석사 학위논문

Changes in β-Cryptoxanthin Contents of *Citrus unshiu* Markovich Fruits Ripened in Greenhouse versus Open field Cultivation

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2005년 12월

Changes in β-Cryptoxanthin Contents of *Citrus unshiu* Markovich Fruits Ripened in Greenhouse versus Open field Cultivation

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(Supervised by professor Chan-Shick Kim)

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF AGRICULTURE

DEPARTMENT OF AGRICULTURAL CHEMISTRY
GRADUATE SCHOOL
CHEJU NATIONAL UNIVERSITY

December. 2005

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SUMMARY

β-Cryptoxanthin contents were determined from *Citrus unshiu* Markovich fruits grown in a greenhouse and open field of Jeju Island, off the southern coast of Korea. In greenhouse and open field, the β-Cryptoxanthin content in the peel of was greatly increased by harvesting citrus fruits at the late season from August thought November. However, β-Cryptoxanthin content in the flesh was gradually increased and was superior to that of the citrus fruits grown in a greenhouse. β-Cryptoxanthin was efficiently purified from the flesh of citrus fruits harvested in the late harvesting season in November. The β-Cryptoxanthin content in the peel and flesh of citrus fruits harvested from greenhouse in November were 0.89 mg% and 0.35 mg% respectively, and in that obtained from an open field were 1.12 mg% and 0.35 mg%, respectively.

Ⅰ. INTRODUCTION

Citrus are the most widely cultivated fruit trees in the world (Abkenar *et al*., 2004), and citrus fruits have been used as valuable ingredient for oriental medicine and functional foods (Whang *et al*., 1995). *Citrus unshiu* Markovich fruits are representative Citrus for a greenhouse cultivation in Jeju Island, and begins the pigmentation from August and fully ripens on the middle of November. It was reported that citrus fruits contain various bioactive compounds such as flavonoid and carotenoids. (Kris-Etherton *et al.*, 2002).

Carotenoids are isoprenoid molecules that are widespread as natural pigments in nature. In addition to their obvious contribution to food quality as natural pigments, they have been shown to play vital physiological roles (Wilberg *et al.*, 1995). Over recent years there has been considerable interest in dietary carotenoids with respect to their potential in alleviating chronic diseases in humans (Fraser *et al*., 2004). Carotenoids present in fruit and vegetable are widely believed to protect human health, preventing some cancers (Nishino *et al*., 2000; Riso *et al*., 1997) and function as quenchers of single oxygen, as antioxidants (Setiawan *et al*., 2001). Various natural carotenoids, being important biological precursor of vitamin A, have been associated with reduced risk of cardiovascular and other chronic diseases (Su Q *et al*., 2002). Some epidemiological studies have confirmed an inverse relation between carotenoid consumption and the

development of some cancers (Slattery *et al.*, 2000; Cooper *et al*., 1999).

More than 600 carotenoids have been identified in foods, but most nutrition research has focused on α-carotene, β-carotene, lycopene, lutein, and β-cryptoxanthin (Holden *et al.*, 1999). In particular, β -cryptoxanthin is present in human serum and tissues and plays a protective role against human disease (Sumida *et al*., 1999; Krinsky et al., 1990). β-cryptoxanthin has demonstrated anti-breast cancer activity and an inhibitory effect on osteoclast-like cell formation in mouse marrow cultures *in vitro*(Dorgan *et al.*, 1998; Uchiyama *et al*., 2004). It was reported that β-cryptoxanthin from Satsuma Mandarin (*Citrus unshiu* Marc.) had inhibitory effects on colon carcinoma of rats (Sumida *et al*., 1999).

In particular, β-cryptoxanthin has received research attention as on of the target carotenoids because of its great biological activity for human health. To evaluate the nutritive and biological value of food carotenoids, the absolute concentrations needed to be determined (Wilberg *et al.*, 1995). There has been particular emphasis on obtaining more accurate data on concentrations of various carotenoids in foods for various health and nutrition activities (Nam *et al*., 2002). In addition, accurate characterization of the association between carotenoid intake and various chronic diseases has been required (Holden *et al*., 1999). Generally, the accumulation of β-carotenoid in citrus fruits could be varied by the cultivar type and environment, including climate, cultivation condition. Therefore, it is necessary to

distinguish the compositional difference between citrus fruits cultivated in a greenhouse and open field. Such a comparison will provide valuable information for obtaining citrus fruits fortified with a higher β-cryptoxanthin content.

The purpose of this study was to evaluate the contents of β-cryptoxanthin in citrus fruits harvested from a greenhouse or open field during ripening season.

1. β-Cryptoxanthin

Carotenoides, with their highly reactive conjugated double bonds, act as free radical traps or antioxidants. based on the ability of carotenoides to protect plants and bacteria against photosensitivity, trials have been carried out in the use of these pigments for the treatment of erythropoietic porphyria, a condition in which patients suffer from an extreme degree of photosensitivity (Mathews-Roth, 1981). Carotenoides in citrus fruits is widely believed to protect human health (Nishino *et al*., 1996). Various natural carotenoids were proven to have anticarcinogenic activity (Nishino *et al*., 2000). Some epidemiological studies have confirmed an inverse relation between carotenoid consumption and the development of some cancers (Slattery *et al*., 1997; Cooper *et al*., 1999). Anticancer activity of carotenoids from citrus fruits was due to mainly their antioxidants function (Tee and Lim, 1991).

Recently, carotenoids have attracted attention due to reported beneficial health effects (Rauscher *et al*., 1998; Nishino *et al*., 1998). In particular, β-cryptoxanthin functions as protective role against human disease (Hart and Scott, 1995; Sumida *et al*., 1999). β -cryptoxanthin was suggested to stimulate the expression of an anti-oncogene (Nishino *et al*., 2000). Dorgan et al. reported that β -cryptoxanthin shows the effect of anticancer activity on breast cancer (Dorgan *et al*., 1998). *In vivo* experiments for preventing skin cancer or colon cancer with mouse, β-cryptoxanthin shows higher anticancer activity than that of β-carotene.(Nishino *et al.*, 1998) In recent years, there has been particular emphasis on obtaining more accurate data on

the types and concentrations of various carotenoids in foods for various health and nutrition activities (Tee and Lim, 1991). The data on the individual carotenoid content of citrus fruits has become increasingly important. A wide variety of separation and detection and quantization procedures have been used in studies of carotenoids (Tee and Lim, 1991). Nishio et al. reported that *Citrus unshiu* Marcovitch contained great amount of β-cryptoxanthin than those of grapefruits, lemons and oranges cultivated in America. (Nishino *et al*., 1998)

