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**Identification and quantitative analysis of β-cryptoxanthin and β-citraurin esters in Satsuma mandarin fruit during the ripening process**

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## ABSTRACT

In this study, to investigate the xanthophyll accumulation in citrus fruits, the major fatty acid esters of β-cryptoxanthin and β-citraurin were identified, and changes in their contents were investigated in two Satsuma mandarin varieties, 'Miyagawa-wase' and 'Yamashitabeni-wase', during the ripening process. The results showed that β-cryptoxanthin and β-citraurin were mainly esterified with lauric acid, myristic acid, and palmitic acid in citrus fruits. During the ripening process, β-cryptoxanthin laurate, myristate, and palmitate were accumulated gradually in the flavedos and juice sacs of the two varieties. In the flavedo of 'Yamashitabeni-wase', β-citraurin laurate, myristate, and palmitate were specifically accumulated, and their contents increased rapidly with a peak in November. In addition, functional analyses showed that CitCCD1 and CitCCD4 efficiently cleaved the free β-cryptoxanthin, but not the β-cryptoxanthin esters *in vitro*. The substrate specificity of CitCCDs towards free β-cryptoxanthin indicated that β-cryptoxanthin esters might be more stable than free β-cryptoxanthin in citrus fruits.

Keywords: Esterification, Flavedo, Juice sacs, Miyagawa-wase, Xanthophyll, Yamashitabeni-wase

## 1. Introduction

Xanthophylls are oxygenated carotenoids widely distributed in nature, and fulfill critical functions in plant growth and development. As xanthophylls contain hydroxyl groups, they are often acylated with different fatty acids and present in esterified forms in plants. The esterification by fatty acids does not affect the antioxidant activities of xanthophylls on superoxide anion, hydroxide radical and singlet oxygen, but it improves their stability towards heat treatment and UV light (Subagio, Wakaki, & Morita, 1999; Schweiggert, Kurz, Schieber, & Carle, 2007; Fu, Xie, Gan, Ma, Zhu, & Pan, 2010). Recent studies suggested that xanthophyll esters were more stable than the free xanthophyll in fruits during storage and process (Bunea, Socaciu, Pintea, 2014). In addition, the esterification of xanthophyll plays important roles in regulating plant growth and development. In tomato flowers, the xanthophyll esterification promoted the sequestration of carotenoids in the chromoplast, which was important for normal chromoplast development (Ariizumi et al., 2014). In fruits and vegetables, the esterification degree of xanthophyll is species-specific (Bunea, Socaciu, Pintea, 2014). In green vegetables, such as spinach and broccoli, no xanthophyll esters are detected. In contrast, xanthophyll esters are abundant in certain fruits, such as papaya, mango, orange, and apple (Schweiggert, Steingass, Esquivel, & Reinhold, 2012; Ornelas-Paz, Yahia, & Gardea-Bejar, 2007; Delgado-Pelayo, Gallardo-Guerrero, & Hornero-Méndez, 2014).

β-Cryptoxanthin is an important xanthophyll accumulated in many fruits and vegetables. Some epidemiological studies reported that dietary intake of β-cryptoxanthin reduced the risks of certain diseases, especially cancers, diabetes and rheumatism because of its antioxidant activity (Sugiura et al., 2011; Takayanagi et al., 2011; Yamaguchi 2012; Iskandar et al., 2013). In the recent years, the accumulation of β-cryptoxanthin esters has

been reported in several fruits and vegetables. It was found that β-cryptoxanthin is mainly esterified with saturated fatty acids such as lauric, myristic and palmitic acids in chili, papaya, peach, and persimmon (Breithaupt & Bamedi, 2001). Additionally, as the fatty acids can be efficiently hydrolyzed from β-cryptoxanthin esters before intestinal absorption in human's body, the bioavailability of β-cryptoxanthin esters is comparable with the unesterified form (Breithaupt, Weller, Wolters, & Hahn, 2003).

Citrus fruits are a rich source of dietary β-cryptoxanthin, which accounts for more than 80% of total carotenoid in the juice sacs of Satsuma mandarin (Kato et al., 2004). In several citrus varieties, β-cryptoxanthin can be specifically cleaved by carotenoid cleavage dioxygenase4 (CitCCD4), and the cleavage of β-cryptoxanthin led to the formation of β-citraurin (Ma et al., 2013; Rodrigo et al., 2013). In the citrus fruits, it has been found that β-cryptoxanthin and β-citraurin are present in both free and esterified forms (Philip, Chen, & Nelson, 1989; Wada et al., 2013). In the past decade, the accumulation of xanthophylls has been extensively investigated in citrus fruits during the ripening process (Kato, Ikoma, Matsumoto, Sugiura, Hyodo, & Yano, 2004; Rodrigo, Marcos, & Zacarías, 2004; Rodrigo & Zacarías 2007; Ma et al., 2013; Wei et al., 2014). However, since the analysis of carotenoids in citrus fruits was usually performed on saponified extracts, in which the fatty acids were hydrolyzed from the xanthophylls, the accumulation of the β-cryptoxanthin and β-citraurin esters was not reflected in the previous studies. In the present study, to reveal the accumulation of native xanthophyll in citrus fruits, the major fatty acid esters of β-cryptoxanthin and β-citraurin were identified, and their accumulation were investigated in the two varieties of Satsuma mandarin, 'Miyagawa-wase' and 'Yamashitabeni-wase' during the ripening process. The results presented in this study provided novel information on xanthophyll accumulation in citrus

fruits, which will contribute to further elucidating the mechanism of carotenoid biosynthesis in citrus fruits.

