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Rapid and Convenient Method for Preparing Aurapten-Enriched Product from Hassaku Peel Oil: Implications for Cancer-Preventive Food Additives

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Aurapten (7-geranyloxycoumarin) has been reported to be an effective inhibitor of chemical carcinogenesis in some rodent models. In the present study, a method for preparing an aurapten-enriched agricultural product has been established. Out of 17 Rutaceae varieties, the aurapten content in hassaku (*Citrus hassaku* Hort ex Y. Tanaka) fruit peel was marked, as well as that in natsumikan (*C. natsudaidai*) and grapefruit (*C. paradisi*). The aurapten content in hassaku peel was most abundant in April. Hassaku fruit peel oil, which was dissolved by heating precipitates including aurapten which had formed after freezing the peel oil at −20 °C, was used. After adsorbing aurapten from peel oil onto synthetic adsorbent SP70, the adsorbent was washed with 40% (v/v) ethanol in water to remove essential oils and pigments remaining on the adsorbent. Aurapten was then eluted with 80% (v/v) ethanol. In a laboratory-scale test, the recovery rates of aurapten and total carotenoids from the eluates were 74.3 and 4.6%, respectively. In a pilot-scale test, the recovery rate of aurapten in the aurapten-enriched preparation from dissolved hassaku oil was 91.0%, and its concentration was 64.1% (w/w). When stored for 180 days under sunlight, aurapten in powder form remained at 88.0−89.0% of the initial level, but only 31.3−43.8% in ethanol. The stability of aurapten in the aurapten-enriched preparation was higher than that of purified aurapten. These results suggest that aurapten is readily recovered from hassaku peel oil using SP70, and thus may be used as a food additive.

KEYWORDS: Aurapten; hassaku peel oil; *Citrus hassaku*; stability; Rutaceae

INTRODUCTION

It has recently been reported that aurapten (7-geranyloxycoumarin, **Figure 1**), found in the hassaku (*Citrus hassaku* Hort ex Y. Tanaka) fruit peel, has cancer chemopreventive activities in mouse skin (*1*), rat colon (*2*, *3*), and rat tongue carcinogenesis models (*4*).

On the other hand, hesperidin in the citrus fruit peel has been shown to have cholesterol lowering effects in the rat (*5*, *6*) and antioxidant activity in vitro (*7*, *8*). *d*-Limonene has chemopreventive activity in rodent mammary models (*9*, *10*), and citrus pectin shows antiinflammatory activity in rat colon (*11*) and cholesterol lowering effect in human volunteers (*12*).

Some 71600 tons of hassaku fruit were produced in Japan in the year 2000 alone, and 40300 tons were produced in Wakayama prefecture. The fruit weighs about 250−350 g, and the peel color is yellow, similar to that of mature lemons. Processed hassku juice is bitter like grapefruit juice, but removal of the bitterness has been achieved by Wakayama Agricultural Cooperatives Momoyama fruit juice factory (Wakayama, Japan). The dried fruit has been used in natural medicines for stomach ailments.

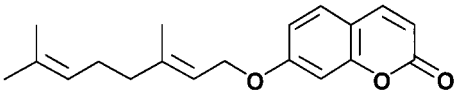

Figure 1. Chemical structure of aurapten (7-geranyloxycoumarin).

It is notable that citrus purification methods for compounds other than aurapten have been well established, and are already being used for the preparation of food additives (*13*). Therefore, the present study was designed to establish a rapid and convenient method for preparing an aurapten-enriched product from hassaku fruit.

MATERIALS AND METHODS

Chemicals and Reagents. Hassaku fruit was supplied by Wakayama Prefectural Fruit Tree Experiment Station (Wakayama, Japan). Hassaku fruit peel oil and juice were supplied by Wakayama Agricultural Cooperatives Momoyama fruit juice factory. Aurapten was purified from hassaku peel oil as previously reported (purity > 99%) (*1*). SEPABEADS SP70 adsorbent was purchased from Mitsubishi Chemical