Ⅱ. MATERIALS AND METHODS

1. Reagents and Material

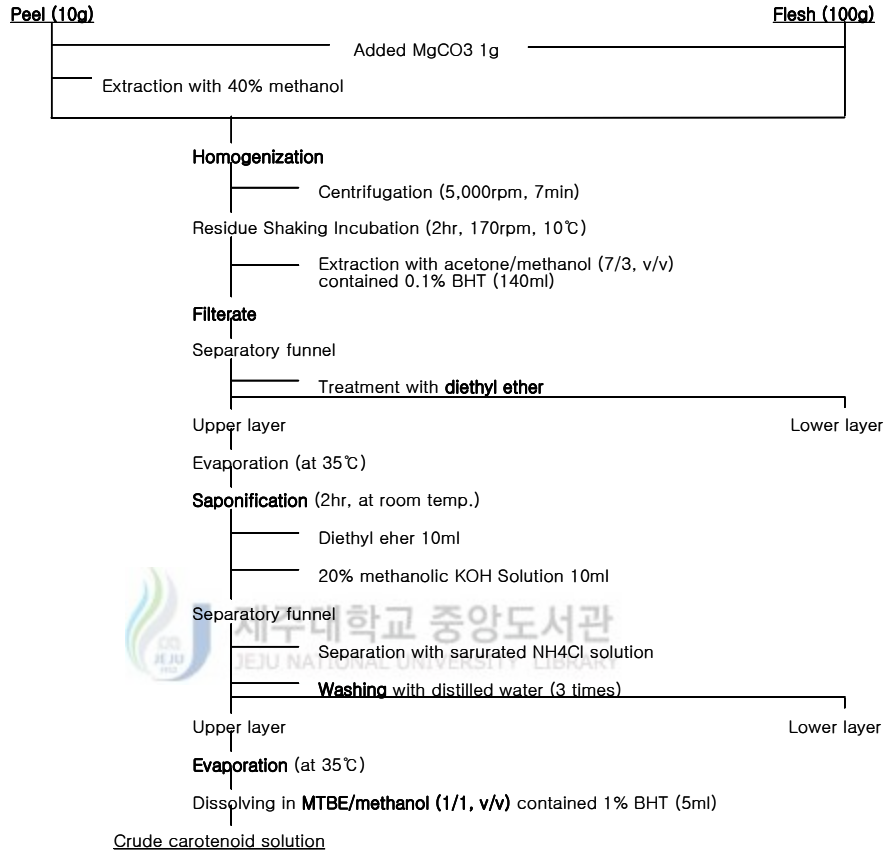
Citrus fruits (*Citrus unshiu* Markovich) were cultivated in a greenhouse and open field of the National Institute of Subtropical Agriculture in Jeju Island and were harvested through August to November 2003. The peel and flesh from citrus fruits were separated and sliced. Each part of citrus fruit was stored at -70 ℃ deep freezer. The frozen samples were thawed in refrigerator before the carotenoid extraction. β-Cryptoxanthin as a standard and butylated hydroxy toluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other chemicals used were HPLC or analytical grade. A TLC plate was purchased from Merck Co. (Silica gel 60 F254, Merck, USA). A Bondapak^{TM} C18 reverse phase column (3.9 x 300 mm, particle size 10 mm) was obtained from Waters Co. (Waters Chromato-graphy, Milford, MA, USA).

2. Extraction of crude carotenoids from Citrus fruits

The extraction of carotenoids was performed using the method reported by Ko et al. A 10 g peel (or 100 g flesh) was mixed with 70 mL of 40 % methanol containing 1 g of $MgCO_3$ using juice mixer (LG, Korea). The carotenoid pigments were collected by phase separation using the mixture of deionized water and ethyl ether and then was concentrated using vacuum evaporator at 35 ℃. Crude carotenoids were dissolved in 20 mL of ethyl ether containing 20 % methanolic KOH. The saponification was conducted at room temperature in the dark for 2 hr, and then was reduced to dryness using a rotary evaporator at 35 ℃ The saponified samples were dissolved in 5 mL of MTBE/methanol (1/1, v/v) containing 1 % BHT and was filtered through 0.45 ㎛ PTFE filter (Micro Filtration System, CA, USA).

3. Separation of carotenoid by silica gel TLC.

A concentrated solution of each sample extract in MTBE/methanol (1/1, v/v) was chromatographed on silica gel TLC plates (Silica gel 60 F254, Merck, USA). A solution containing hexane(75%) and acetone (25%)and allowed to condition for 10min. The plate was then lowered into the solvent and the chromatogram developed for 30min in darkness at room temperature.



Scheme 1. Extract scheme of carotenoids in peel and flesh of Citrus

4. Chromatography

The carotenoids extracted from peel or flesh of citrus fruit was analyzed using silica gel TLC plates. For the separation of pigment compounds, hexane/acetone (3/1, v/v) was used as the mobile solvent. The separation of components was accomplished for 30 min in darkness at room temperature. β-Cryptoxanthin as a standard carotenoid was used for determining the R_f value. The quantitative separation of carotenoids was also performed by HPLC. HPLC equipment included a Spectra-Physics (Spectra-SYSTEM) consisted of P4000 pump (Spectra-Physica Analytical, Inc., CA, USA), UV1000 UV/Vis detector (Spectra-Physics Analytical, Inc., CA, USA). A Bondapak^{TM} C18 reverse phase column (3.9 x 300 mm, particle size 10 m) was used and column temperature was maintained at 35℃. As mobile phase HPLC-grade methanol, water and analytical grade methyl tert-butyl ether (MTBE) were mixed for gradient conditions from (95:1:4) to (25: 71: 4) for 13 min, respectively. Each solvent was filtered through a 0.5 m PTFE membrane filter (Advantec MFS, Inc., CA, USA) and then passed through a solvent degasser (A0099-504, Spectra-physics Analytical, Inc.). Each sample was injected onto the column via an automatic sampler (AS3500, Thermo Separation Productions Inc., USA) equipped with a sample loop (20㎕). An operation was performed for 30 min with 1 mL/min of flow rate, and then peak responses were determined by measuring absorbance at 445 nm. A 5 mg of β-cryptoxanthin as a reference was dissolved in 25 ml of methyl tert-butyl ether containing 1 % BHT and methanol (1:1, v/v). The concentrations for a carotenoid standard ranged from 0.1 -

0.5 μg/mL. The linearity of the calibration between concentration of β-cryptoxanthin and absorbance was determined. The retention time of β-cryptoxanthin was used for the identification of β-cryptoxanthin from the extract of citrus fruits. Duplicate samples were injected for each extract for HPLC analysis.

Table 1. Operating condition of HPLC for carotenoids.

Solvent Degassers	: Spectra-Physics Analytical, Inc. part number A0099-504
Gradient pumps	: Spectra-Physics Analytical, Inc. part number A0099-510 (P4000)
Autosamplers	: Thermo Separation Products Inc. part number A0099-587 (AS1000)
UV/Vis Detectors	: Spectra-Physics Analytical, Inc. 8/91 part number A0099-540 (UV2000)
Column	: μ BondapakTM C18 125Å 10 μ m 3.9×300mm HPLC Column, Waters

Mobile phase

A solvent = Methanol : Methyl tert-buthyl ether : H_2O = 95 : 1 : 4

B solvent = Methanol : Methyl tert-buthyl ether : H_2O = 25 : 71 : 4

Injection volume	: 20㎕
Column temperature	: 35℃
Flow rate	: 1ml/min
Wave length	: 445 nm

Gradient table

Time(min)	A(%)	B(%)
0	100	0
12	100	0
25	0	100
30	100	0
35	100	0

Ⅲ. RESULTS AND DISCUSSION

1. TLC analysis of carotenoids in citrus fruits.

Crude carotenoids were extracted from peel and flesh of citrus fruits grown in a greenhouse and open field of Jeju Island. The composition of crude carotenoids prepared by solvent extraction was analyzed by TLC method. As shown in Fig. 1, crude carotenoids extracted from peel were composed of several carotenoid compounds, including β-cryptoxanthin with R_f value of 0.4. It was reported that β-cryptoxanthin from citrus fruits produced in Jeju Island had R_f value of 0.39 (Ko KC, *et al.,* 2000) It was expected that β-carotene was separated with R_f of 0.97. The β-cryptoxanthin content in the flesh and peel was increased as the harvest season for the citrus fruits was delayed from August (coloring season) to November (harvesting). It was concluded that carotenoids containing β-cryptoxanthin were successfully expected from both the peel and flesh of citrus fruits.