## 2. Materials and methods

### *2.1. Plant Materials*

Two varieties of Satsuma mandarin (*Citrus unshiu* Marc.), 'Miyagawa-wase' and 'Yamashitabeni-wase', cultivated at the Fujieda Farm of Shizuoka University (Shizuoka, Japan) were used as materials. Fruit samples were collected periodically from September to January. According to the changes of colors in the flavedo, the ripening process was separated into three stages: green stage (September), transition stage (from October to November), and mature stages (from December to January; Supplemental Fig. 1). The flavedos and juice sacs were separated from the sampled fruits, immediately frozen in liquid nitrogen, and stored at -80 ˚C until analysis.

### *2.2. Extraction of Carotenoids*

The extraction of carotenoids was conducted according to the methods described by Kato et al. (2004). Carotenoids were extracted from the samples using an hexane:acetone:ethanol (2:1:1 [v/v]) solution containing 0.1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and 10% (w/v) magnesium carbonate basic. The extraction procedure was repeated three times until complete extraction of pigments. The carotenoid extraction was centrifuged at 3,000 rpm for 10 min at 10˚C, and the supernatants were collected and evaporated to dryness. After the organic solvents had been completely evaporated, the extracts containing carotenoids esterified to fatty acids were saponified with 20% (w/v) methanolic KOH. Water-soluble extracts were then removed by adding NaCl-saturated water. The pigments repartitioned into the diethyl ether phase were recovered and evaporated to dryness. Subsequently, the residue was redissolved in 5 mL of a TBME:

methanol (1:1 [v/v]) solution, and analyzed by using HPLC.

To analyze the esterified forms of β-cryptoxanthin and β-citraurin, carotenoid extraction was carried out without saponification. Samples were extracted three times with an hexane:acetone:ethanol (2:1:1 [v/v]) solution containing 0.1% (w/v) 2,6-di-*tert*-butyl-4-methylphenol and 10% (w/v) magnesium carbonate basic. After centrifuged at 3,000 rpm for 10 min at 10°C, the supernatants were collected and evaporated to dryness. The residue was redissolved in 5 mL of a TBME: methanol (1:1 [v/v]) solution and analyzed by using HPLC.

*2.3. HPLC analyses of carotenoids*

Quantitative analysis of carotenoids was carried out by using HPLC according to the method of Kato et al. (2004). An aliquot (20 μL) was injected into a reverse-phase HPLC system (Jasco, Tokyo, Japan) fitted with a YMC C-30 carotenoid S-5 column of 250-× 4.6-mm-i.d. (Waters, Milford, MA) at a flow rate of 1 mL $min^{-1}$. The eluent was monitored by a photodiode array detector (MD-2015, Jasco). The standard of β-cryptoxanthin was obtained from Sokenkagaku (Tokyo). The standard of β-citraurin was prepared according to the methods described by Ma et al. (2013). As esterification of hydroxyl group does not modify the chromophore, xanthophyll esters exhibits identical UV-visible spectrum with those unesterified xanthophyll. In the present study, β-cryptoxanthin and β-citraurin esters were identified by comparison the UV-visible spectra with the standards in the HPLC chromatograms. The eluent of each β-cryptoxanthin and β-citraurin ester was further isolated, and subjected to mass analysis. To identify β-cryptoxanthin esters, fast atom bombardment mass spectrometry (FAB-MS) analysis was performed with a JMS-770V high-resolution mass spectrometer (JEOL, Tokyo, Japan). To identify β-citraurin esters, direct analysis in real time mass spectrometry (DART-MS) analysis was conducted

using a JMS-T100LP high-resolution mass spectrometer (JEOL). The contents of free β-cryptoxanthin and β-citraurin were estimated by the standard curves and expressed as micrograms per gram fresh weight (μg $g^{-1}$ FW). The contents of β-cryptoxanthin and β-citraurin esters were determined according to the method of Schiedt & Liaaen-Jensen (1995). The calculation of xanthophyll esters was based on molar extinction coefficient ε ($\varepsilon = E_{1cm}^{1\%} \times \frac{\text{molecular weight}}{10}$; $E_{1cm}^{1\%}$, specific extinction coefficient), which was identical between free xanthophyll and xanthophylls esters. Carotenoid quantification was performed in three replicates

*2.4. Functional analyses of CitCCD1 and CitCCD4 in vitro*

The recombinant plasmids pGEX-6P-1-*CitCCD1* and pCold I-*CitCCD4* constructed previously were used to investigate the substrate specificity of CitCCDs towards free and esterified β-cryptoxanthin (Kato et al., 2006; Ma et al., 2013). The protein expression and purification was conducted according to the methods described by Kato et al. (2006). The recombinant plasmids pGEX-6P-1-*CitCCD1* and pCold I-*CitCCD4* were transformed into XL1-Blue cells, respectively. The culture solution of each clone was incubated at 27 ˚C until an $OD_{600}$ of 0.7 was reached. The expression of proteins was induced with 0.1 mM IPTG, and the cultures were grown at 27 ˚C (pGEX-6P-1-CitCCD1) or 15 ˚C (pCold I-CitCCD4) for an additional 14 h. The cells were harvested and resuspended in 2.5 ml of extraction buffer (0.1 M Tris-HCl, pH 7.2, 30 mM Na-ascorbate, 5 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.05% (v/v) Triton X-100). After sonication and centrifugation, the protein was purified using a PD-10 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

