Abstract of Doctor Thesis

Title: **Hesperidin Accumulation during Fruit and Leaf Development in Satsuma Mandarin (*Citrus unshiu*)**

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In this thesis, experiments were performed to validate efficient extraction method of hesperidin from fruit peel of *C. unshiu*, and to understand how hesperidin accumulates during development of the fruit and leaf.

In Chapter 1, simultaneous extraction by microwave-irradiation and crystallization were performed in the same pot of solvent of 70% (v/v) aqueous ethanol for isolation of hesperidin from thinned immature fruit peels of *Citrus unshiu* as refining of *Citrus* waste biomass. The hesperidin content in immature fruits peels was about 3.2-fold higher than that of mature fruit. After microwave-assisted extraction (MAE), the yield of hesperidin reached 58.6 mg/g, which was comparable to the amount obtained after extraction using DMSO:methanol (1:1, v/v) as a solvent for 30 min at room temperature. Heating temperature and time for isolation of hesperidin crystallites were optimized as 140°C and 8 min by using response surface methodology. Under this optimal condition, 86.8% (47.7 mg/g) of total hesperidin was isolable by MAE and low-temperature storage (5°C, 24 h).

In Chapter 2, the morphology and distribution of crystals found in *C. unshiu* peels were observed microscopically. The crystals were more abundant in immature peel and were observed particularly in areas surrounding vascular bundles, around the border between the flavedo and albedo layers and just below the epidermal cells. In the morphological analysis by SEM, needle-shaped crystals aggregated and formed clusters

of spherical crystals. Raman spectra of the crystals in the peel sections were consistent with those of the hesperidin standard. It was demonstrated that the main component of the crystals was identified using Raman microscopy to be hesperidin for the first time.

In Chapter 3, detection of hesperidin crystals and clarification of quantitative dynamics of hesperidin were performed in the current-year branch of *C. unshiu* by Raman microscopy, HPLC and LC-MS. Needle-shaped crystals were observed in leaf, petiole and stem, of young summer flush shoots although few crystals were found in these portions of grown spring flush shoots. They were identified as hesperidin at the accumulated site. Concentration of hesperidin was higher in the young shoot than in the grown shoot. The concentration in young stem decreased with a steep gradient from the tip to the base. Quantitative dynamics of hesperidin during leaf development had two different phases in the young shoot; phase (I) with a high and constant concentration and a linear increase in total content per leaf of hesperidin, and phase (II) with the stable content and a linear decrease in the concentration. The two phases in quantitative dynamics of hesperidin accumulation during leaf development in young shoots suggests a significant change in hesperidin biosynthesis during the transition phase.

Hesperidin is supposed to be involved in stress resistance against pathogen infection, UV-B irradiation and nutrient deficiencies. In this thesis, it was demonstrated that hesperidin accumulates rapidly during early developmental stages in fruits and leaves. It is plausible that hesperidin functions as a defensive compound against environmental stresses in stress-sensitive young tissues that lack physical barriers composed of thick surface structures, such as cuticles and waxes in mature tissues.