

原著論文

Quantitation of Carotenoids in Astaxanthin Supplement with
Modified HPLC-UV/Vis MethodMitsuhiro WADA¹⁾ and Kenichiro NAKASHIMA^{2)*}

¹⁾Department of Pharmacy, School of Pharmaceutical Sciences, Kyushu University of Health and Welfare, ²⁾Department of Pharmacy, Faculty of Pharmaceutical Sciences, Nagasaki International University, *Corresponding author)

改良 HPLC-UV/Vis 法によるアスタキサンチン
サプリメント中のカロテノイド類の定量和田 光 弘¹⁾, 中 島 憲一郎^{2)*}

(¹⁾九州保健福祉大学 薬学部 薬学科、²⁾長崎国際大学 薬学部 薬学科、*連絡対応著者)

要 旨

サプリメント中のアスタキサンチン (AX) およびカロテノイド (α -および β -カロテン, α -および β -クリプトキサンチン) の HPLC-UV/Vis 法による定量について記載した。AX または他のカロテノイドは C30 カラム (Combi-RP 5; 50×4.6mm, i.d.) で少なくとも35分以内に分離が達成された。AX および他のカロテノイドの定量には異なったケン化条件が必要であった。さらに、提案した方法は AX サプリメントにうまく適用することができた。AX と β -カロテンの定量値はサプリメントの栄養表示とよく一致した。

キーワード

アスタキサンチン、サプリメント、 α -および β -カロテン、 α -および β -クリプトキサンチン、ケン化

Abstract

A quantitation method for astaxanthin (AX) and other carotenoids (α - and β -carotenes, α - and β -cryptoxanthins) in supplements with HPLC-UV/Vis method was described. The separation of AX or other carotenoids at least within 35 min was achieved using a C30 column (Combi-RP 5; 50×4.6 mm, i.d.). Different saponification conditions for AX and other carotenoids were needed to their quantification. Furthermore, the proposed method could be successfully applied to AX supplements. The quantitation data for AX and β -carotene were well-agreed with on nutrition fact of the supplements.

Key words

Astaxanthin, supplement, α - and β -carotene, α - and β -cryptoxanthin, saponification

Introduction

Carotenoids are naturally occurring tetraterpenes found in various fruits, vegetables, plants, algae and bacteria. They can be divided into two main groups: (a) carotenes or

hydrocarbon carotenoids, such as α -carotene and β -carotene, and (b) xanthophylls that are oxygenated hydrocarbon derivatives, such as α -, β -cryptoxanthins and astaxanthin (AX).¹⁾ Since carotenoids act as antioxidants

through a free radical mechanism by quenching singlet oxygen and oxidizing species in the prevention of cellular damages, they are suggested to be effective as therapeutic agents for cancer, heart attacks and coronary artery diseases.²⁻⁴⁾

The AX is widely distributed, especially in marine bacteria, green algae, crustaceans, fish, and bird,^{3, 4)} and its antioxidant activity has been reported more effective than those of other carotenoids such as β -carotene, lutein, canthaxanthin, and zeaxanthin. Therefore, AX has been used in pharmaceutical products, functional foods, nutraceuticals, cosmetics, and food and feed industries.^{4, 5, 6)} One of the natural sources of AX is green algae, *H. pluvialis*, which is believed to be the richest source of natural AX, cultivated at industrial scale and brought to worldwide business.^{3, 4)} Commercially cultured *H. pluvialis* contains between 1.5% and 3.0% AX, which consists approximately of 70% monoester, 25% diester and 5% free form, and also contains other carotenoids such as β -carotene, lutein and canthaxanthin.^{7, 8)}

High-performance liquid chromatography (HPLC) has been employed as a powerful tool to analyze AX in the green algae. Most analyses of AX in different green algae have been performed using C18 columns.^{9, 10)} However, in comparison with the classical C18 column, the C30 column is much more hydrophobic, and is beneficial for isolating long-chain carotenoids and their esters with a less polarity. Recently, several reports demonstrated that the C30 column could provide better resolutions and higher separation efficiency than the C18 column.¹¹⁻¹⁴⁾

In *H. pluvialis*, AX exists mainly as AX esters of various fatty acids and elute over

broad range of retention time. A number of HPLC methods for separation and quantitation of AX and its ester of *H. pluvialis* have been reported,^{9, 10, 15, 16)} a few are considered ideal to quantitate total AX. Therefore, the saponification of algae extracts is recommended for reducing the number of chromatographic peaks and thus simplifies the quantitation.^{17, 18)} The existence of esterified AX in the supplements sometimes hampers accurate quantitation because AX is alkaline-labile carotenoid.¹⁹⁾ Therefore, optimal condition for saponification of its esters is necessary to be carried out for total AX quantitation.

In our previous report, an HPLC-UV/V is method for quantitation of the 4 carotenoids (α - and β -carotenes, α - and β -cryptoxanthins) in Buah Merah oil using combination of C30 and C18 columns was developed.²⁰⁾ Although this method achieved the separation of α - and β -cryptoxanthin, analytical run time was more than 60 min. Therefore the first aims of this study is to optimize the saponification condition for hydrolysis of AX ester from AX supplements and develop an HPLC-UV/Vis method for total AX quantification. The second one is to modify HPLC-UV/Vis method with shorten analytical run time by gradient elution for the quantification of α -, β -cryptoxanthin and α -, β -carotene, which was useful to gain information on other carotenoid contents in AX supplement.

