Journal of Health Science, **51**(6) 738–743 (2005)

Relationship between Serum β-Cryptoxanthin and Circulating Bone Metabolic Markers in Healthy Individuals with the Intake of Juice (*Citrus unshiu*) Containing β-Cryptoxanthin

Masayoshi Yamaguchi,*,[a] Aki Igarashi,[a] Seiichi Morita,[b] Takashi Sumida,[b] and Kuniaki Sugawara[b]

[a]*Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan and* [b]*Research & Development, Ehime Beverage, Inc., 478 Anjyoji-machi, Matsuyama, Ehime 791–8603, Japan*

(Received September 1, 2005; Accepted September 12, 2005)

The relationship between the serum β-cryptoxanthin concentration and circulating biochemical markers of bone metabolism in healthy individuals with the intake of juice prepared from Satsuma mandarin (*Citrus unshiu* MARC.) containing β-cryptoxanthin was investigated. Twenty volunteers (10 men and ten women) were divided into two groups of 10 volunteers (5 men and 5 women) each, and each group was sequentially given juice (192 ml) containing two different amounts of β-cryptoxanthin once a day for 56 days as follows: either regular juice with naturally occurring β-cryptoxanthin 802 μg/100 ml or reinforced juice containing β-cryptoxanthin 1500 μg/100 ml. γ-Carboxylated osteocalcin, which is a marker of bone formation, and bone tartrate-resistant acid phosphatase (TRAP), which is a marker of bone resorption, were assayed. The serum β-cryptoxanthin concentration was significantly increased with the intake of regular juice for 56 days. This increase was significantly enhanced by the intake of β-cryptoxanthin-reinforced juice. The intake of regular juice or of β-cryptoxanthin-reinforced juice for 56 days caused a significant increase in serum γ-carboxylated osteocalcin and a significant decrease in serum bone TRAP activity. A positive relationship between serum β-cryptoxanthin and circulating γ-carboxylated osteocalcin concentrations was found using the value obtained from all groups for before intake and with the intake of regular juice and β-cryptoxanthin-reinforced juice. A negative relationship between serum β-cryptoxanthin concentration and circulating TRAP activity was observed. This study shows that a relationship between serum β-cryptoxanthin and circulating bone metabolic markers is found in healthy individuals with the intake of juice containing β-cryptoxanthin.

Key words —— β-cryptoxanthin, γ-carboxylated osteocalcin, bone tartrate-resistant acid phosphatase, bone metabolic marker, osteoporosis

*To whom correspondence should be addressed: Laboratory of Endocrinology and Molecular Metabolism, Graduate School of Nutritional Sciences, University of Shizuoka, 52–1 Yada, Suruga-ku, Shizuoka 422–8526, Japan. Tel. & Fax: +81-54-264-5580; E-mail: yamaguch@u-shizuoka-ken.ac.jp

INTRODUCTON

Aging induces a decrease in bone mass, and osteoporosis with its accompanying decrease in bone mass is widely recognized as a major public health problem.[1–3] A decrease in bone mass leads to bone fracture. Bone loss with increasing age may be due to decreased bone formation and increased bone resorption. Pharmacologic and nutritional factors may prevent bone loss with aging.[4,5] Chemical compounds in food that act on bone metabolism, however, are poorly understood.

Micronutrients and phytochemicals are found in vegetables and fruit. Recent studies have shown that isoflavones (including genistein and daidzein), which are contained in soybeans,[5–8] and menaquinone-7, an analogue of vitamin K_2 which is abundant in fermented soybeans,[9–11] have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption *in vitro*, thereby increasing bone mass.[12,13]

Carotenoids are also present in fruit and vegetables and have been shown to play a possible biological role in cancer prevention.[14] The preventive effect on osteoporosis, however, has not been fully clarified. Vitamin A is known to have a detrimental effect on bone at high doses. High levels of vitamin A lead to accelerated bone resorption, bone fractures, and osteoporotic bone lesions in animals.[15–17]

β-Cryptoxanthin is a carotenoid abundant in Satsuma mandarin orange (*Citrus unshiu* MARC.) and it is enzymatically converted from β-carotene (provitamin A) in plants. Of the various carotenoids (including β-cryptoxanthin, lutein, lycopene, and β-

carotene) and rutin (quercetin-3-rutinoside), β-cryptoxanthin has been found to have a unique anabolic effect on bone calcification *in vitro*.[18] β-Cryptoxanthin has stimulatory effects on bone formation and inhibitory effects on bone resorption in rat femoral tissue culture *in vitro*.[19] β-Cryptoxanthin can stimulate cell proliferation and mineralization in osteoblastic cells *in vitro*[20,21] and it can inhibit osteoclast-like cell formation induced by various bone-resorbing factors in mouse marrow cultures *in vitro*.[22] Thus β-cryptoxanthin has been demonstrated to have stimulatory effects on osteoblastic bone formation and inhibitory effects on osteoclastic bone resorption *in vitro*.