Four substrates, free β-cryptoxanthin, β-cryptoxanthin laurate, myristate, and palmitate were prepared from the flavedo of Satsuma mandarin. The four substrates were separated

and collected by HPLC, respectively. The purity of each standard obtained was more than 95%, which was calculated based on the peak area at 452 nm by HPLC. The enzymatic activities of the recombinant CitCCD1 and CitCCD4 protein were assayed in a reaction mixture consisting of 0.1 M Tris-HCl, pH 7.2, 30 mM Na-ascorbate, 50 μM $FeSO_4$, 20 μg catalase, 0.05% (v/v) Triton X-100, 20% (v/v) glycerol, 1 mM substrate and 5 μg of the recombinant protein in a total volume of 200 μl at 27 ˚C for 3 h. After the incubation, 1 ml of water was added to the reaction mixture. The reaction products were partitioned three times into 1.2 ml of ethyl acetate, evaporated to dryness, and dissolved in methanol. An aliquot (20 μl) was injected into a reverse-phase HPLC system (Jasco) fitted with a YMC C-30 carotenoid S-5 column of 250-×4.6-mm-i.d. (Waters) at a flow rate of 1 ml $min^{-1}$. The eluent was detected with a photodiode array detector (MD-2015, Jasco). To assay CitCCD1 activity, the gradient elution schedule consisted of 20% (v/v) methanol and 80% (v/v) water (0–5 min), a linear gradient to 100% (v/v) methanol (5–25 min), and 100% (v/v) methanol (25–40 min). To assay CitCCD4 activity, the gradient elution schedule consisted of 95% (v/v) methanol, 1% (v/v) TBME, and 4% water (0–30 min), and 6% (v/v) methanol, 90% (v/v) TBME, and 4% water (30–90 min).

*2.5. Statistical analysis*

All values are shown as the mean ± SE for three replicates. The data were analyzed. Tukey's HSD test (at $P<0.05$) and student's *t*-test (at $P<0.05$ and $P<0.01$) were used to compare the treatment means.

**3. Results**

*3.1. Isolation and identification of β-cryptoxanthin esters from citrus fruits*

In Satsuma mandarin, β-cryptoxanthin is a major carotenoid accumulated in the juice sacs, which accounts for approximately 80% of total carotenoid, and its content is much

higher than other xanthophylls in the juice sacs (Kato et al., 2004; Fig. 1A and B). In this study, the isolation and identification of the β-cryptoxanthin esters were conducted using the juice sacs as materials. As shown in Fig. 1A and B, three major xanthophyll esters (peak 1, eluted at 68.3 min; peak 2, eluted at 71.5 min; peak 3, eluted at 75.7 min) were detected in the juice sacs, which were observed in the HPLC chromatogram of the non-saponified carotenoid extracts, but disappeared in the HPLC chromatogram of the saponified carotenoid extracts. The analyses of UV-Vis spectrum showed the absorption maxima of the three xanthophyll esters were identical with that of β-cryptoxanthin (426, 452, 478 nm; Table 1). To further confirm they were the β-cryptoxanthin esters, the eluents of the three peaks (peak 1, eluted at 68.3 min; peak 2, eluted at 71.5 min; peak 3, eluted at 75.7 min) were isolated and saponified with 20% (w/v) methanolic KOH. After removing the fatty acids by saponification, free β-cryptoxanthin was detected in the eluents of the three peaks by HPLC. Thus, these results suggested that the three peaks (eluted at 68.3 min, 71.5 min, and 75.7 min) were the β-cryptoxanthin esters. To identify the compositions of the β-cryptoxanthin esters, the eluents of the three peaks was subjected to mass analysis. The mass spectra showed a molecular ion at m/z 734.6 ($[M+H]^+$) for peak 1, m/z 762.6 ($[M+H]^+$) for peak 2, and m/z 790.6 ($[M+H]^+$) for peak 3 (Table 1). The absorption maxima and mass spectrum analyses suggested that the peak 1, 2 and 3 were β-cryptoxanthin laurate, myristate, and palmitate, respectively. In addition, β-cryptoxanthin laurate, myristate, and palmitate were also detected as the major β-cryptoxanthin esters in the flavedos of 'Miyagawa-wase' and 'Yamashitabeni-wase' (Fig. 1C and D).

*3.2. Isolation and identification of β-citraurin esters from citrus fruits*

β-Citraurin, which is the cleavage product of β-cryptoxanthin, was specifically

accumulated in the flavedo of 'Yamashitabeni-wase' (Ma et al., 2013). In the present study, three peaks (peak 4, at 57.2 min; peak 5, 61.1 min; peak 6, 66.0 min), which exhibited identical absorption maximum with β-citraurin (462 nm; Table 1), were detected in HPLC chromatogram of the non-saponified carotenoid extracts (Fig. 1E). The eluents of the three peaks (peak 4, at 57.2 min; peak 5, 61.1 min; peak 6, 66.0 min) were isolated and saponified with 20% (w/v) methanolic KOH. After saponification, free β-citraurin was detected in the eluents of the three peaks by HPLC, which confirmed that peak 4, 5 and 6 were β-citraurin esters (Fig. 1E). The mass spectra of the three β-citraurin esters showed a molecular ion at m/z 614.9, ($[M+H]^+$) for peak 4, m/z 642.5 ($[M+H]^+$) for peak 5, and m/z 671.1 ($[M+H]^+$) for peak 6 (Table 1). The absorption maximum and mass spectrum analyses suggested that peak 4, 5, and 6 were β-citraurin laurate, myristate, and palmitate, respectively (Fig. 1E).