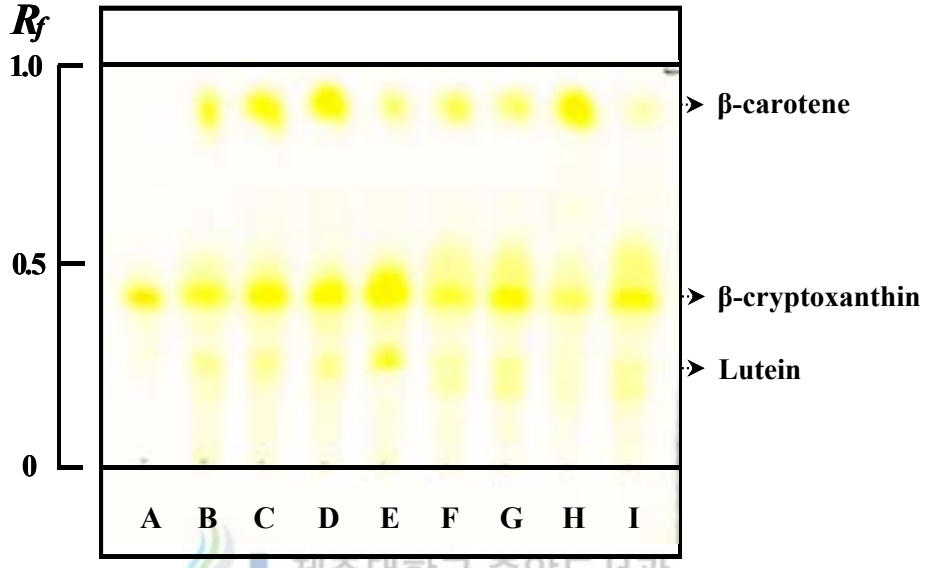


Figure 1. TLC chromatograms of a standard of β -cryptoxanthin and carotenoids from flesh of citrus fruits.

A: standard of β-cryptoxanthin

B-E: citrus fruits from open field; F-I: citrus fruits from a greenhouse; B, F: Aug., C, G: Sep., D, H: Oct., E, I: Nov.

TLC conditions : 1) Plate; Silica gel 60 F254 TLC (Merck),

2) Solvent system; Hexane/Acetone (75/25, v/v)

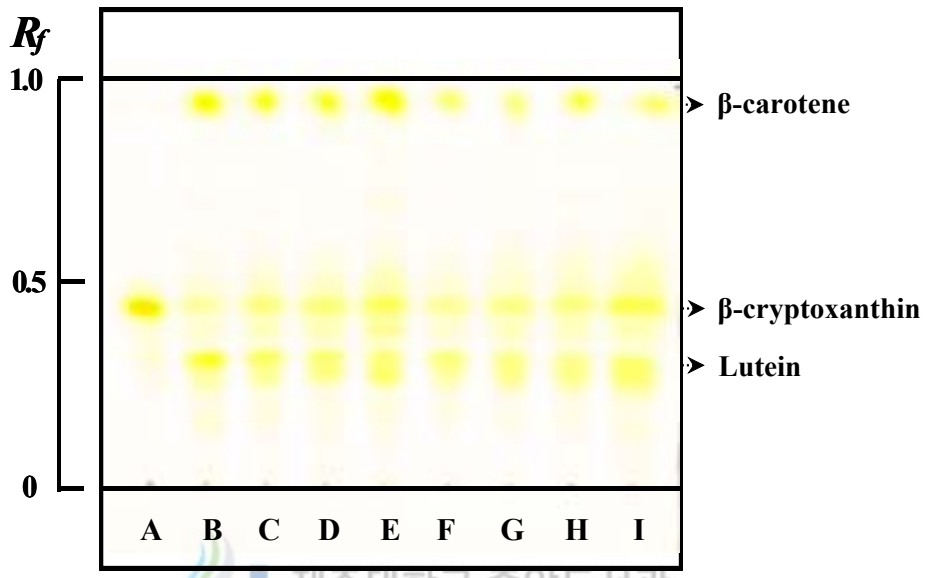


Figure 2. TLC chromatograms of a standard of β -cryptoxanthin and carotenoids from peel of citrus fruits.

A: standard of β-cryptoxanthin

B-E: citrus fruits from open field; F-I: citrus fruits from a greenhouse; B, F: Aug., C, G: Sep., D, H: Oct., E, I: Nov.

TLC conditions : 1) Plate; Silica gel 60 F254 TLC (Merck), 2) Solvent system; Hexane/Acetone (75/25, v/v)

2. Analysis of β-cryptoxanthin by HPLC

To determine the β-cryptoxanthin content quantitatively, HPLC analysis was performed. The retention time of β-cryptoxanthin was about 19 min. The β-cryptoxanthin in the flash was gradually increased and purified effectively as the harvesting season of citrus fruits was delayed from August to November. However, β -cryptoxanthin in the crude carotenoids was less purified compared to those of the flesh from citrus fruits, including various carotenoid components. Based on this previous HPLC experiment, it was considered that crude carotenoids from the peel contained xanthophyll-like compounds with a retention time of 4-9 min, along with β-carotene and lutein with retention time of 24min and 8.5min, respectively. As shown in Fig. 12, β-cryptoxanthin content was greatly changed by the harvesting season and cultivation method of the citrus fruits. In citrus fruits harvested from an open field, β-cryptoxanthin contents of flesh and peel were increased from 0.06 mg% and 0.05 mg%(August) to 0.35 mg% and 1.12 mg% (November), respectively.

In citrus fruits grown in a greenhouse, β-cryptoxanthin contents of peel were 0.05 mg% (August), 0.17 mg% (September), 0.53 mg% (October), and 0.89 mg% (November), and β-cryptoxanthin contents of the flesh were gradually increased with 0.24 mg% (August), 0.27 mg% (September), 0.34 mg% (October) and 0.45 mg% (November). Previously, it was recognized that β-cryptoxanthin content in the peel of citrus fruit was higher than that of flesh (Tee and Lim, 1991, Ko KC, *et al.,* 2000). It was concluded that the β-cryptoxanthin contents

in both peel and flesh increased as the citrus fruits fully matured. β-cryptoxanthin contents from peel and flesh of citrus fruits cultivated in November in an open field were 1.12 mg% and 0.35 mg%, respectively, and greenhouse were 0.89 mg% and 0.45 mg%, respectively.