Oral administration of β-cryptoxanthin has been shown to have anabolic effect on bone components in young and aged rats *in vivo*.[23,24] Oral administration of β-cryptoxanthin has preventive effect on bone loss in streptozotocin-diabetic rats.[25] Moreover, the intake of β-cryptoxanthin -reinforced juice has been shown to have stimulatory effects on bone formation and inhibitory effects on bone resorption in healthy individuals as estimated based on serum biochemical markers of bone metabolism *in vivo*.[26] The intake of dietary β-cryptoxanthin may have a preventive effect on osteoporosis.

This study was undertaken to determine whether there is a relationship between serum β-cryptoxanthin concentration and circulating bone metabolic markers in healthy individuals following the intake of juice containing β-cryptoxanthin.

MATERIALS AND METHODS

Materials —— Juice prepared from Satsuma mandarin oranges (*Citrus unshiu* MARC.) was supplied by Ehime Beverage, Inc. (Matsuyama, Japan), in which β-cryptoxanthin is present in a volume of 802 μg/100 ml of juice. Reinforced juice with increased β-cryptoxanthin content was prepared with a β-cryptoxanthin supplement isolated from Satsuma mandarin. The content of β-cryptoxanthin in the reinforced juice was 1500 μg/100 ml of juice.

Experimental Procedures —— Twenty adults, aged 23–47 years (10 men and 10 women), who were judged to be healthy with no abnormal liver and kidney function as assessed by standard biochemical data, were enrolled as volunteers in this study. Informed consent was obtained from all before enrollment. The intake of other foods with an abundance of β-cryptoxanthin was prohibited during the period of the experiment. The period for washout or intake of each type of juice was 7 and 56 days, respectively. The 20 volunteers were divided into two groups of 10 volunteers (5 men and 5 women) each. Each group was sequentially given, 192 ml of juice containing β-cryptoxanthin 1540 or 2880 μg/192 ml of juice once daily for 56 days. The intake of juice occurred between 10:00 and 12:00 (noon). Blood samples were collected from each at 15:00 and 17:00 on the day prior to intake (control), and at 28 and 56 days after the start of intake. Serum samples were obtained by centrifugation (2500 rpm for 5 min) between 30 and 60 min after blood sampling and then stored at –20°C until assayed.

Analytical Procedures —— Serum β-cryptoxanthin concentrations were measured using the procedure of Peng *et al*.[27] To each tube containing 0.25 ml of serum, 250 μl of ethanol solution containing 1% sodium dodecylsulfate and 0.1% butylhydroxytoluene (BHT) and *n*-hexane containing 0.1% BHT were added. After the samples were mixed, they were incubated at 37°C for 5 min and then centrifuged for 5 min at 2500 rpm. Samples of the hexane-extracted phase were dried with nitrogen gas, and then mobile phases for the high pressure liquid chromatography (HPLC) system (Amersham Pharmacia Biotechnology, U.S.A.) were added. To separate β-cryptoxanthin, a mobile phase at a flow rate of 1.3 ml/min was used. Mobile phases consisted of acetonitrile, methanol, and 0.1% triethylamine-containing tetrahydrofuran (20 : 75 : 5). Elution was monitored at 451 nm.

The serum γ-carboxylated osteocalcin concentration was assayed using Gla-type Osteocalcin (Gla-OC) EIA kit (Takara Shuzou, Shiga, Japan).[28] Serum bone tartrate-resistant acid phosphatase (TRAP) activity was assayed using the Bone TRAP Assay EIA kit (SBA Sciences, Turku, Finland).[29]

Serum albumin, γ-GTP, nitrogen urea, amylase, glucose, high-density lipoprotein (HDL) cholesterol, triglyceride, and zinc levels were determined using kits (Wako Pure Chemicals, Osaka, Japan).