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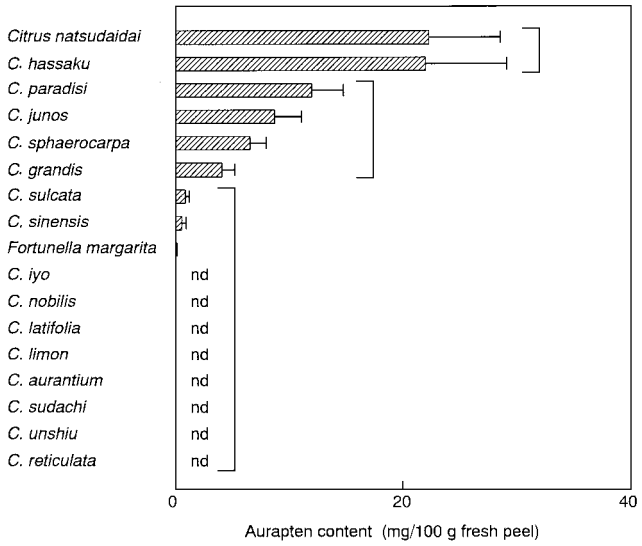

Figure 2. Aurapten contents in fruit peels of Rataceae varieties. Data are expressed as the mean ($n = 6$) ± standard deviation from three experiments.

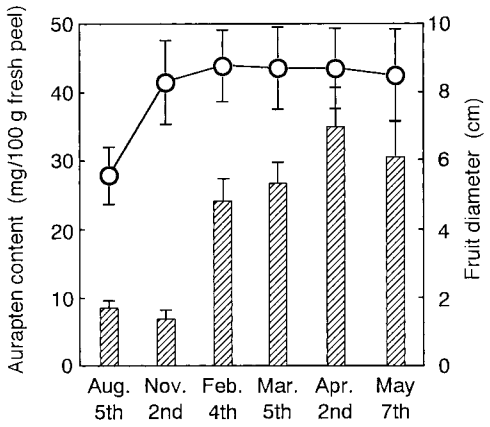

Figure 3. Aurapten content in hassaku fruit peels and fruit diameters. Twenty fruit were used from August–November, and 10 fruit were from January–May in Wakayama Prefecture, Japan. Data are expressed as the mean ($n = 10$) ± standard deviation: (○) fruit diameter (cm); (slashed bars) aurapten contents (mg/100 g fresh peel).

Industries Ltd (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), and were used without further purification.

Fruit Collection and Sample Preparation. Hassaku fruit ($n = 10-20$) was harvested during August (immature) to May (mature), and 17 Rutaceae varieties ($n = 6$, each) were harvested in Wakayama Prefecture, on January 9, 1998. The collected fruits were divided into peel and sarcocarp portions. A 2-g portion each of peel and sarcocarp samples were homogenized with 10 mL of water, and each sample including 5 g of juice was extracted 3 times with 50 mL of chloroform at room temperature. The extracts were centrifuged for 10 min at 1700*g*. The chloroform fractions of lower layers were finally concentrated in vacuo. The dried samples obtained were dissolved in 0.5 mL of 99.7% ethanol, and then filtered through a 0.45-μm filter for HPLC analysis as described below.

Aurapten-Enriched Preparation. Aurapten content in the peel oil supplied by the juice factory was 4.5%(w/w) when it was determined by the above-mentioned method. Precipitates, including aurapten, formed after maintaining the oil at −20 °C for a week. The precipitates were dissolved by heating the oil at 70 °C, and the concentration of aurapten in the dissolved oil was measured to be 16.0% (w/w). In a laboratory-scale test, the dissolved oil was saturated in 30 mL of SP70 (styrene−divinylbenzene synthetic adsorbent; average particle size 450 μm; dry volume 3.1 mL/g). The determination of ethanol concentration was carried out by washing (or eluating) the adsorbent with 1200 mL of 0, 20, 40, 60, 80, and 99.7% (v/v) ethanol. Elution times were determined by washing (or eluating) the adsorbent at concentrations of 40, 60, and 80% (v/v) ethanol.

