

Original Article

Association between 24 hour urinary α -tocopherol catabolite, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman (α -CEHC) and α -tocopherol intake in intervention and cross-sectional studies

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The objective is to determine the association between the 24 hour urinary α -tocopherol catabolite, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman (α -CEHC) and α -tocopherol intake in an intervention and a cross-sectional studies. In the 4-weeks intervention study, Japanese men (n = 10) consumed the test diet in week 1, and the test diet plus varying amounts of α -tocopherol in the three subsequent weeks: 21 μ mol/d α -tocopherol in week 2, 63 μ mol/d in week 3, and 125 μ mol/d in week 4. A significant association between α -tocopherol intake and urinary α -CEHC was observed in this strictly controlled experiment ($r = 0.99$, $p < 0.001$). In the cross-sectional study, all foods consumed over 4 consecutive days were recorded in 76 free-living young subjects (18-33 years). The association was weak, but a significant relationship was observed ($r = 0.29$, $p < 0.05$) even in the cross-sectional study. In the cross-sectional study adults, mean estimated α -tocopherol intake calculated by urinary α -CEHC and the excretory ratio was 91% of their mean intake over the 4 days. The results show that urinary α -CEHC level reflected recent α -tocopherol intake in free-living young Japanese adults, and could be used as a measure of intake during the previous few days, both for group means and for individual rankings within a group.

Key Words: α -tocopherol, catabolism, CEHC, urine, biomarker

INTRODUCTION

Measurements of food intake are widely used for surveys of nutritional assessment. However, there are limitations in assessing only information from food surveys,¹ and methods that measure biological parameters can reveal new information. Urine, which is a noninvasive bio-sample, might overcome the limitations of nutritional assessment by food survey. For example, 24-hour urinary nitrogen level is established as a marker for protein intake,² urinary potassium level as a marker for potassium intake,³ and urinary sugar level as a marker for sugar intake.^{4,5} In previous studies, we investigated the relationship between water-soluble vitamin intake and their urinary excretion of these nutrients. We clarified that urinary water-soluble vitamin levels are strongly correlated with their intake.⁶⁻⁹ These studies⁶⁻⁹ have indicated that 24-h urinary excretion of water-soluble vitamins is a potential biomarker for recent vitamin intake in both intervention and cross-sectional studies.

Generally, fat-soluble vitamins are not excreted in urine. However, In 1995, Schultz *et al.*,¹⁰ reported that a catabolite of α -tocopherol, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman (α -CEHC), which is a metabo-

lite with an intact chroman ring, is excreted in urine. Previously, α -CEHC was proposed as a potential excretion product of α -tocopherol in 1965 by Schmandke *et al.*,¹¹ but had not been described again until Schultz's report. Schultz *et al.*,¹⁰ suggested that α -CEHC excretion indicates the saturated binding capacity of α -tocopherol in the plasma, and thus may be considered to be a marker of optimum α -tocopherol intake. This proposal was strengthened by Shuelke *et al.*,¹² who found that α -CEHC was excreted into the urine of patients with α -tocopherol binding protein defects regardless of the plasma α -tocopherol concentration, whereas it was not excreted by healthy subjects until the plasma α -tocopherol concentration

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surpassed 40 $\mu\text{mol/L}$. These findings indicate that the urinary content of α -CEHC in the Japanese is almost below detection, because the average plasma concentration of α -tocopherol in the Japanese is around 20 $\mu\text{mol/L}$.¹³ However, as Schultz *et al*,¹⁰ pointed out in their paper, their results were obtained with only seven participants, and therefore, further research is necessary to allow the results to be applied generally.

In 2003, Morinobu *et al*,¹⁴ reported a straightforward and reliable method of determining α -CEHC, which was later modified by Stahl *et al*¹⁵ and Lodge *et al*.¹⁶ Morinobu *et al*¹⁴ also reported that α -CEHC was detected in healthy adult male Japanese volunteers ($n = 14$). Therefore, it is probable that α -CEHC is excreted into urine in Japanese individuals who take an ordinary amount of α -tocopherol.

The aim of the present study was to determine the possibility that α -CEHC can be used as a biomarker of α -tocopherol status in young Japanese adults. We examined the association between 24 h urinary α -CEHC levels and the intakes of α -tocopherol in strictly controlled-living and in free-living participants.