Statistical Analysis —— Differences in values before and after the intake of each type of juice was estimated using Student's *t*-test. A paired *t*-test was used for differences in values before and after the intake of each juice or between the two groups after each intake period. *p*-Values less than 0.05 were considered to represent statistically significant differences.

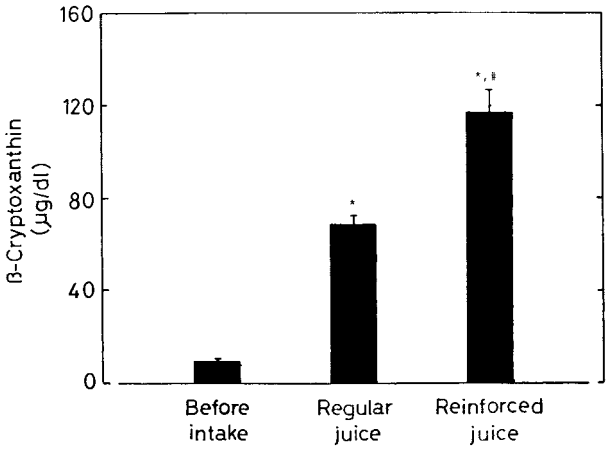

Fig. 1. Changes in Serum β-Cryptoxanthin Concentrations Following Intake of Juice Containing β-Cryptoxanthin in Healthy Individuals

Juice from Satsuma mandarin oranges containing β-cryptoxanthin was given to volunteers for 56 days. Twenty persons (before intake) were divided into two groups of 10 persons for the intake of regular juice and 10 persons for the reinforced β-cryptoxanthin juice. Each value is mean ± S.E.M. of 10 to 20 persons. $*p < 0.01$ compared with the value before intake. $\#p < 0.01$ compared with the value obtained after regular juice intake.

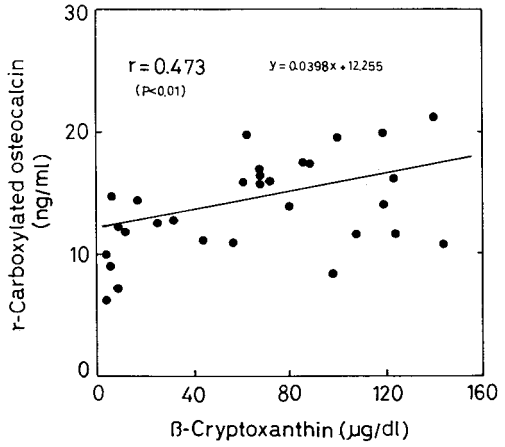

Fig. 2. Relationship between Serum β-Cryptoxanthin and γ-Carboxylated Osteocalcin Concentrations following Intake of Juice Containing β-Cryptoxanthin in Healthy Individuals

The procedure for the intake of juice containing β-cryptoxanthin is described in the legend to Fig. 1. Each plot is the value obtained from all individuals examined.

RESULTS

Changes in serum β-cryptoxanthin concentrations in healthy individuals following the 56-day intake of regular juice or of β-cryptoxanthin-reinforced juice prepared from Satsuma mandarin was examined (Fig. 1). Serum β-cryptoxanthin concentrations increased significantly after the intake of regular juice for 56 days as compared with that before intake. This increase was significantly enhanced by the intake of the β-cryptoxanthin-reinforced juice for 56 days.

When changes in serum bone metabolic markers were examined, serum γ-carboxylated osteocalcin concentration, a marker of bone formation, was significantly increased after the intake of regular juice or β-cryptoxanthin-reinforced juice as compared with that before intake, as reported previously.[26] Serum TRAP activity, a marker of bone resorption, was significantly decreased after the intake of regular juice or β-cryptoxanthin-reinforced juice, as reported previously.[26] The relationship between serum β-cryptoxanthin and γ-carboxylated osteocalcin or TRAP activity concentrations in all groups before intake and after regular juice and β-cryptoxanthin-reinforced juice intake was examined. A significant positive relationship between β-cryptoxanthin and γ-carboxylated osteocalcin concentrations was found