*3.3. Changes in the contents of free and esterified β-cryptoxanthin in citrus fruits during the ripening process*

In the present study, to reveal the accumulation of the native β-cryptoxanthin composition during the ripening process, the changes in the contents of the free β-cryptoxanthin and three major β-cryptoxanthin esters were investigated using the carotenoid extracts without saponification. As shown in Fig. 2A, the contents of β-cryptoxanthin laurate, myristate, and palmitate increased rapidly, and researched a peak in December in the flavedo of 'Miyagawa-wase'. In 'Yamashitabeni-wase', β-cryptoxanthin laurate, myristate, and palmitate accumulated gradually in the flavedo during the ripening process. The content of free β-cryptoxanthin increased and researched a peak in November in the flavedos of 'Miyagawa-wase' and 'Yamashitabeni-wase'. In the flavedo, the contents of the free and esterified β-cryptoxanthin in 'Yamashitabeni-wase'

were lower than those in 'Miyagawa-wase' in November and December. In addition, the esterification degree of β-cryptoxanthin increased gradually in the flavedos of 'Miyagawa-wase' and 'Yamashitabeni-wase' during the ripening process (Supplemental Fig. 2). In December, more than 90% of β-cryptoxanthin was present in esterified forms in the flavedos of 'Miyagawa-wase' and 'Yamashitabeni-wase', and β-cryptoxanthin laurate, myristate, and palmitate accounted for 33%, 27%, and 31% of total β-cryptoxanthin, respectively.

In the juice sacs, the contents of free β-cryptoxanthin, β-cryptoxanthin laurate, myristate, and palmitate increased gradually during the ripening process in the two Satsuma mandarin varieties. Moreover, the contents of free and esterified β-cryptoxanthin in 'Yamashitabeni-wase' were lower than those in 'Miyagawa-wase' (Fig. 2B). Similar to the flavedo, over 80% of β-cryptoxanthin was present in esterified forms in the juice sacs of 'Miyagawa-wase' and 'Yamashitabeni-wase' during the ripening process. In December, β-cryptoxanthin laurate, myristate, and palmitate accounted for 29%, 38%, and 18% of total β-cryptoxanthin in the juice sacs of ‘Miyagawa-wase’ and ‘Yamashitabeni-wase’ (Supplemental Fig. 3).

*3.4. Changes in the content and composition of β-citraurin in citrus fruits during the ripening process*

In 'Miyagawa-wase', β-citraurin was undetectable throughout the ripening process. In 'Yamashitabeni-wase', the contents of free β-citraurin, β-citraurin laurate, myristate, and palmitate increased significantly, and reached a peak in November in the flavedo (Fig. 3). Moreover, most β-citraurin was esterified in the flavedo of 'Yamashitabeni-wase' (Supplemental Fig. 4). In November, 96% of β-citraurin was present in ester forms in the flavedo, and β-citraurin laurate, myristate, and palmitate accounted for 28%, 38%, and

30% of total β-citraurin, respectively.

*3.5. Substrate specificities of CitCCD1 and CitCCD4 towards free and esterified β-cryptoxanthin*

CitCCD1 and CitCCD4 are two carotenoid cleavage dioxygenases that cleave β-cryptoxanthin producing different apocarotenoids (Kato et al., 2004; Ma et al., 2013) In the present study, the substrate specificities of CitCCD1 and CitCCD4 towards free and esterified β-cryptoxanthin were further investigated (Figs. 4 and 5). The results showed that free β-cryptoxanthin was cleaved by both CitCCD1 and CitCCD4 *in vitro*. In the case of CitCCD1, free β-cryptoxanthin was cleaved at the 9,10 and 9',10' positions, producing 3-hydroxy-β-ionone and β-ionone, which were detected at 300 nm, and C14 dialdehyde, which was detected in 400 nm. In the case of CitCCD4, free β-cryptoxanthin was cleaved at 7,8 or 7',8' position producing β-citraurin and *trans*-β-8'-carotenal. In contrast to free β-cryptoxanthin, no cleavage products were observed when the three β-cryptoxanthin esters were used as substrates, indicating that CitCCD1 and CitCCD4 were specific for free β-cryptoxanthin instead of its esters.

## 4. Discussion

In the recent years, the profile of β-cryptoxanthin esters has been characterized in several fruits and vegetables. It has been reported that β-cryptoxanthin laurate, myristate, and palmitate were present as the main β-cryptoxanthin esters in chili, papaya, peach, and persimmon (Breithaupt & Bamedi, 2001). In papaya, the content of β-cryptoxanthin laurate, which was around 8.92 μg $g^{-1}$, was much higher than other plant species. In the present study, the results showed that β-cryptoxanthin laurate, myristate, and palmitate were the major β-cryptoxanthin esters in the two varieties of Satsuma mandarin, 'Miyagawa-wase' and 'Yamashitabeni-wase' (Fig. 1). Similarly, the predominant

accumulation of β-cryptoxanthin laurate, myristate, and palmitate was also observed in other citrus varieties, such as orange, ponkan, and kiyomi (Wada et al., 2013). β-Citraurin, which is a breakdown product of β-cryptoxanthin, is only accumulated in a few citrus verities. Recently, the accumulation of β-citraurin esters has been reported in some tropical fruits (Mercadante, Britto, & Rodriguez-Amaya, 1998; Murillo et al., 2013). However, the information on β-citraurin esters in citrus fruits was still limited (Philip, Chen, & Nelson, 1989). In this study, β-citraurin esters was isolated from the fruits of Satsuma mandarin, and the results showed that the fatty acids that esterified with β-citraurin were identical with those of β-cryptoxanthin esters. In 'Yamashitabeni-wase', β-citraurin laurate, myristate, and palmitate, were the major β-citraurin esters accumulated in the flavedo. In Satsuma mandarin, it has been reported that the main members of the fatty acids were oleic acid (C18:1), linoleic acid (C18:2), palmitic acid (C16:0) and linolenic acid (C18:3), which accounted for 39%, 26%, 19%, and 10% of total fatty acid, respectively (Wada et al., 2011). However, in the present study, the results showed that β-cryptoxanthin and β-citraurin were mainly esterified with lauric acid (C12:0), myristic acid (C14:0), and palmitic acid (C16:0) in the two varieties of Satsuma mandarin, indicating that the formation of β-cryptoxanthin and β-citraurin esters might be independent of the concentrations of fatty acids. These results suggested that the biosynthesis of β-cryptoxanthin and β-citraurin esters was substrate specific; enzymes that catalyze β-cryptoxanthin and β-citraurin esterification preferentially used lauric acid (C12:0), myristic acid (C14:0), and palmitic acid (C16:0) as substrates in citrus fruits. The similar phenomenon was also observed in *Sarsaparilla* Berries (Delgado-Pelayo & Hornero-Méndez, 2012).