MATERIALS AND METHODS

Both studies were reviewed and approved by the ethics committee of The University of Shiga Prefecture. The purpose and protocol of this study was explained to all participants before joining the study, and written informed consent was obtained from each participant.

Subjects and experimental design

Intervention study group

We recruited students from a registered dietician department. All subjects (male Japanese college students, $n = 10$) were housed in the same facility and given the same diet. The experimental period was 4 weeks. They did not have regular use of medications or dietary supplements, or habitual alcohol or cigarette consumption. Age, body weight, height, and body mass index (mean \pm SD) were 22.1 ± 2.3 years (18–25 years), 63.6 ± 5.2 kg, 174 ± 5 cm, and 21.0 ± 1.6 kg/m², respectively.

Cross-sectional study group

A total of 102 healthy, free-living Japanese females, aged 18–33 years, voluntarily participated in this study. The exclusion criteria were: presence of cold or influenza, and use of multivitamin supplements at least once during the previous month. In addition, we excluded participants whose 24-hour urine collection or dietary records were considered as incomplete, with a collection time outside the 22–26 h range, a urine volume of <250 ml, creatinine excretion in relation to body weight outside the 10.8–25.2 mg/kg range,^{17,18} or extremely low or high energy intake ($<2,090$ or $>16,700$ kJ/d).¹⁹ After these exclusions, 76 of the 102 female students were found to be eligible and were enrolled into the group.

Dietary records

Intervention study group

The diet given to the participants consisted of a breakfast of bread, margarine, ham, yoghurt, tomato, lettuce, and milk; a lunch of rice, toasted and seasoned laver (a type of seaweed), luncheon meat, boiled egg, raw cabbage,

Table 1. Daily intake of energy and nutrients from the basal diet in intervention study

Energy and nutrients ¹	Amount
Energy (kJ)	11,100
Protein (g)	97.5
Fat (g)	86.7
Carbohydrates (g)	361
α -Tocopherol (mg)	8.7 (20.2 μmol)

¹Nutrients were calculated from the Standard Tables of Food Composition in Japan.²⁰

miso soup, and Japanese tea; and an evening meal of rice, soy sauce, seasonal Pacific saury (a type of fish), tofu (soybean curd), boiled spinach leaves, kiwi fruit and Japanese tea, with a midnight snack of cheese and jelly fruit mix. The daily intakes of energy and nutrients from the basal diet are shown in Table 1. Nutrients were calculated by using Standard Tables of Food Composition in Japan, (fifth revised and enlarged edition).²⁰ The subjects ate this diet on days 1–5 of each week over the experimental period of 4 weeks and were free to eat what they wanted on days 6 and 7. In the latter three weeks, the participants took α -tocopherol acetate in addition to the diet: 21 $\mu\text{mol/d}$ in week 2, 63 $\mu\text{mol/d}$ in week 3, and 125 $\mu\text{mol/d}$ in week 4.

Cross-sectional study group

This group underwent a 4-day dietary assessment in which the participants were living freely in the university and consuming their normal diet. The 4-day assessment began on a Monday (day 1) and ended on Thursday (day 4). All food consumed during the 4-day period was recorded using a weighed food recording method.²¹ A digital cooking scale capable of weighing in 1 g increments (Tanita Inc., Tokyo, Japan), a set of dietary record forms, a dietary record manual, and a disposable camera were distributed to the participants in advance. In the dietary record, the ingested food was described (eg “raw”, “boiled”, “cooked”, “skin present”, “a part of cooking ingredients”, or “with or without seasoning”), and coded according to the Standard Tables of Food Composition in Japan (fifth revised and enlarged edition) as for the intervention group.²⁰ The participants took photographs with a disposable camera of the dishes before and after eating. Several experienced dietitians used the photographs to complete the data, and asked the participants to resolve any discrepancies or to obtain further information when needed. The food that remained after eating was measured on the digital scales and was deducted from the dietary record. Food, nutrient and energy intakes were calculated using SAS statistical software (version 6.12; SAS Institute, Cary, NC, USA), based on the Standard Tables of Food Composition in Japan.