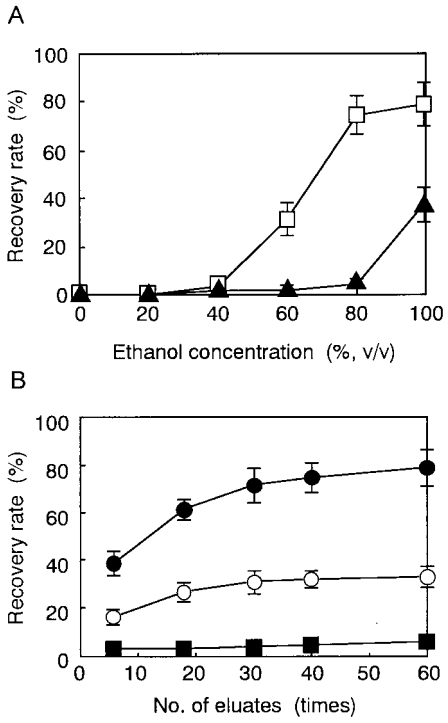

Figure 4. (A) Influence of ethanol concentration on the recovery rates of aurapten and total carotenoids: (□) aurapten; (▲) beta-carotene. (B) Effects of ethanol concentration and the number of eluates on the recovery rate of aurapten: (●) 80% (v/v) ethanol in water; (○) 60% (v/v) ethanol in water; (■) 40% (v/v) ethanol in water. Calculation formula for the recovery rate of aurapten: aurapten content in elution/aurapten content in dissolved oil × 100 (%). Data are expressed as the mean ± standard deviation from three experiments.

In a pilot-scale test, the dissolved oil including aurapten (2900 g) was adsorbed onto 7.5 L of SP70 adsorbent in a stainless steel container. The adsorbent was washed using 51 L (17 L × 3) of 40% (v/v) ethanol and was then filtered through No. 2 filter paper (diameter 30 cm; pore size 5 μm) (TOYO ROSHI, Tokyo, Japan), followed by elution with 120 L (24 L × 5) of 80% (v/v) ethanol. Both the 40% (v/v) ethanol and 80% (v/v) ethanol eluates were combined and concentrated to dryness in vacuo using a film type evaporator SD-167 (Shibata Scientific Inc., Tokyo, Japan).

Stability Tests. Aurapten (500 mg each) in powder form was packaged in polyethylene bags (film thickness 40 μm; volume 0.6 mL), and aurapten (1 and 100 mg each) dissolved in 99.7% ethanol (500 mg each) were put into glass bottles (volume 10 mL). These samples were stored in the following lighting conditions: a dark room (5 °C or 37 °C, <0.1 lux), fluorescent light (25 °C, 500−800 lux), sunlight through a window (23−33 °C, max. 30000 lux), or ultra-violet light (22−24 °C, 70−80 lux) for 180 days.

Aurapten Analysis. After both oil and eluate samples were filtered through 0.45-μm filters, the concentrations of aurapten were determined by high-performance liquid chromatography (HPLC) on a TSK-gel ODS-120T column (250 × 4.6 mm) (TOSOH, Tokyo, Japan). The mobile phase was methanol/water (95:5, v/v) at a flow rate of 0.3 mL/min. Detection wavelength and retention time were 320 nm and 16.0 min, respectively. The concentration of aurapten was calculated from a standard curve for aurapten.

Carotenoid Analysis. Oil (1 g) and eluate samples were extracted 3 times with 10 mL of *n*-hexane at room temperature. The extract was concentrated to 5 mL and the sample obtained was examined by spectrophotometry using a U-best 55 (JASCO Corporation, Tokyo,

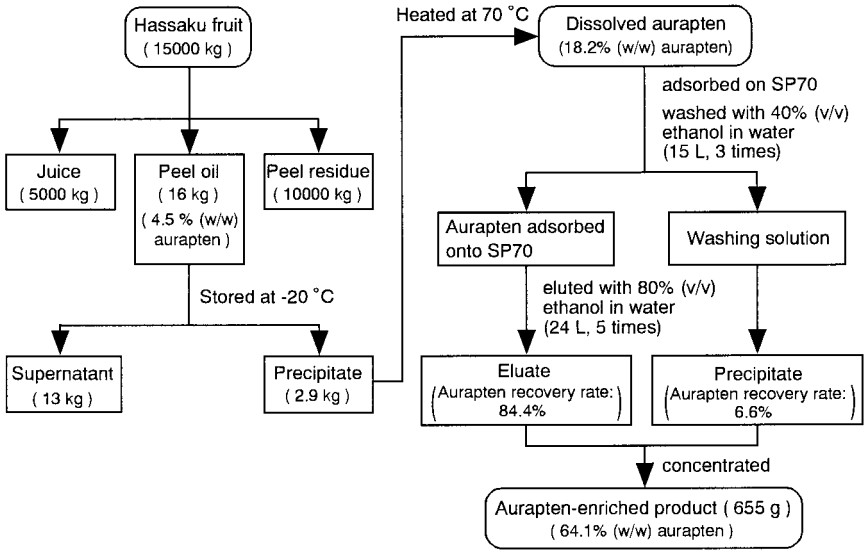

Figure 5. Schematic procedure for aurapten-enriched preparations from hassaku fruit.