In the previous studies, it has been reported that β-cryptoxanthin and β-citraurin

accumulated gradually in citrus fruits during the ripening process (Kato et al., 2004; Ma et al., 2013; Ma et al., 2016). However, the analyses of the β-cryptoxanthin and β-citraurin contents in these studies were performed using the saponified carotenoid extracts. In the saponification process, fatty acids were removed from β-cryptoxanthin and β-citraurin, and the contents of β-cryptoxanthin and β-citraurin was calculated as a sum of free and esterified forms. Thus, the native accumulation of β-cryptoxanthin and β-citraurin was not reflected in the previous studies. In the present study, to further investigate the accumulation of native xanthophyll composition in citrus fruits, the analyses of β-cryptoxanthin and β-citraurin contents were performed using the carotenoid extracts without saponification. The results showed that the free β-cryptoxanthin, β-cryptoxanthin laurate, myristate, and palmitate were simultaneously accumulated in the flavedos and juice sacs of 'Miyagawa-wase' and 'Yamashitabeni-wase' during the ripening process (Fig. 2). In the flavedo of 'Yamashitabeni-wase', where β-citraurin is specifically accumulated, the contents of free β-citraurin, β-citraurin laurate, myristate, and palmitate increased with a peak in November during the ripening process. In addition, we found that more than 80% of β-cryptoxanthin and β-citraurin were present in esterified forms in the fruits of 'Miyagawa-wase' and 'Yamashitabeni-wase', and the esterification degree of β-cryptoxanthin increased gradually in the flavedos during the ripening process. In plants, the xanthophyll esterification occurs during the conversion of chloroplast to chromoplast, and it promotes the sequestration of carotenoids in chromoplast. The biosynthesis of xanthophyll esters in tomato was important for chromoplast development and carotenoid accumulation during the ripening process (Ohmiya, 2013; Ariizumi et al., 2014). In the present study, the massive accumulation of β-cryptoxanthin and β-citraurin esters in the flavedo and juice sacs indicated that β-cryptoxanthin and β-citraurin were preferentially

to be esterified in citrus fruits, and biosyntheses of β-cryptoxanthin and β-citraurin esters might contribute to carotenoid accumulation and the pigmentation of citrus fruits during the ripening process. In addition, since β-cryptoxanthin esters exhibited comparable bioavailability with the free β-cryptoxanthin, β-cryptoxanthin esters that accumulated in citrus fruits also serve as an important source of dietary xanthophylls for humans (Breithaupt, Weller, Wolters, & Hahn, 2003).

CCDs are the key enzymes that catalyze the oxidative cleavage of carotenoids with strict regional and substrate specificity. In Arabidopsis, it has been reported that AtCCD1 preferred to cleave the apocarotenoids rather than bicyclic carotenoids, while AtCCD4 was specific to all-*trans*-configured bicyclic and monocyclic carotenoids (Schmidt, Kurtzer, Eisenreich, & Schwab, 2006; Ilg, Yu, Schaub, Beyer, & Al-Babili, 2010; Bruno et al., 2016). To date, however, the information on the substrate specificity of CCDs towards free and esterified xanthophylls was still limited. In the present study, the results showed that CitCCD1 and CitCCD4 efficiently cleaved the free β-cryptoxanthin *in vitro*, but not the β-cryptoxanthin esters. This result was in agreement to Ariizumi et al. (2014), which reported that NCED might specifically target free-form 9-*cis*-xanthophylls instead of their esters in tomato petals. In the recent years, the crystallographic structures of two CCD enzymes, ACO and VP14, have been characterized, which provided insights into elucidating the substrate specificity of CCD enzymes (Messing et al., 2010; Sui, Zhang, Golczak, Palczewski, &Kiser, 2016). In the structure of ACO, there was a tunnel that entered the reaction centre. Kloer & Schulz (2006) reported that larger substrates, such as β-carotene, cannot pass through the narrow tunnel, thus they can not be cleaved by ACO. In the present study, it was presumed that as structures of β-cryptoxanthin esters were different from free β-cryptoxanthin, β-cryptoxanthin esters might not be able to enter the

active center of the CCDs enzymes, and as a result they were not cleaved by CitCCD1 or CitCCD4. The results presented herein indicated that esterified β-cryptoxanthin might be more stable than the free β-cryptoxanthin in citrus fruits. Moreover, the substrate specificity of CitCCDs towards free β-cryptoxanthin instead of its esters might contribute to the predominant accumulation of β-cryptoxanthin esters in citrus fruits.

Recently, several efforts have been devoted to elucidate formation of xanthophyll esters in plants. In tomato, a carotenoid modifying gene *Pale Yellow Petal 1* (*PYP1*) was identified, which was an essential factor in xanthophyll esterification and yellow flower pigmentation in flowers (Ariizumi et al., 2014). In wheat, it has been reported that two loci on chromosomes 7D and $7H^{ch}$ were important for lutein esterification, and a GDSL-like gene on the short arm of chromosome 7D was required for the esterification of lutein (Ahmad et al., 2015; Mattera, Hornero-Méndez, & Atienza, 2017). In citrus, however, the molecular mechanism by which xanthophylls are esterified with fatty acids is completely unknown. In the future research, the identification of the key enzymes that catalyze the xanthophyll esterification will be of great importance and contribute to further elucidating the accumulation of xanthophyll in citrus fruits.