Collection of 24 hour urine sample

For the intervention study group, the 24 hour urine samples were collected from the second urination on day 4 to the first urination after 06:30 hours (wake-up time) on day 5 in each week.

For the cross-sectional group, a single 24-hour urine sample was collected on day 4 to measure the α -

tocopherol metabolite, α -CEHC. In the morning, participants were asked to discard the first specimen and to record the time on the sheet. The following morning, participants were asked to collect a specimen at the same time as the discarded specimen from the previous morning and to record the time on the sheet.

After the urine samples were collected, the sample volumes were measured, and aliquots of the urine were stabilized to avoid destruction of α -CEHC. All treated urine samples were then stored at -20°C until analysis.

Chemicals

α -CEHC was purchased from Cayman Chemical Co., Ltd (Ann Arbor, Michigan, USA). β -Glucuronidase derived from *Escherichia coli* was obtained from Nacalai Tasque Co., Ltd (Kyoto, Japan). All other chemicals used were of the highest purity available from commercial sources.

Analysis of α -CEHC

The concentrations of α -CEHC in urine were measured by high performance liquid chromatography with electrochemical detection (HPLC-ECD), as described by Morinobu *et al.*¹⁴ β -Glucuronidase (25,000 units) was dissolved in 2.5 ml of 0.1 mol/L sodium acetate-acetate buffer (pH 4.5) on ice and used to hydrolyze the conjugate immediately after preparation. Urine (1 ml) was placed into a tube with 1,000 U (100 μl) of β -glucuronidase, 100 μl of 57 mmol/L ascorbic acid, and 20 μl of 0.015% dibutylhydroxytoluene (BHT). After which the mixture was incubated for 4 hours at 37°C to achieve hydrolysis, 50 μl of 6 mol/L HCl and 2 ml of diethylether were added to stop the reaction. After mixing by vortex and separating by centrifugation at $1,800\times g$ for 10 min, 1 ml of the diethylether layer was collected and evaporated to dryness. The residue was dissolved in 200 μl of 0.015% BHT, and a 20 μl aliquot was injected into the HPLC-ECD.

Statistics

SPSS software (version 16 for Windows; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Values

were presented as means \pm SD. The daily measurements of urinary α -CEHC and the dietary α -tocopherol intakes were not normally distributed, therefore, the data were converted logarithmically. Pearson correlation coefficients were calculated to determine the association between urinary and dietary measurements. The value of the urinary excretory ratio (%) was calculated as follows: [α -CEHC excretion in the fourth day ($\mu\text{mol/d}$)/the average α -tocopherol intake during 4-days ($\mu\text{mol/d}$)] $\times 100$. An analysis of variance (ANOVA) random effects model was used to quantify inter- and intra-individual percentage coefficient of variance (% CV), which was used to estimate the variability in α -tocopherol intake.

RESULTS

Relationship between the intake of α -tocopherol and the urinary excretion of α -CEHC

Intervention study group

Figure 1 shows the relationship between the intake of α -tocopherol and the urinary excretion of α -CEHC. A strong significant association was observed ($r = 0.99$, $p = 0.0043$). The average urinary α -CEHC concentration at baseline (week 1) was 0.74 $\mu\text{mol/d}$, which increased to 0.94, 1.89, and 3.34 $\mu\text{mol/d}$ after supplementation at the doses of 21, 63 and 125 $\mu\text{mol/d}$, respectively. The excretory ratio of α -CEHC was 3.7 ± 1.3 , 2.3 ± 0.7 , 2.3 ± 1.1 , and $2.3 \pm 0.6\%$ for α -tocopherol intakes of 20, 41, 83 and 146 $\mu\text{mol/d}$, respectively.