In both a greenhouse and open field, β-cryptoxanthin content was greatly increased in citrus fruits harvested on November (Fig. 12), which suggests that the harvesting of *Citrus unshiu* Markovich fruits in November is quite reasonable for increased content of β-cryptoxanthin. In particular, β-cryptoxanthin of flesh from citrus fruits grown in a greenhouse was higher than that of open field during ripening season, being indicating 0.45 mg% (greenhouse) and 0.35 mg% (open field) (Fig. 12). However, in the peel of citrus fruits, β-cryptoxanthin was gradually increased until September, and was higher than that of an open field. β-cryptoxanthin of peel was reached 1.12 mg% (open field) and 0.89 mg% (greenhouse) in November as the harvesting season. Considering higher content of β-cryptoxanthin in the peel of citrus fruits, it is necessary to utilize the peel effectively as a functional ingredient. Generally, the β-cryptoxanthin contents varied according to various citrus cultivars. It was reported that the amount of β-cryptoxanthin ranged from 0.3 to 2.1 mg% in the peel of domestic citrus cultivars (Nam TS, *et al.,* 2002). Miyagawa wase exhibited 5.26 mg% in the peel and 0.78 mg% in flesh (Whang HJ, *et al.,* 1995) However, the β-cryptoxanthin contents in lemon and Grapefruit were considerably lower than those found in other citrus cultivars, containing below 0.1 mg% in both peel and flesh (Nam TS,

et al., 2002). Based on the comparison of β-cryptoxanthin content, *Citrus unshiu* Markovich fruit harvested from a greenhouse on November contained reasonably higher β-cryptoxanthin in both peel (0.89 mg%) and flesh (0.35 mg%), which confirms that *Citrus unshiu* Markovich fruits are suitable Citrus for greenhouse cultivation in Jeju Island. The accumulation of β-cryptoxanthin could be affected by various environmental conditions as well as by genetic manipulation. It is expected that improve-ments of culture environment and cultivar will increase the levels of β-cryptoxanthin in *Citrus unshiu* Markovich fruit. For this approach the basic evaluation of β-cryptoxanthin content from *Citrus unshiu* Markovich fruit will provide important information to improve the citrus culticar in the future.

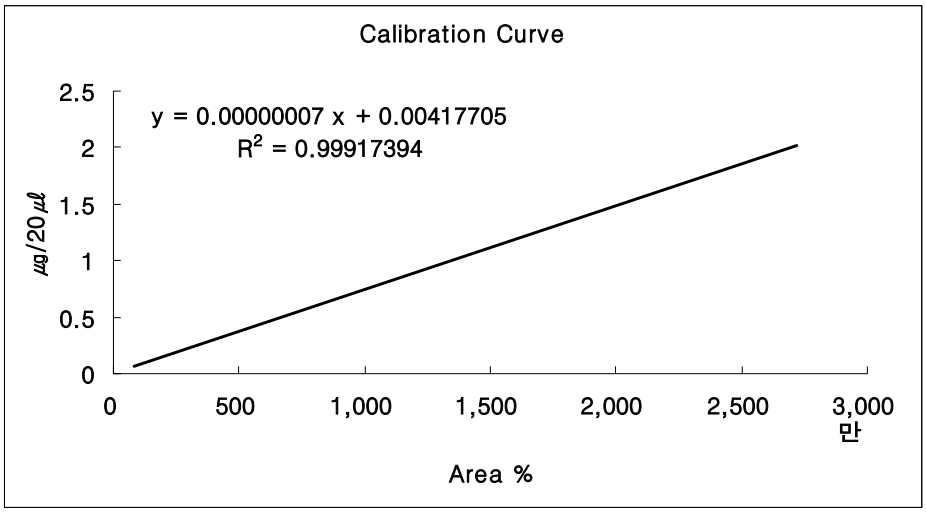


Figure 3. Standard Calibration of β-cryptoxanthin by HPLC

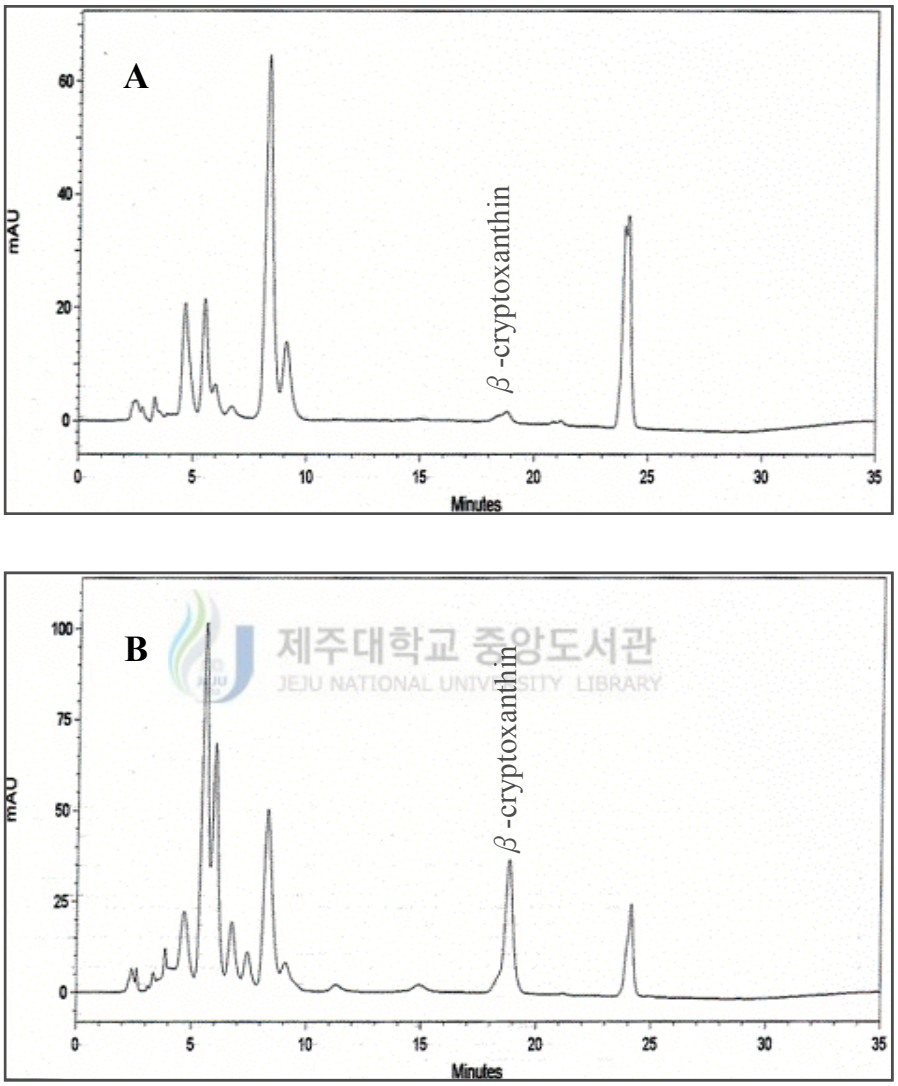


Figure 4. HPLC elution profiles of carotenoid pigments extracted from peel of citrus.