## 5. Conclusion

In the present study, the major fatty acid esters of β-cryptoxanthin and β-citraurin were characterized in the two varieties of Satsuma mandarin, 'Miyagawa-wase' and 'Yamashitabeni-wase'. The results showed that β-cryptoxanthin and β-citraurin were mainly esterified with lauric acid, myristic acid, and palmitic acid in citrus fruits. During the ripening process, the β-cryptoxanthin and β-citraurin esters were predominantly accumulated in citrus fruits. In addition, functional analyses showed that CitCCD1 and CitCCD4 specifically cleaved the free β-cryptoxanthin. The substrate specificity of

CitCCDs towards free β-cryptoxanthin indicated that esterified xanthophylls might be more stable than the free xanthophylls in plants. The results presented in this study provided more information on the accumulation of carotenoid in citrus fruits, which will contribute to further elucidating the mechanism of carotenoid metabolism in plants.

**Studied compounds**

1. β-Cryptoxanthin
2. β-Citraurin
3. β-Cryptoxanthin laurate,
4. β-Cryptoxanthin myristate
5. β-Cryptoxanthin palmitate
6. β-Citraurin laurate
7. β-Citraurin myristate
8. β-Citraurin palmitate
9. 3-Hydroxy-β-ionone
10. β-Ionone

**Conflict of interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version.

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**Table 1.** UV/Vis and MS spectra characteristics of β-cryptoxanthin and β-citraurin esters

| Peak | Carotenoids | Retention time | UV/Vis λmax(nm) | $[M+H]^+$ |
|---|---|---|---|---|
| 1 | β-cryptoxanthin laurate | 68.3 | 426,452,478 | 734.6 |
| 2 | β-cryptoxanthin myristate | 71.5 | 426,452,478 | 762.6 |
| 3 | β-cryptoxanthin palmitate | 75.7 | 426,452,478 | 790.6 |
| 4 | β-citraurin laurate | 57.2 | 456 | 614.9 |
| 5 | β-citraurin myristate | 61.1 | 456 | 642.5 |
| 6 | β-citraurin palmitate | 66.0 | 456 | 671.1 |

## Figure legends

**Fig. 1.** Typical HPLC chromatograms of a saponified and non-saponified carotenoid extracts. A, juice sacs of 'Yamashitabeni-wase' at 452 nm; B, juice sacs of 'Miyagawa-wase' at 452 nm; C, flavedo of 'Yamashitabeni-wase' at 452 nm; D, flavedo of 'Miyagawa-wase' at 452 nm; E, flavedo of 'Yamashitabeni-wase at 510 nm. Peak assignment: free β-cryptoxanthin (RT, 53. 3 min); 1, β-cryptoxanthin laurate (RT, 68.3 min); 2, β-cryptoxanthin myristate (RT, 71.5 min); 3, β-cryptoxanthin palmitate (RT, 75.7 min); free β-citraurin (RT, 21.5 min); 4, β-citraurin laurate (RT, 57.2 min); 5, β-citraurin myristate (RT, 61.1 min); 6, β-citraurin palmitate (RT, 66.0 min). RT, retention time. mAU, Miliabosorbance units.

**Fig. 2.** Changes in the content and composition of β-cryptoxanthin in the flavedos (A) and juice sacs (B) of 'Yamashitabeni-wase' and 'Miyagawa-wase' during the ripening process. The results shown are means ± SE for triplicate samples. Total was calculated as the sum of free and esterified forms. Sep, September; Oct, October; Nov, November; Dec, December; Jan, January.

**Fig. 3.** Changes in the content and composition of β-citraurin in the flavedo of 'Yamashitabeni-wase' and 'Miyagawa-wase' during the ripening process. The results shown are means ± SE for triplicate samples. Total was calculated as the sum of free and esterified forms. Sep, September; Oct, October; Nov, November; Dec, December; Jan, January.

**Fig. 4.** Substrate specificity analysis of CitCCD4 enzyme *in vitro*. HPLC analysis of the cleavage products from the induction of recombinant CitCCD4 with β-cryptoxanthin (A), β-cryptoxanthin laurate (B), β-cryptoxanthin myristate (C), β-cryptoxanthin palmitate (D). mAU, Miliabosorbance units.

**Fig. 5.** Substrate specificity analysis of CitCCD1 enzyme *in vitro*. HPLC analysis of the cleavage products from the induction of recombinant CitCCD1 with β-cryptoxanthin (A), β-cryptoxanthin laurate (B), β-cryptoxanthin myristate (C), β-cryptoxanthin palmitate (D). mAU, Miliabosorbance units.