Cross-sectional study group

The basic characteristics of the 76 young women are presented in Table 2. Each values were similar to those reported for young adults female aged 18–22 years.¹⁹ In brief, the participants were considered as typical female university students in Japan, characterized by relatively low BMI (20.2 kg/m^2), and low intake of fat (28.4%). During the experimental period, all participants were living freely, and none of the participants were drinking or smoking. Average intake of α -tocopherol in participants was $13.7 \pm 3.8 \mu\text{mol/d}$, and daily intake of α -tocopherol

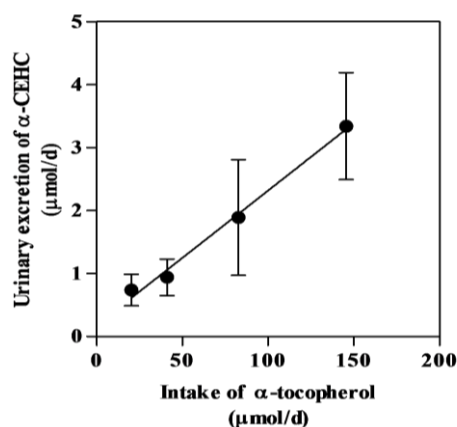


Figure 1. Relationship between urinary excretion of α -CEHC and the intake of α -tocopherol in strictly controlled participants (intervention study). Individual average intake of α -tocopherol is plotted on the x-axis, and the 24-h urinary excretion of α -CEHC, a catabolite of α -tocopherol, is plotted on the y-axis. In total, 10 healthy male Japanese college students aged 18–25 years were enrolled. Values are mean \pm SD. A significant correlation ($r = 0.99$, $p = 0.0043$) was obtained. Regression line: $y = 0.0214 (\pm 0.0014) x + 0.180 (\pm 0.122)$.

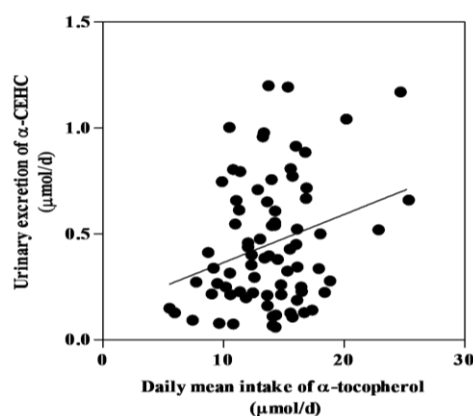


Figure 2. Relationship between urinary excretion of α -CEHC and intake of α -tocopherol in young adults' female (cross-sectional study). Measurements were taken of food intake on 4 consecutive days. The urine samples were collected at day 4. Individual average intake of α -tocopherol is plotted on the x-axis, and the urinary excretion of α -CEHC on day 4 is plotted on the y-axis. In total, 76 healthy, free-living, female college students aged 18–33 years were enrolled. A significant correlation ($r = 0.29$, $p = 0.0147$) was obtained. Regression line: $y = 0.0228 (\pm 0.0091) x + 0.137 (\pm 0.130)$.

Table 2. Characteristics and dietary intakes of female subjects

Variables	Young adult female (n = 76)
Anthropometric variable	
Age (years)	20.1 ± 2.3
Body height (cm)	158 ± 5
Body weight (kg)	50.6 ± 5.4
Body mass index (kg/m ²)	20.2 ± 1.8
Dietary mean intake at days 1-4	
Total energy (kJ/d)	6950 ± 1260
Protein (% of energy)	13.8 ± 2.2
Fat (% of energy)	28.4 ± 4.5
Carbohydrate (% of energy)	56.5 ± 4.9
α-Tocopherol intake (μmol/d)	13.7 ± 3.8
Inter-individual variations on the vitamin E intake (% CV) ¹	41.5
Intra-individual variations on the vitamin E intake (% CV) ¹	55.3
Urinary α-CEHC (μmol/d)	0.444 ± 0.292
Excretory ratio ² of vitamin E (%)	3.58 ± 3.39

¹% CV, percentage coefficient of variance.

²Calculated by the formula [α -CEHC excretion on day 4 (μmol/d) / the average α -tocopherol intake over the 4 days (μmol/d)] × 100.

Table 3. Mean dietary α-tocopherol intake and 24-hour urinary α-CEHC, excretory ratio, and estimated mean α-tocopherol intake in young adult females

	Young adult female (n = 76)
Mean α-tocopherol intake ¹ (μmol/d)	13.7 ± 3.8
Excretory ratio ² (%)	3.58 ± 3.39
Estimated mean α-tocopherol intake ³ (μmol/d)	12.4 ± 8.2
% ratio ⁴	91

¹Calculated by average α-tocopherol intake over the 4 days for each individual.