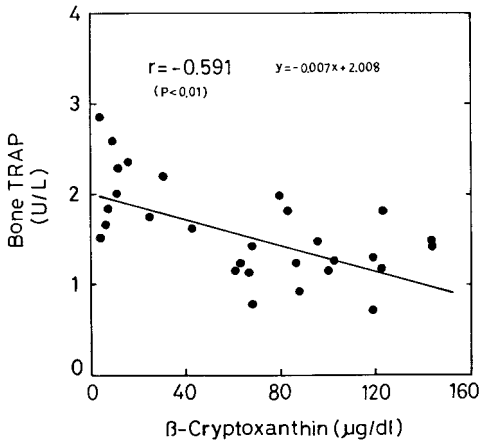

Fig. 3. Relationship between Serum β-Cryptoxanthin and TRAP Activity Following Intake of Juice Containing β-Cryptoxanthin in Healthy Individuals

The procedure for the intake of juice containing β-cryptoxanthin is described in the legend to Fig. 1. Each plot is the value obtained from all individuals examined.

(Fig. 2). A significant negative relationship between β-cryptoxanthin concentration and TRAP activity was observed (Fig. 3).

Other serum biochemical results following the intake of regular juice or β-cryptoxanthin-reinforced juice did not change significantly after the intake of juice for 56 days as compared with before intake (Table 1).

Table 1. Serum Metabolic Findings Following Intake of β-Cryptoxanthin-Containing Juice in Healthy Individuals

Serum level	Control (baseline)	Regular juice	Reinforced juice
Albumin (g/dl)	4.31 ± 0.086	4.28 ± 0.11	4.15 ± 0.071
γ-GTP (U/l)	23.2 ± 2.83	19.5 ± 3.19	16.5 ± 3.09
Nitrogen urea (mg/dl)	14.5 ± 0.72	14.9 ± 0.99	14.0 ± 0.56
Amylase (U)	85.7 ± 11.6	92.6 ± 8.95	74.7 ± 13.7
Glucose (mg/dl)	96.0 ± 9.01	85.3 ± 4.71	101.8 ± 18.6
HDL cholesterol (mg/dl)	49.7 ± 1.99	50.3 ± 3.22	45.4 ± 2.13
Triglyceride (mg/dl)	79.0 ± 12.0	56.0 ± 5.83	50.3 ± 15.9
Zinc (μg/dl)	87.7 ± 2.57	89.0 ± 4.56	84.6 ± 2.86

The procedure for the intake of juice containing β-cryptoxanthin is described in Fig. 1. Each value is the mean ± S.E.M. of 10 to 20 persons. Differences were not significant.

DISCUSSION

The intake of regular juice containing β-cryptoxanthin for 56 days was found to increase serum β-cryptoxanthin concentrations in healthy individuals. This increase was significantly enhanced by the intake of β-cryptoxanthin reinforced juice. Serum β-cryptoxanthin concentrations were 1.63×10^{-7}, 1.23×10^{-6}, and 2.12×10^{-6} M before intake, after regular juice intake, or after β-cryptoxanthin-reinforced juice, respectively. It has been reported that the serum concentration of β-cryptoxanthin increases due to the consumption of vegetable juice in women, in the range of 1.3×10^{-7} to 5.3×10^{-7} M.[30] The anabolic effects of β-cryptoxanthin on bone components was observed at 10^{-7} and 10^{-6} M in rat femoral tissues *in vitro*.[18,19] The intake of β-cryptoxanthin-containing juice may have anabolic effects on bone metabolism in healthy individuals.

The intake of β-cryptoxanthin containing juice caused a significant increase in serum markers of bone formation and a corresponding decrease in serum markers of bone resorption in healthy individuals, suggesting that the intake of β-cryptoxanthin-containing juice has anabolic effects on bone mass. Serum γ-carboxylated osteocalcin is expressed in osteoblastic cells,[28] which stimulate bone formation. Serum γ-carboxylated osteocalcin concentrations were increased after the intake of regular or reinforced juice containing additional β-cryptoxanthin in healthy individuals.[26] Serum bone TRAP is a specific marker enzyme in osteoclasts.[29] The intake of β-cryptoxanthin-containing juice caused a significant decrease in serum bone TRAP activity in healthy individuals,[26] suggesting that bone resorption is suppressed by the intake of β-cryptoxanthin-containing juice. We found that an increase in serum β-cryptoxanthin concentration elevated circulating γ-carboxylated osteocalcin and correspondingly decreased circulating bone TRAP activity in healthy individuals. A significant relationship between serum β-cryptoxanthin concentration and circulating bone metabolic markers was thus observed in healthy individuals.