Japan) at 451 nm. The concentration of carotenoid was tentatively calculated from a standard curve for beta-carotene.

Flavor Analysis. Analytical gas chromatography was performed with a Hitachi model G-7000 (Hitachi Co., Tokyo, Japan) equipped with a flame ionization detector and a silica fused capillary column Thermon 600T (0.25 mm i.d. × 25 m) (Gas-Chro Kogyo, K. K., Tokyo, Japan) with He at 1.0 mL/min and an 80:1 injector split. Detector and injector were maintained at 250 °C and the column was programmed to be heated from 70 to 230 °C at 3 °C/min. All peak areas on chromatograms were integrated, and the concentration of total flavor was calculated from a standard curve for *d*-limonene.

RESULTS AND DISCUSSION

Aurapten Contents in the Fruit Peels of Rutaceae Plants. We first analyzed the aurapten content of various citrus fruits which are consumed in some countries, including Japan, to identify appropriate sources for aurapten-enriched preparations. As shown in **Figure 2**, marked levels were found in *C. natsudaidai* (natsumikan, 22.5 mg/100 g peel) and *C. hassaku* (hassaku, 22.1 mg/100 g peel), and moderate levels in *C. paradisi* (grapefruit, 12.0 mg/100 g peel), *C. junos* (yuzu, 8.7 mg/100 g peel), *C. sphaerocarpa* (kabosu, 6.6 mg/100 g peel), and *C. grandis* (buntan, 4.0 mg/100 g peel). Other varieties examined showed only trace amounts (<0.7 mg/100 g peel).

Seasonal Changes of Aurapten Contents in Hassaku Peel. We then examined the aurapten content throughout the growing season. As shown in **Figure 3**, aurapten levels in hassaku peel were correlated with growth of the fruit. Aurapten contents were highest in April (34.9 mg/100 g fresh peel), and fruit diameters were maximal in February (8.8 cm). On the other hand, aurapten content in the sarcocarp portion was less than 0.3 mg/kg fresh weight throughout the growing season. Because hassaku fruit, in general, is prepared by juice extractors from late February to middle April, such seasonal changes in the aurapten content may be beneficial to the preparation of aurapten-enriched products.

Preparation of Aurapten-Enriched Products from Hassaku Peel Oil. Because precipitates including aurapten were formed when peel oil was maintained at −20 °C for one week, the precipitates were separated from the oil and were then dissolved by heating at 70 °C. We selected SP70 adsorbent out of 18 ion-exchange resins and 4 adsorbents, because the adsorption rate of aurapten in SP70 was marked (data not shown). As shown in **Figure 4A**, the recovery rates of aurapten with 80 and 99.7% (v/v) ethanol were 74.3 and 78.7%, respectively, and those of total carotenoids were 4.6 and 39.0%, respectively. As the concentration of ethanol increased, the recovery rates of aurapten and total carotenoids were higher. As indicated above, however, the use of 80% (v/v) ethanol was best for selective aurapten elution. As shown in **Figure 4B**, the recovery rate of aurapten with 80% (v/v) ethanol increased as elution times increased with notable saturation following 30−60 times.

Table 1. Recovery of Aurapten, Total Carotenoids, and Total Flavors in Pilot-scale Tests

	aurapten (g)	total carotenoids (g)	total flavors (g)
dissolved aurapten[a]	527	1.44	1950
eluate with 80% (v/v) ethanol in water			
first	178	0.02	n.a.[b]
second	140	0.02	n.a.
third	70	< 0.01	n.a.
fourth	35	< 0.01	n.a.
fifth	22	< 0.01	n.a.
aurapten-enriched product	420	0.05	<0.2

[a] Dissolved aurapten was dissolved precipitate in peel oil obtained from 15000 kg of hassaku fruit as described in Materials and Methods and as shown in **Figure 5**. [b] n.a., not analyzed.