**Fig. 1.**

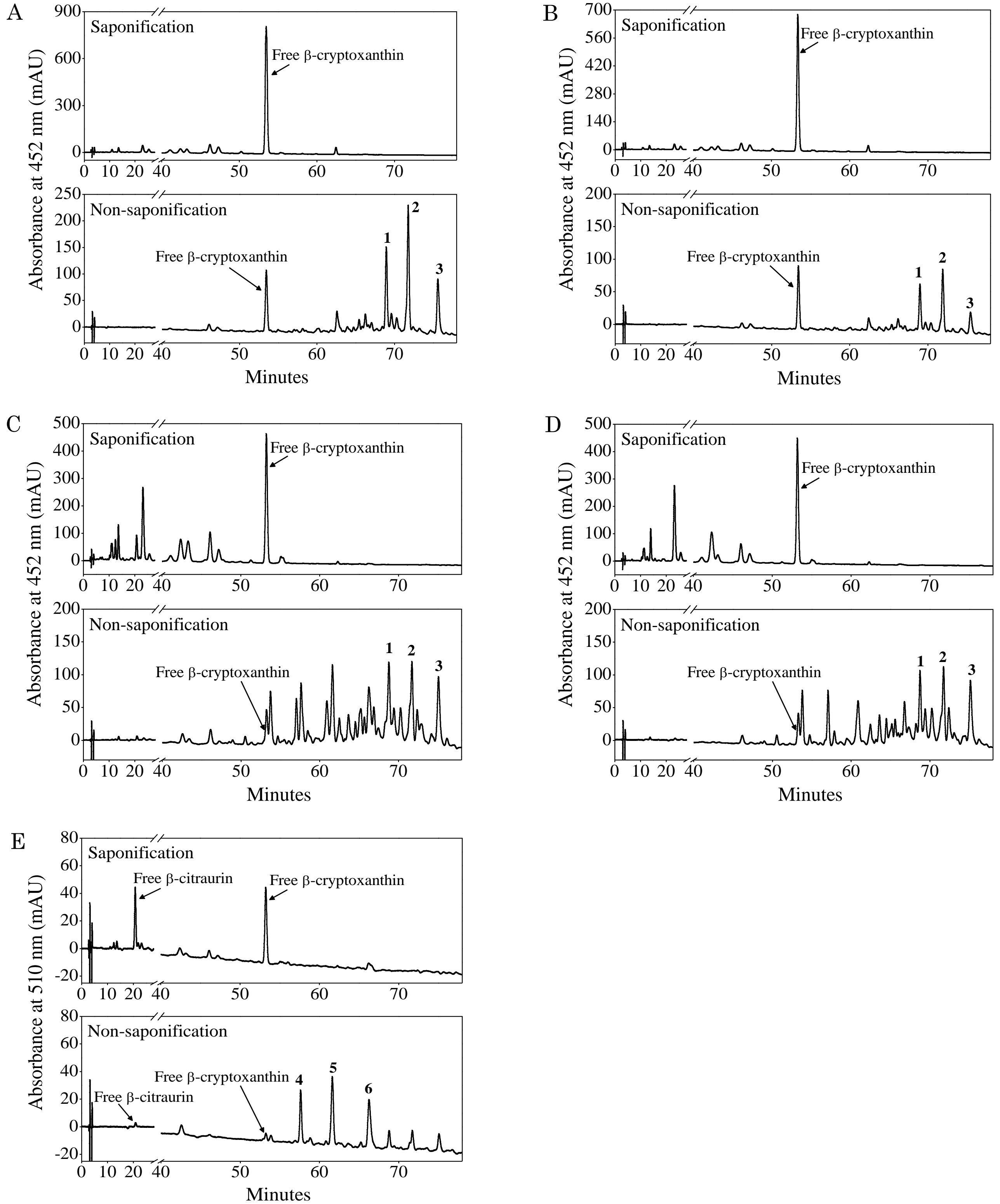
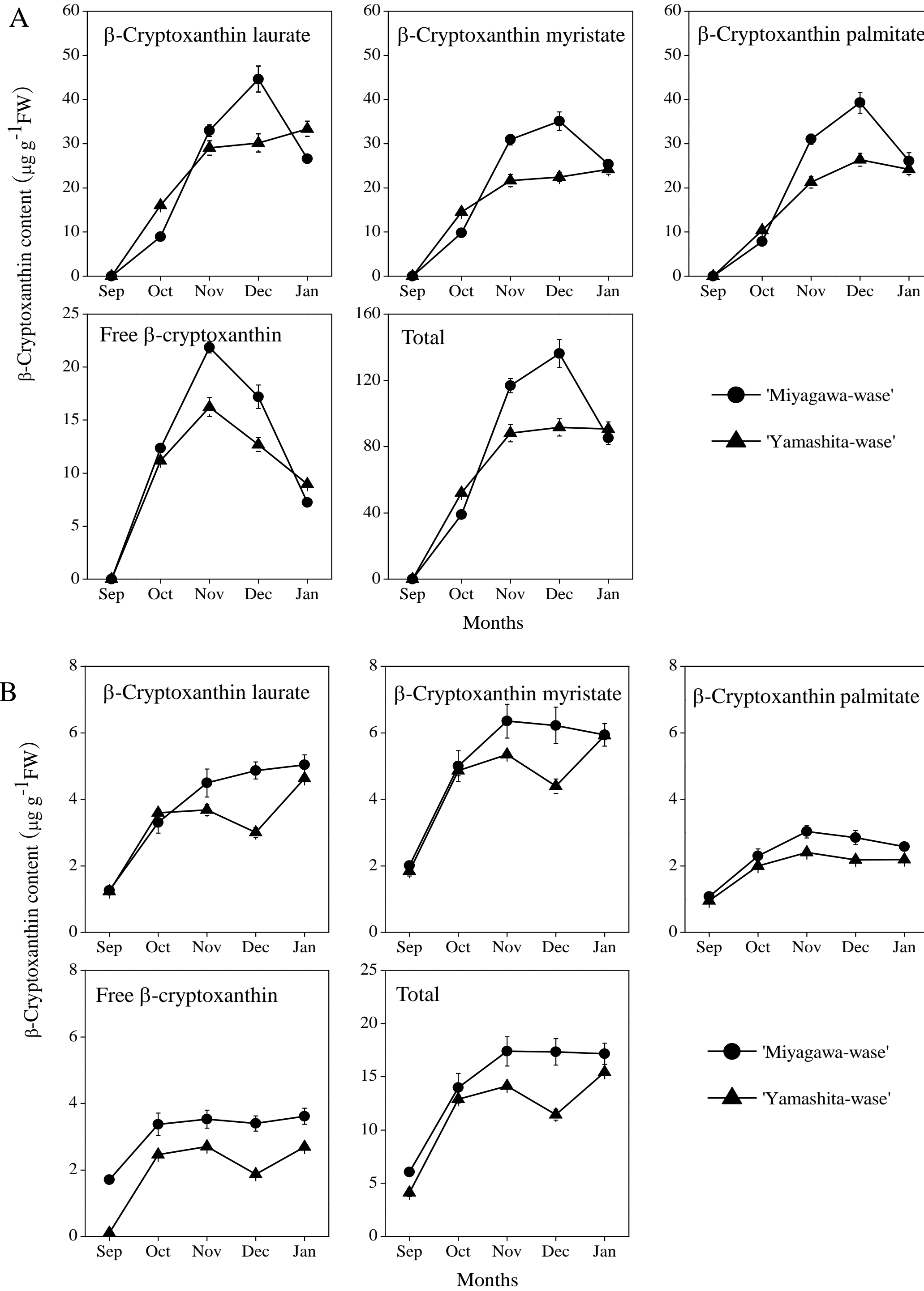