A : Aug. Greenhouse, B: Sep. Greenhouse

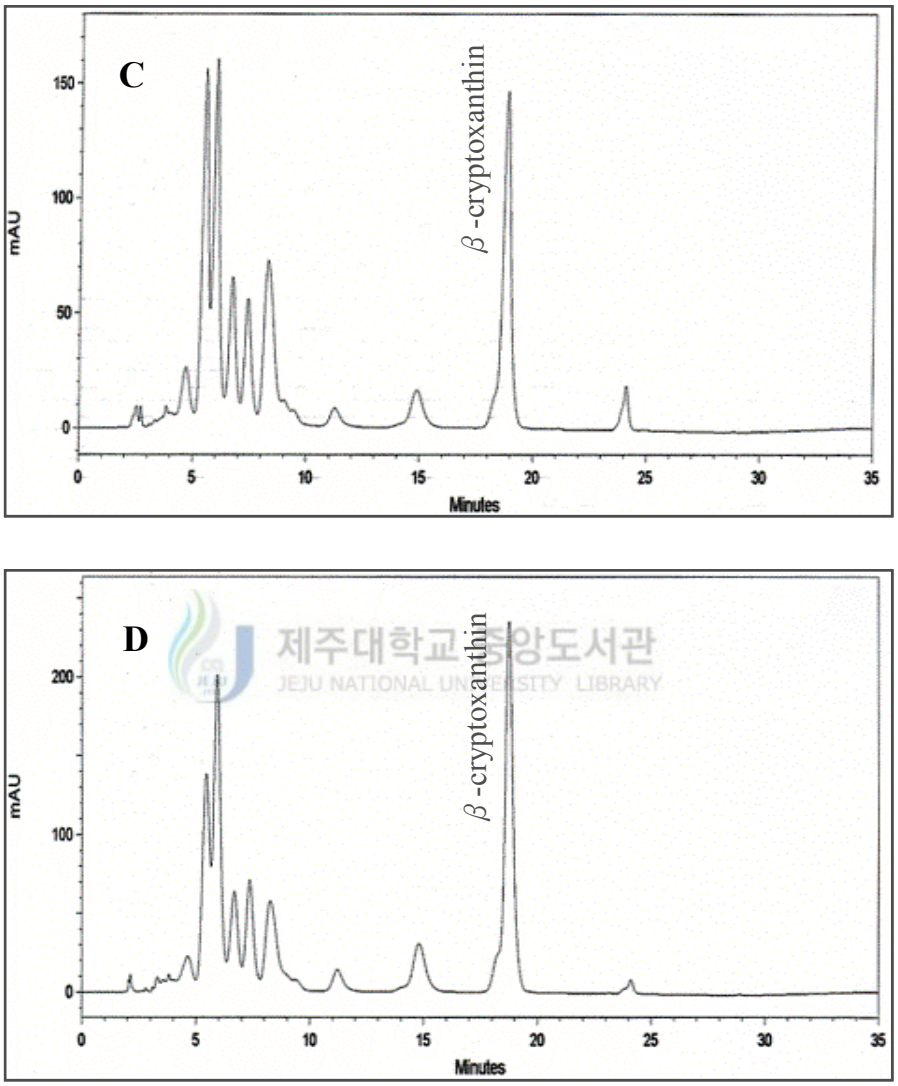


Figure 5. HPLC elution profiles of carotenoid pigments extracted from peel of citrus.

C: Oct. Greenhouse, D: Nov. Greenhouse

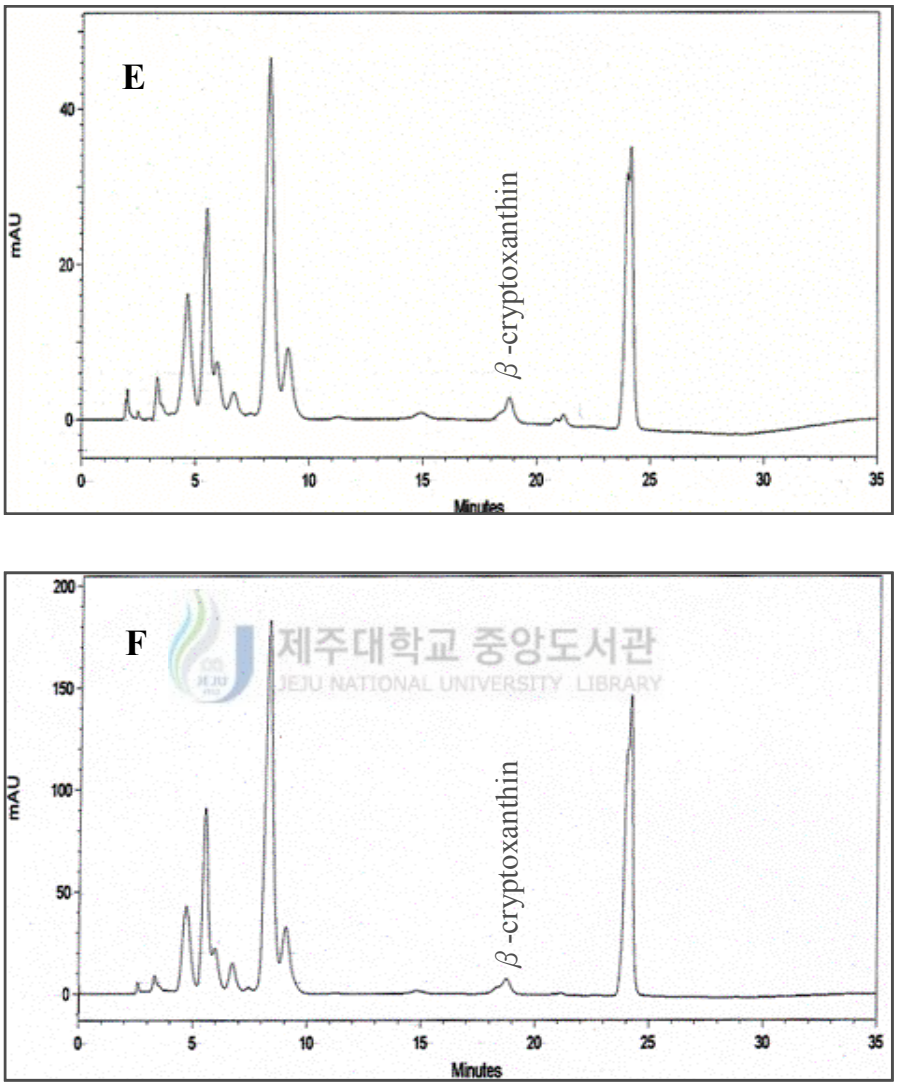


Figure 6. HPLC elution profiles of carotenoid pigments extracted from peel of citrus.