²Calculated by the formula [α -CEHC excretion on day 4 (μmol/d) / the average α-tocopherol intake over the 4 days (μmol/d)] × 100.

³The estimated α-tocopherol intake in young adult was calculated by the individual value of the 24-h urinary excretion of α-CEHC and the excretory ratio of 3.58 %, and the estimated mean α-tocopherol intake was calculated by the resulting estimated α-tocopherol intake.

⁴%Ratio between the mean and the estimated mean α-tocopherol intake.

was similar to adequate intake for Japan.²⁰ Urinary excretion of α-CEHC was twofold higher in elderly subjects than healthy peoples. The value of the excretory ratio of vitamin E in elderly subjects was 1.4-fold higher than in healthy individuals. Intra and inter-individual variations in α-tocopherol intakes, the values were both around 50% (Table 2).

Correlations between 24-hour urinary excretion of α-tocopherol metabolite, α-CEHC on day 4 and the average α-tocopherol intake on 4 consecutive days are shown in Figure 2. A significant association was observed in young adult females.

Estimated mean α-tocopherol intake calculated by urinary excretion of α-CEHC and the excretory ratio in the cross-sectional study

Mean dietary α-tocopherol intake and 24-hour urinary α-CEHC, excretory ratio, and estimated mean α-tocopherol intakes in young women are shown in Table 3. The excre-

tory ratio was determined from the urinary excretion of α-CEHC and the average α-tocopherol intake over 4 days. The individual estimated α-tocopherol intake was calculated by average excretory ratio and the individual urinary α-CEHC value. The estimated mean α-tocopherol intake in young women was 91% of the real mean α-tocopherol intake over 4 days in young adult females.

DISCUSSION

Alpha-CEHC, a urinary metabolite of α-tocopherol, was described in 1995 by Schultz *et al*¹⁰ Excretion of α-CEHC is considered to reflect saturation of α-tocopherol in the body, because it is not a metabolite of the α-tocopherol consumed for antioxidant defense. In other words, the detection of α-CEHC in urine generally indicates a better α-tocopherol nutritional status. Therefore, it is generally considered that the content of α-CEHC is below the limit of detection under an ordinary dietary habitant. Morinobu *et al*,¹⁴ however, reported that α-CEHC was detected in healthy adult male Japanese volunteers. Therefore, it is probable that α-CEHC is excreted into urine in Japanese individuals who consume an ordinary amount of α-tocopherol. But, the data are based on only seven persons. Further research is necessary to allow the results to be applied generally.

In the present first study, the intervention study was performed to determine whether urinary α-CEHC excretion correlates with intake of α-tocopherol. We found a significant positive correlation between urinary α-CEHC and intake of α-tocopherol in healthy young male Japanese adults who consumed a strictly controlled diet with doses of α-tocopherol ranging from 20 to 145 μmol/d.

In the second experiment, to determine the usefulness of urinary α-CEHC as a biomarker for α-tocopherol nutritional status, a cross-sectional study was performed on free-living subjects. A weak but significant correlation was found between urinary α-CEHC and the mean α-tocopherol intake. These results indicate that α-CEHC levels in 24-h urine reflect dietary α-tocopherol intakes over the past few days, and suggest that α-tocopherol intake can be estimated from urinary α-CEHC values in free-living subjects.

This phenomenon might be only applicable for the Japanese and East-Asian populations. The subjects ate a lot of vegetable and were of relatively low BMI and had low intake of fat. The requirement of α -tocopherol is dependent on the intakes of polyunsaturated fatty acids. The average intakes of the polyunsaturated fatty acids were around 10 g/d. The optimum ratio of α -tocopherol (mg/d) to polyunsaturated fatty acids (g/d) is reported to be 0.60.²² Its average ratio was about 0.60 in the present subjects. This is a reason why a significant association was observed even with low intake of α -tocopherol for the Japanese.