Serum biochemical findings associated with liver, kidney, and pancreatic functions were not significantly altered by the intake of regular juice or of β-cryptoxanthin-reinforced juice for 56 days in healthy individuals. Serum glucose and triglyceride concentrations, which are metabolic markers, were not significantly changed after the prolonged intake of β-cryptoxanthin-containing juice. These observations indicate that the prolonged intake of β-cryptoxanthin-containing juice does not influence metabolic functions of the liver, kidney, and pancreas in such individuals. It is assumed that β-cryptoxanthin-containing juice is a safe and healthy dietary supplement.

In conclusion, it has been shown that there is a relationship between serum β-cryptoxanthin concentration and circulating bone metabolic markers in normal individuals following the prolonged intake of juice containing β-cryptoxanthin.

REFERENCES

1) Schapira, C., Slinn, S., Sarid, M., Mokadai, S., Kabara, A. and Silibermann, M. (1995) Calcium and vitamin D enriched diets increase and preserve vertebral mineral content in aging laboratory rats. *Bone*, **16**, 575–582.
2) Wild, R. A., Buchamain, J. R., Meyers, C. and Demera, L. M. (1987) Declining adrenal androgen:

an association with bone loss in aging women. *Proc. Soc. Exp. Biol. Med*., **186**, 335–360.
3) Cooper, C. and Melton, J., III (1992) Epidemiology of osteoporosis. *Trends Endocrinol. Metab*., **3**, 224–229.
4) Bonjour, J.-P., Schurch, M.-A. and Rizzori, R. (1996) Nutritional aspects of hip fractures. *Bone*, **18**, 1395–1445.
5) Yamaguchi, M. (2002) Isoflavone and bone metabolism: Its cellular mechanism and preventive role in bone loss. *J. Health Sci*., **48**, 209–222.
6) Yamaguchi, M. and Gao, Y. H. (1998) Anabolic effect of genistein and genistin on bone metabolism in the femoral-metaphyseal tissues of elderly rats: The genistein effect is enhanced by zinc. *Mol. Cell. Biochem*., **178**, 377–382.
7) Sugimoto, E. and Yamaguchi, M. (2000) Stimulatory effect of daidzein in osteoblastic MC3T3-E1 cells. *Biochem. Pharmacol*., **59**, 471–475.
8) Ono, R., Ma, Z. J. and Yamaguchi, M. (2000) Prolonged intake of fermented soybean diets with supplementation of isoflavone and saponin prevents bone loss in ovariectomized rats. *J. Health Sci*., **46**, 70–74.
9) Yamaguchi, M., Sugimoto, E. and Hachiya, S. (2001) Stimulatory effect of menaquinone-7 (vitamin K_2) on osteoblastic bone formation *in vitro*. *Mol. Cell. Biochem*., **223**, 131–137.
10) Yamaguchi, M., Kakuda, H., Gao, Y. H. and Tsukamoto, Y. (2000) Prolonged intake of fermented soybean (*natto*) diets containing vitamin K_2 (menaquinone-7) prevents bone loss in ovariectomized rats. *J. Bone Miner. Metab*., **18**, 71–76.
11) Tsukamoto, Y., Ichise, M. and Yamaguchi, M. (2000) Prolonged intake of dietary fermented soybean (*natto*) with the reinforced vitamin K_2 (menaquinone-7) enhances circulating γ-carboxylated osteocalcin concentration in normal individuals. *J. Health Sci*., **46**, 317–321.
12) Ono, R., Ma, Z. J. and Yamaguchi, M. (2000) Prolonged intake of fermented soybean diets with supplementation of isoflavone and saponin prevents bone loss in ovariectomized rat. *J. Health Sci*., **46**, 70–74.
13) Yamaguchi, M., Taguchi, H., Gao, Y. H., Igarashi, A. and Tsukamoto, Y. (1999) Effect of vitamin K_2 (menaquinone-7) in fermented soybean (*natto*) on bone loss in ovariectomized rats. *J. Bone Miner. Metab*., **17**, 23–29.