E : Aug. Open field, F: Sep. Open field

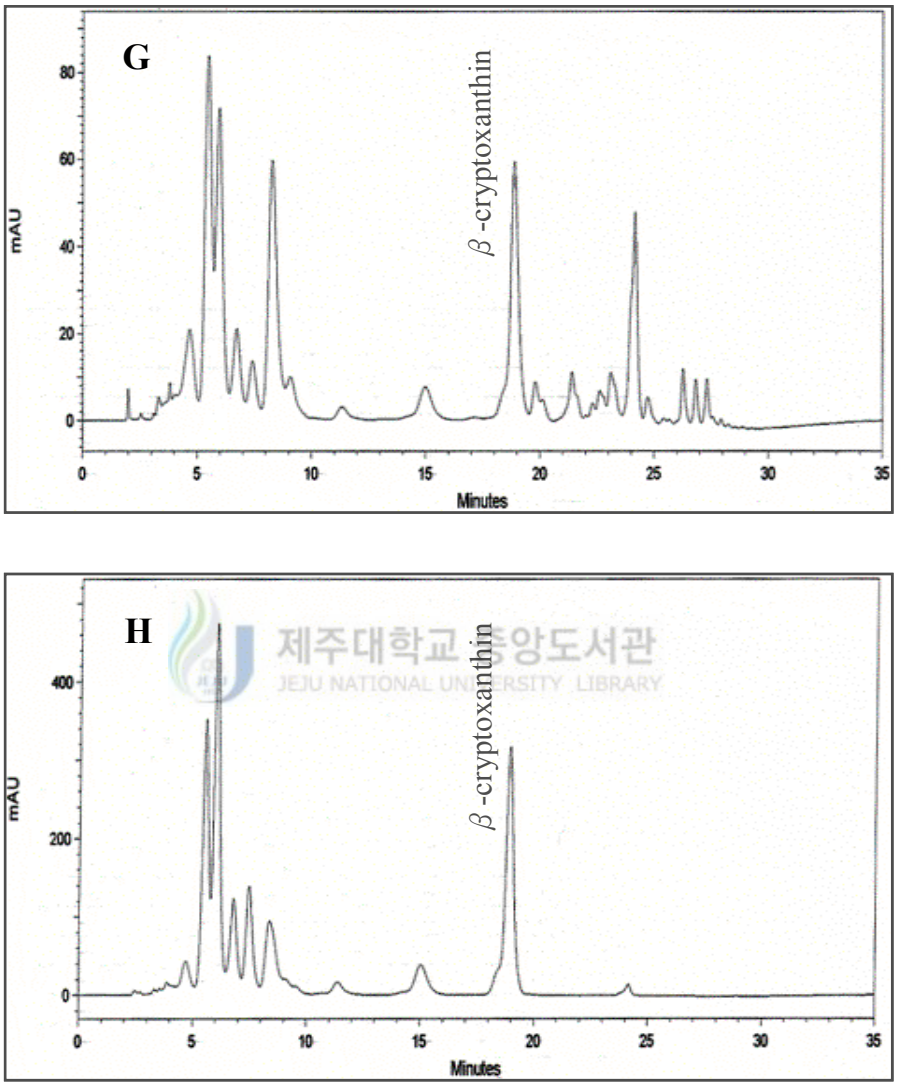


Figure 7. HPLC elution profiles of carotenoid pigments extracted from peel of citrus.

G: Oct. Open field, H: Nov. Open field

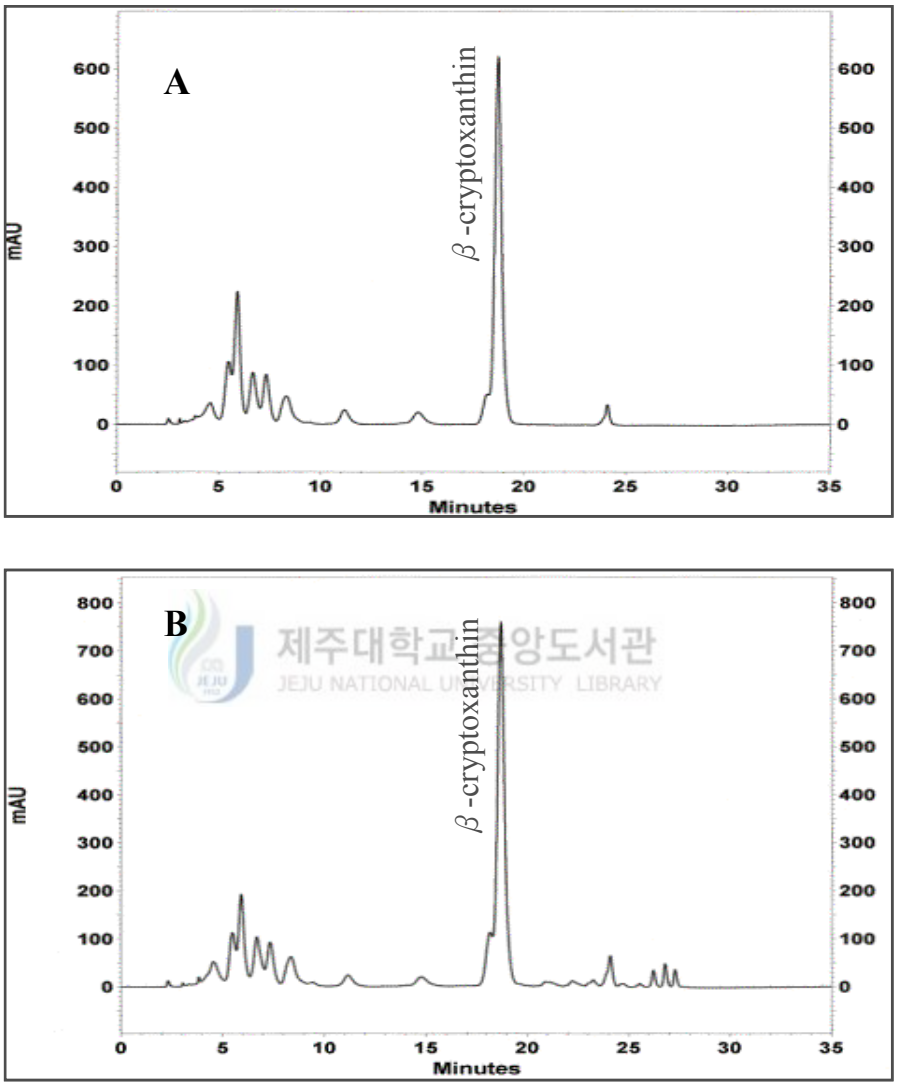


Figure 8. HPLC elution profiles of carotenoid pigments extracted from flesh of citrus.