Metabolism of α -CEHC from α -tocopherol is somewhat different between Japanese, America and European populations. For example, the metabolic activity of α -tocopherol to α -CEHC might be higher in the Japanese than in the Americans and Europeans. The precise regulatory mechanism of post-absorption α -tocopherol elimination is not clear. The current knowledge is only of a pathway involving cytochrome P450-mediated ω -hydroxylation of the α -tocopherol phytol side chain, followed by stepwise removal of two or three carbon molecules such as acetyl-CoA or propionyl-CoA, ultimately yielding the α -CEHC that is excreted in urine.²³ Low fat intakes in the Japanese might bring about surplus ability to the β oxidation system, which results in increased activity of α -tocopherol to α -CEHC. Sesamin increases tissue α -tocopherol concentration by inhibiting the α -tocopherol oxidation pathway;²⁴ sesame oil, which contains sesamin, is often used in Japanese cooking, which might bring about the saving effect of α -tocopherol. Compared to Caucasian, Asian population had significantly lower plasma vitamin E levels in the same environment.²⁵ However, there are no data regarding its metabolism or immune functions among two populations. In Asian populations, they may be easily excreted into urine as α -CEHC compared to Caucasians. This is also a reason why the significant association was observed even with low intake of α -tocopherol for the Japanese.

The limiting factor of the transport capacity appears to be plasma lipid concentration. In general, subjects with a low total plasma lipid concentration did not accumulate as high concentration of α -tocopherol, as those with a higher total lipid content; and they started to excrete α -CEHC earlier¹⁰. If the urinary excretion of α -CEHC is related to the α -tocopherol content of plasma lipid, the range of thresholds of excretion is much wider and the correlation between plasma concentration and urinary excretion becomes more pronounced. In the present cross-sectional study, we did not measure plasma lipid concentration, and how plasma lipid affected urinary excretion of α -CEHC is unclear.

In terms of the completeness of the dietary assessment in the present study, there are several limitations in terms of using a weighed food record method. To reduce errors associated with self report, several dietitians reviewed the collated records along with the photographs. The selection of participants from a dietetics course also contributed to reduce reporting errors, as they had nutritional knowledge and were well trained. Another limitation exists in the present food composition table developed for Japan. In a dietary assessment of free-living people, po-

tential errors caused by the quality of this table, such as defects in food composition, are inevitable. These limitations might cause the relatively low correlation in the free-living experiment population compared with that of the intervention study.

In conclusion, a significant relationship between the α -tocopherol intake and urinary excretion of α -CEHC was observed in young Japanese adults.

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AUTHOR DISCLOSURES

None of the authors had any financial or personal conflicts of interest associated with this manuscript.

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Original Article

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24 小時尿液 α -生育醇代謝產物 2,5,7,8-tertranetgyl-2(2'carboxyethyl)-6-hydroxychroman(α -CEHC)與 α -生育醇攝取之介入及橫斷研究之相關

本研究的目的為評估在介入及橫斷研究中，24 小時尿液的 α -生育醇代謝產物 2,5,7,8-tertranetgyl-2(2'carboxyethyl)-6-hydroxychroman (α -CEHC) 與 α -生育醇攝取量之相關性。在四週的介入性研究，日本男性 (n=10) 在第一週攝取測試飲食，後續三週攝取添加不同量 α -生育醇的測試飲食：第二週為 21 $\mu\text{mol}/\text{d}\alpha$ -生育醇、第三週為 63 $\mu\text{mol}/\text{d}\alpha$ -生育醇與第四週為 125 $\mu\text{mol}/\text{d}\alpha$ -生育醇。在這個嚴格控制的實驗中，觀察到 α -生育醇的攝取量與尿液中的 α -CEHC 有顯著的相關 ($r=0.99$, $p<0.001$)。在橫斷性研究，76 名年輕一般研究對象 (18-33 歲) 紀錄連續四天攝取的所有食物。在橫斷性研究這個相關性儘管不強，但是顯著的 ($r=0.29$, $p<0.05$)。在橫斷性研究中的成年人，以尿液 α -CEHC 計算其平均估計 α -生育醇攝取量，其排泄率為四日平均攝取量的 91%。無論組別平均值或是組別中個體的排序結果，都顯示尿液中的 α -CEHC 量可反映年輕日本一般成年人近期的 α -生育醇攝取量，且可以當作過去幾天的攝取量測量方法。

關鍵字： α -生育醇、代謝產物、CEHC、尿液、生物標記