14) Stenmetz, K. A. and Potter, J. D. (1996) Vegetable, fruits, and cancer prevention: a review. *J. Am. Diet Assoc*., **96**, 1027–1033.
15) Forsyth, K. S., Watson, R. R. and Gensler, H. L. (1989) Osteotoxicity after chromic dietary administration of 13-*cis*-retinoic acid, retinyl palmitate or selenium in mice exposed to tumor initiation and promotion. *Life Sci*., **45**, 2149–2156.
16) Rohde, C. M., Manatt, M., Clagett-Dome, M. and DeLuca, H. F. (1999) Vitamin A antagonizes the action of Vitamin D in rats. *J. Nutr*., **129**, 2241–2250.
17) Promislow, J. H. E., Goodman-Gruen, D., Slymen, D. J. and Barret-Connor, E. (2002) Retinol intake and bone mineral density in the elderly: the Rancho Bernardo study. *J. Bone Miner. Res*., **17**, 1349–1358.
18) Yamaguchi, M. and Uchiyama, S. (2003) Effect of carotenoid on calcium content and alkaline phosphatase activity in rat femoral tissues *in vitro*: The unique anabolic effect of β-cryptoxanthin. *Biol. Pharm. Bull*., **26**, 1188–1191.
19) Yamaguchi, M. and Uchiyama, S. (2004) β-Cryptoxanthin stimulates bone formation and inhibits bone resorption in tissue culture *in vitro*. *Mol. Cell. Biochem*., **258**, 137–144.
20) Uchiyama, S. and Yamaguchi, M. (2005) β-Cryptoxanthin stimulates cell proliferation and transcriptional activity in osteoblastic MC3T3-E1 cells. *Int. J. Mol. Med*., **15**, 675–681.
21) Uchiyama, S. and Yamaguchi, M. (2005) β-Cryptoxanthin stimulates cell differentiation and mineralization in osteoblastic MC3T3-E1 cells. *J. Cell. Biochem*., **95**, 1224–1234.
22) Uchiyama, S. and Yamaguchi, M. (2004) Inhibitory effect of β-cryptoxanthin on osteoclast-like cell formation in mouse marrow cultures. *Biochem. Pharmacol*., **67**, 1297–1305.
23) Uchiyama, S., Sumida, T. and Yamaguchi, M. (2004) Oral administration of β-cryptoxanthin induces anabolic effects on bone components in the femoral tissues of rats *in vivo*. *Biol. Pharm. Bull*., **27**, 232–235.
24) Uchiyama, S., Sumida, T. and Yamaguchi, M. (2004) Anabolic effect of β-cryptoxanthinon on bone components in the femoral tissues of aged rats *in vivo* and *in vitro*. *J. Health Sci*., **50**, 491–496.
25) Uchiyama, S. and Yamaguchi, M. (2005) Oral administration of β-cryptoxanthin prevents bone loss in streptozotocin-diabetic rats *in vivo*. *Biol. Pharm. Bull*., **28**, 1766–1769.
26) Yamaguchi, M., Igarashi, A., Uchiyama, S., Morita, S., Sugawara, K. and Sumida, T. (2004) Prolonged intake of juice (*Citrus unshiu*) reinforced with β-cryptoxanthin has an effect on circulating bone biochemical markers in normal individuals. *J. Health Sci*., **50**, 619–624.
27) Peng, Y.-M., Peng, Y.-S., Lin, Y., Moon, T., Roe, D. J. and Ritenbaugh, C. (1995) Concentrations and plasma-tissue-diet relationships of carotenoids, retinoids, and tocopherols in humans. *Nutr. Cancer*,

23, 233–246.
28) Koyama, N., Ohara, K., Yokota, H., Kurome, T., Katayama, M., Hino, F., Kato, I. and Aki, T. (1991) A one step sandwich enzyme immunoassay for antibodies. *J. Immunol. Methods*, **139**, 17–23.
29) Hallen, J. M., Alatalo, S. L., Suminen, H., Cheng, S., Janckila, A. J. and Vaananen, H. K. (2000) Tartrate-resistant acid phosphatase 5b: A novel serum marker of bone resorption. *J. Bone Miner. Res*., **15**, 1337–1345.
30) McEligot, A. J., Rock, C. L., Shanks, T. G., Flatt, A. W., Newman, V., Faerber, S. and Pierce, J. P. (1999) Comparison of serum carotenoid response between women consuming vegetable juice and women consuming raw or cooked vegetable. *Cancer Epidemiol. Biomarkers Prev*., **8**, 227–231.