A: Aug. Greenhouse, B: Sep. Greenhouse

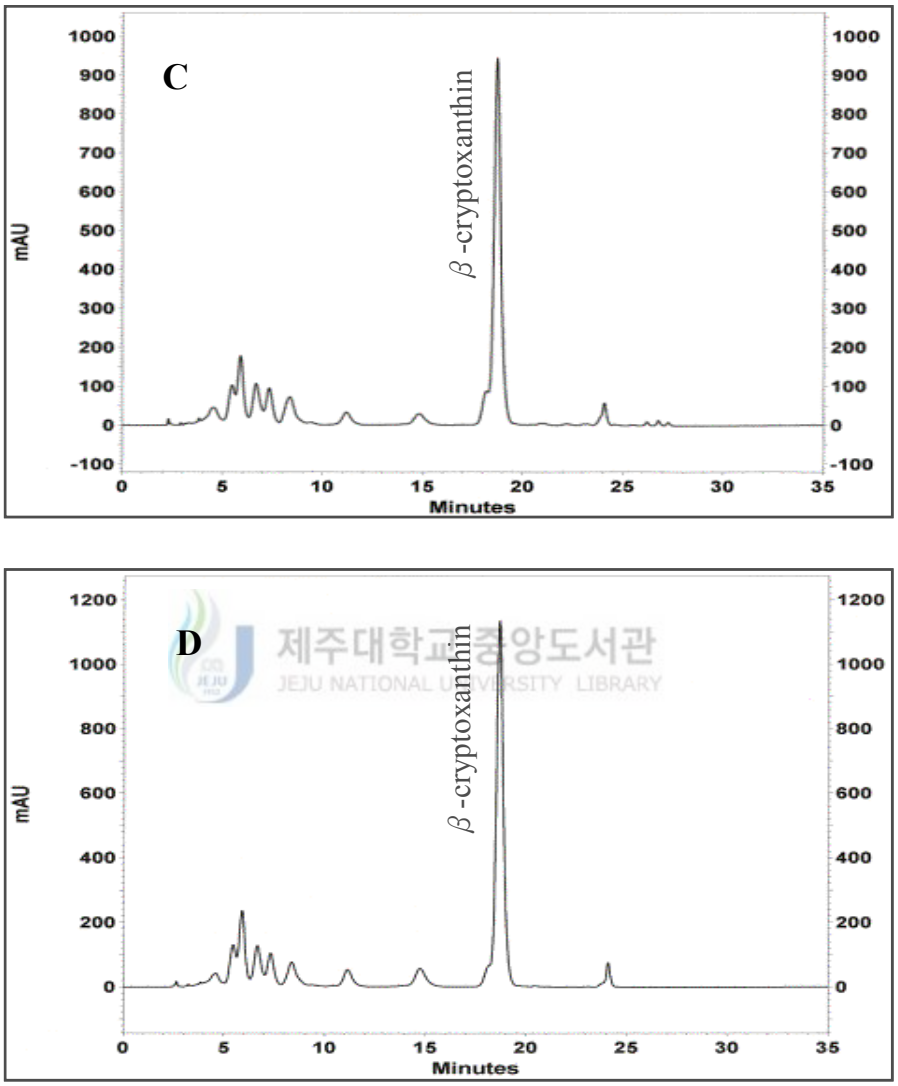


Figure 9. HPLC elution profiles of carotenoid pigments extracted from flesh of citrus.

C: Oct. Greenhouse, D: Nov. Greenhouse

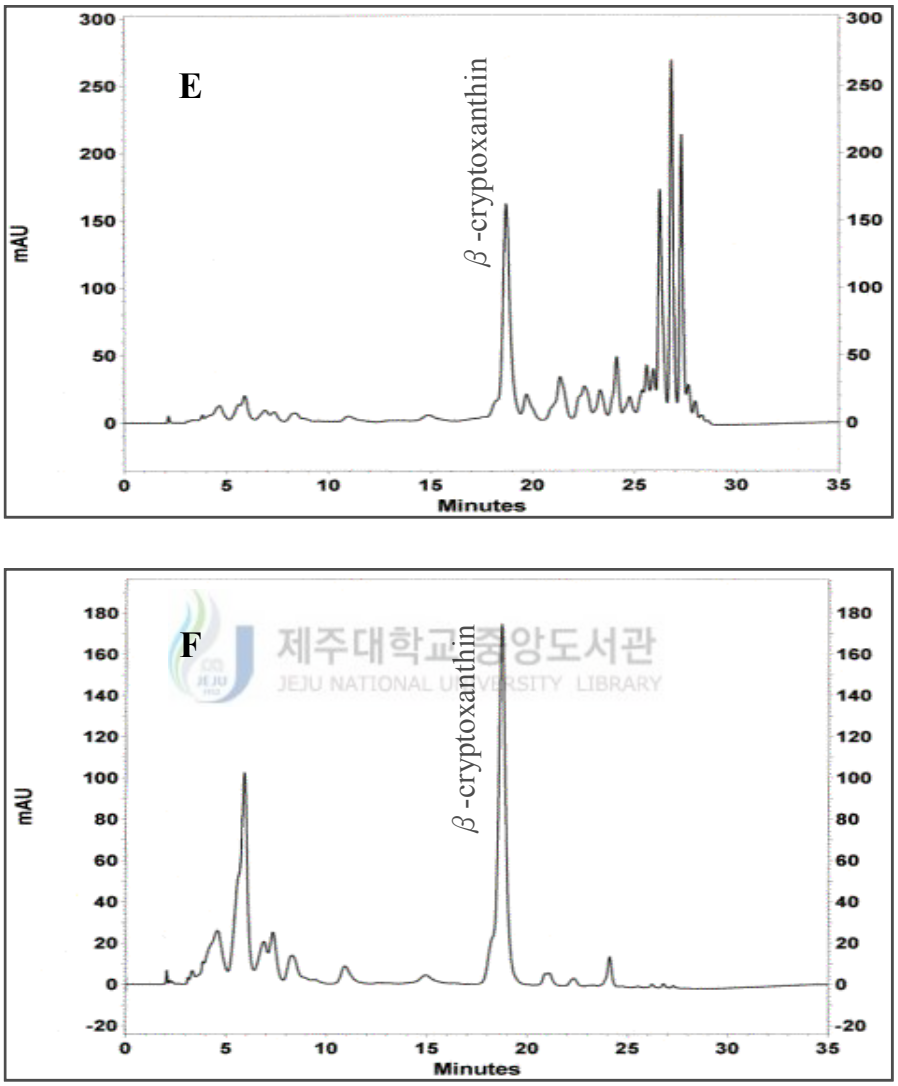


Figure 10. HPLC elution profiles of carotenoid pigments extracted from flesh of citrus.