**Fig. 2.**



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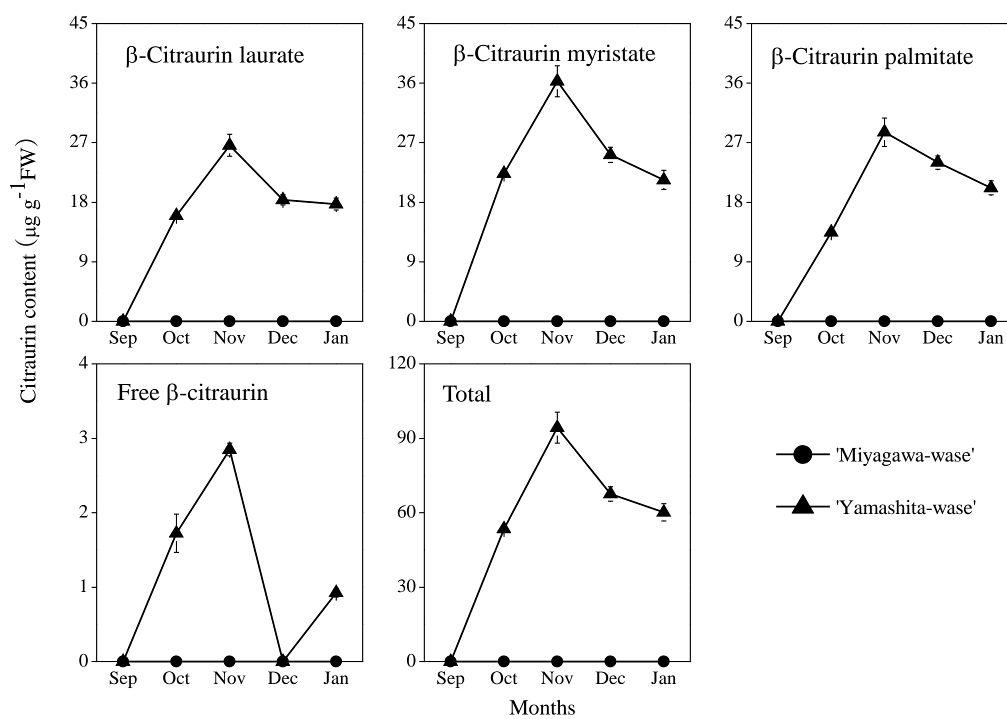

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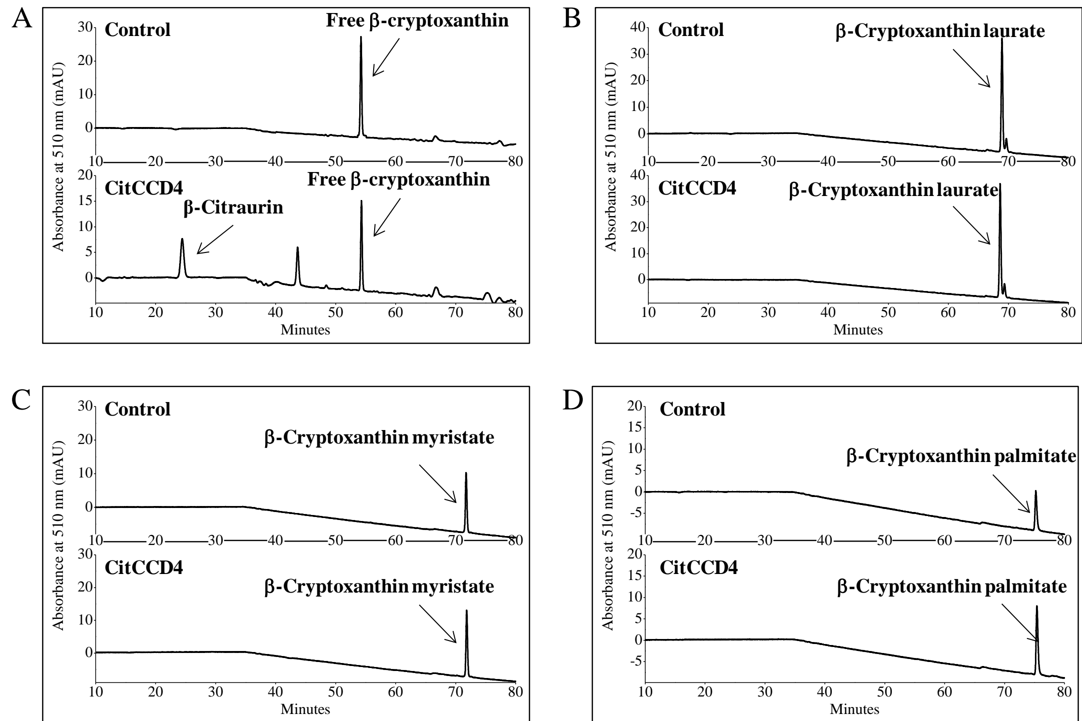

**Fig. 5.**