamin A but inadequate intakes of β-carotene and vitamin E were observed. Therefore, higher dietary consumption of green and yellow vegetables, fruits, vegetable oils and other foods rich in vitamin E are clearly needed for these Vietnamese populations. In order to establish appropriate and adequate recommendations for fat-soluble vitamin intake in Vietnamese populations, further and more detailed studies are necessary.

Acknowledgement. This study was partly supported by the Sasakawa Scientific Research Grant from the Japan Science Society.

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