E: Aug. Open field, F: Sep. Open field

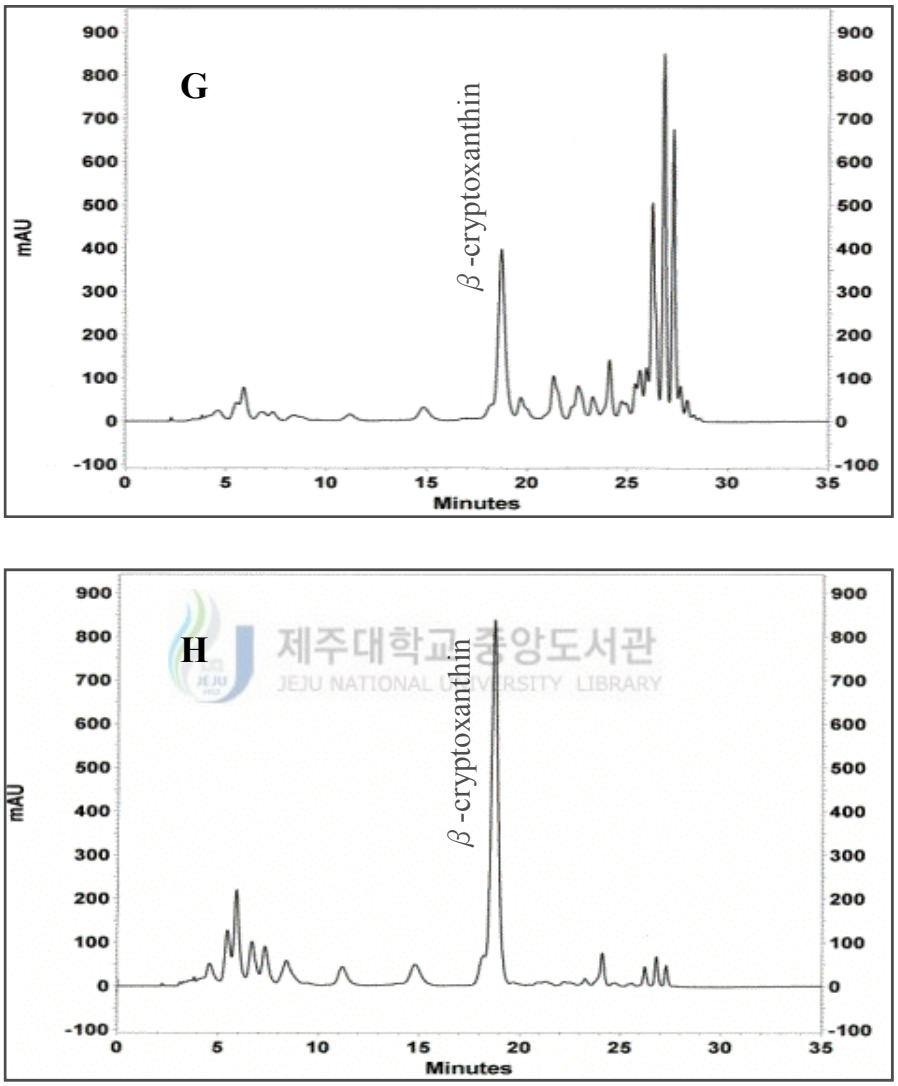


Figure 11. HPLC elution profiles of carotenoid pigments extracted from flesh of citrus.

G: Oct. Open field, H: Nov. Open field

Table 2. β-Cryptoxanthin Content from Peel of Citrus

samples	Content(㎎%)			
	Aug.	Sep	Oct.	Nov.
Open field	0.05	0.06	0.25	1.12
Greenhouse	0.05	0.17	0.53	0.89

Table 3. β-Cryptoxanthin Content from Flesh of Citrus

samples	Content(㎎%)			
	Aug.	Sep.	Oct.	Nov.
Open field	0.06	0.07	0.15	0.35
Greenhouse	0.24	0.27	0.34	0.45

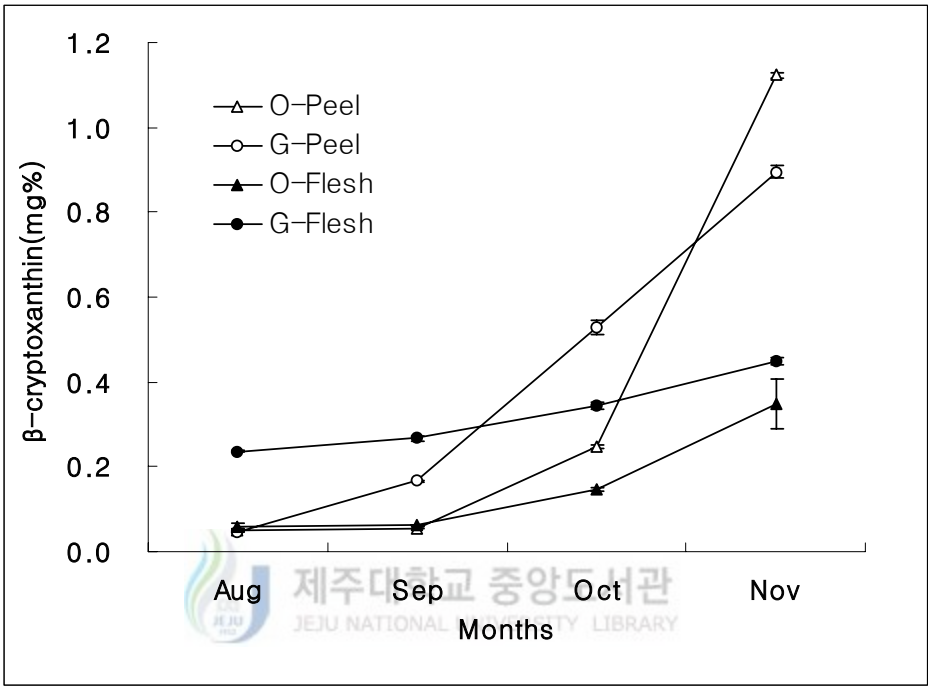


Fig. 12. Comparison of β-cryptoxanthin from peel and flesh of citrus fruits according to the harvesting season. Abbreviation: O-open field, G-greenhouse.

Ⅳ. SUMMARY (국문요약)

제주에서 하우스와 노지에서 각각 재배되고 있는 궁천조생의 β-cryptoxanthin함량을 분석하기위해 하우스와 노지에서 재배되고 있는 과실을 이른 착색기인 8월부터 성숙기인 11월까지 발육단계별로 수확하여 β-cryptoxanthin 추출에 이용하였다.

발육단계별 궁천조생의 과육과 과피에 존재하는 β-cryptoxanthin의 함량은 노지 과피에서 0.05 mg%, 0.0 6 mg%, 0.25 mg%, 1.12 mg%이고, 하우스 과피에서 0.05 mg%, 0.17 mg%, 0.53 mg%, 0.89 mg% 측정되었다. 노지 과육내 함량은 0.06 mg%, 0.07 mg%, 0.15 mg%, 0.35 mg%이고, 하우스 과육내 함량은 0.24 mg%, 0.27 mg%, 0.34 mg%, 0.45 mg%로 측정되었다.

측정된 β-cryptoxanthin함량은 과실이 익어감에 따라 증가하고 과육보다는 과피에 많이 함유되어 있음을 보여주고 있으며, 노지에서 재배된 과실이 보다 많은 β-cryptoxanthin을 포함하고 있었다. 특히 성숙기에 접어든 노지 과피에서는 β-cryptoxanthin함량이 0.25 mg% (10월)에서 1.12 mg% (11월)로 매우 높은 증가를 보였다.

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ACKNOWLEDGEMENT

본 논문이 완성되기까지 끝임 없는 관심과 학문적 가르침으로 이끌어 주신 김찬식 지도교수님께 마음깊이 감사드립니다. 그리고 바쁘신 와중에서도 시간을 내어 미흡한 논문을 다듬어 주신 송관정 교수님과 김인중 교수님께 진심으로 감사드립니다. 또한 학부시절부터 석사과정까지 많은 충고와 가르침을 주신 강순선 교수님, 유장걸 교수님 고정삼 교수님, 류기중 교수님, 현해남 교수님께 감사드립니다.

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마지막으로 오늘날까지 한결같은 믿음과 사랑으로 보살펴주신 부모님과 가족들 그리고 항상 큰 용기와 힘이 되어주던 친구들과 철우형에게 고마움을 전하며 이 논문을 바칩니다.