As summarized in **Figure 5** and **Table 1**, in a pilot-scale test, the recovery rate of aurapten in the first 24 liters of 80% (v/v) ethanol was highest, and the total recovery rate in the eluate of 80% (v/v) ethanol from dissolved aurapten was 84.4%. Aurapten in the washing solution precipitated to show poor solubility in 40% (v/v) ethanol. In aurapten-enriched preparations compared with dissolved aurapten, the removal rates of total carotenoids and total flavors were 96.5 and >99.9%, respectively. Some peaks, other than *d*-limonene, were detected showing trace levels (<0.2 g/655 g final product) in gas chromatograms of the final product. The final product obtained was a pale yellow powder, with a slightly orange-like flavor, and no bitterness.

Stability of Aurapten in Aurapten-Enriched Preparation. Using an aurapten-rich preparation and purified aurapten, the stabilities of each were examined in powder forms and in ethanol solutions. To evaluate the exposure of juice products containing fortified aurapten to sunlight through glass (such as during storage or on sale in stores), although stability of aurapten in

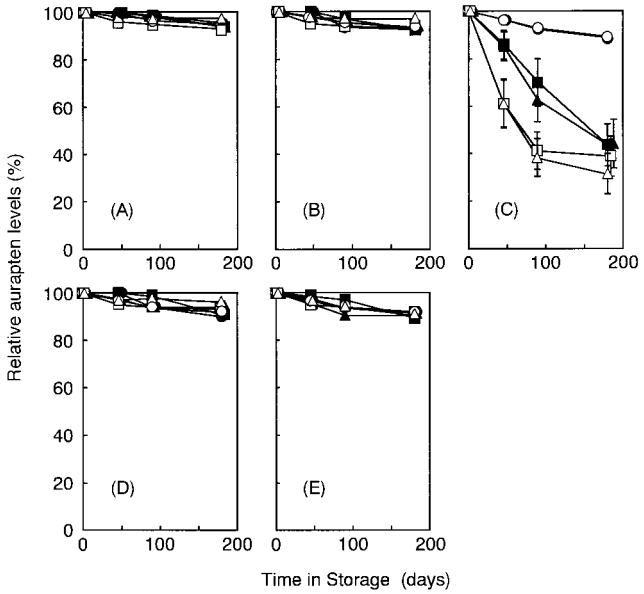

Figure 6. Changes in the aurapten content in powder and ethanol during storage: (A) dark room (5 °C, < 0.1 lux); (B) fluorescent light (25 °C, 500–800 lux); (C) sunlight through a window (23–33 °C, max. 30000 lux); (D) dark room (37 °C, < 0.1 lux); (E) ultraviolet light (22–24 °C, 70–80 lux). (○) Purified aurapten powder, 500 mg/0.6 mL polyethylene bag; (□) purified aurapten in ethanol, 1 mg/10 mL ethanol; (△) purified aurapten in ethanol, 100 mg/10 mL ethanol; (●) aurapten-enriched preparation powder, 500 mg/0.6 mL polyethylene bag; (■) aurapten-enriched preparation in ethanol, 1 mg/10 mL ethanol; (▲) aurapten-enriched preparation in ethanol, 100 mg/10 mL ethanol. Data are expressed as the mean ± standard deviation from three experiments.

juice was not examined, sunlight tests were conducted. As shown in **Figure 6**, aurapten content in 99.7% ethanol containing purified and aurapten-enriched preparations, 1 mg each, decreased to 39.0 and 43.8% of the initial levels, respectively, when exposed to sunlight for 180 days. Similarly, samples containing purified and aurapten-enriched preparations, 100 mg each, also decreased to 31.3 and 42.5%, respectively. These decreased rates were not significantly different ($P > 0.05$) from each other after 180 days, but there was a significant difference ($P < 0.05$) between the sample groups after 45 and 90 days. The degradation product(s) of aurapten, however, have not yet been identified.

CONCLUSION

We have established an efficient method for preparing aurapten from hassaku fruit peel oil. Because the toxicity of aurapten is low (Ames test was negative, and LD50 > 0.75 g/kg body weight in mice, data not shown), aurapten-enriched preparations may be applicable as food additives.

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