Experimental

Reagents and chemicals

The standards used in this study, AX, α -cryptoxanthin, and α -carotene were obtained from Wako Pure Chemicals (Osaka, Japan), while β -cryptoxanthin was obtained from

ChromaDex, Inc. (CA, USA), and β -carotene was obtained from Kanto Chemical Co. (Tokyo, Japan). Other reagents such as NaCl, KOH, ethyl acetate (HPLC Grade) and acetonitrile (HPLC Grade) were obtained from Wako Pure Chemicals. Ethanol (99.5%), hexane and methanol were obtained from Nacalai Tesque (Kyoto, Japan) and ascorbic acid was obtained from Sigma-Aldrich (MO, USA). Water for solution and mobile phase was passed through a pure line WL 21 P (Yamato Scientific Co., Tokyo). Stock standard solution of AX ($50 \mu\text{g/mL}$) was prepared in ethanol, while stock standard solutions of α -, β -cryptoxanthin and α -carotene ($50 \mu\text{g/mL}$) and β -carotene ($100 \mu\text{g/mL}$) were diluted in hexane/ethanol mixture (1:4). All of stock standard solutions were stored at 4°C away from light for at least 1 week. The chemical structures of carotenoids are shown in Fig. 1.

Apparatus

An HPLC system consisted of two LC-

10 ATvp liquid chromatographic pumps (Shimadzu, Kyoto), a Develosil Combi RP-5 column ($50 \times 4.6 \text{ mm}$, i.d., $5 \mu\text{m}$, Nomura Chemical, Tokyo), a SPD-10A detector (Shimadzu), a 7125 injector with a $20 \mu\text{L}$ sample loop (Rheodyne, CA, USA), and an EZChrom Elite Version 3.3.7 J (GL Science Co., Tokyo). There were two different conditions for AX and other carotenoids quantitations. In AX quantitation method, the isocratic elution was used. The mobile phase at a flow rate of 1.0 mL/min was a mixture of acetonitrile and water with a ratio of 99:1 (v/v) containing 0.05% triethylamine (TEA) for chromatographic quantitation (from 0 to 9 min) and a mixture of acetonitrile, methanol and ethyl acetate with a ratio of 68:5:27 (v/v/v) containing 0.05% TEA was used for washing the column (from 9 to 20 min). In four carotenoids quantitation method, the gradient elution program with two different mixtures of mobile phases was used. As mobile phase A, a mixture of acetonitrile and water with a ratio of 95:5

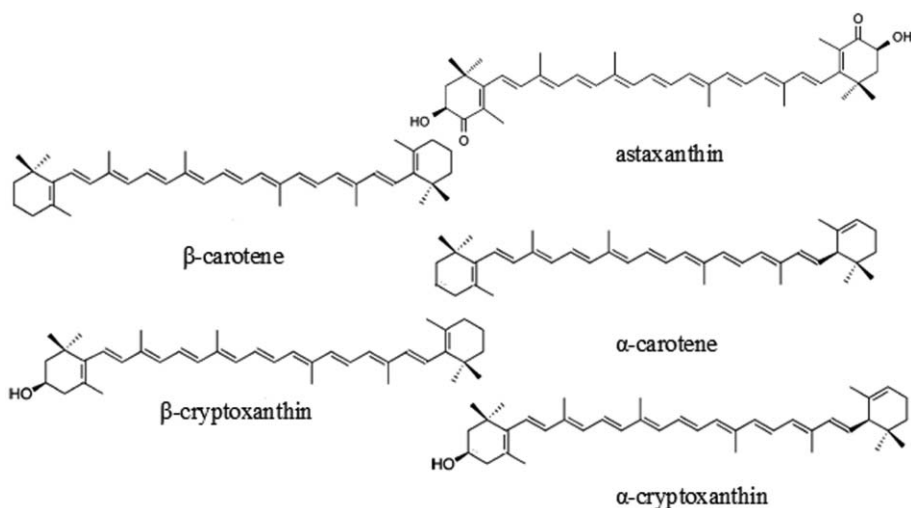


Fig. 1 Chemical structures of carotenoids

(v/v) containing 0.05% TEA was used, and a mixture of acetonitrile, methanol and ethyl acetate with a ratio of 68:5:27 (v/v/v) containing 0.05% TEA was used as mobile phase B. For separation of the carotenoids, the following gradient procedure was used: 1% of mobile phase B for 4 min, followed by linear gradient from 50 to 60% of mobile phase B until 15 min, changed to 100% of mobile phase B until 25 min and return to initial condition until 34 min. The mobile phases were degassed and pumped into the column at a total flow rate of 1.0 mL/min. Peak was monitored at wavelength of 476 nm (for AX) and 450 nm (for other carotenoids) to facilitate the detection.

Pretreatment of AX supplement sample solution

AX supplements used were oil in softgel capsules. Each of capsules was weighed and the shell was cut opened by using forceps and a scissor over a funnel in 25 mL volumetric flask, followed by washing the tools with 5 mL of hexane, sonicated for 5 min, diluted to volume by ethanol and sonicated again for 5 min. The sample was diluted 10 times before placed in amber bottle for analysis. The shell of the capsule was dried and weighed. The oil weight was obtained by reducing the weight of the capsule to weight of the shell. The prepared sample solution was immediately used for HPLC analysis.

For AX saponification, 10 mg of ascorbic acid and 550 μ L of ethanol were added to 200 μ L of sample solution and then vortex-mixed for 20 sec. To this solution, 200 μ L of KOH (0.9 M) was added, vortex-mixed for 30 sec, replaced by N₂ gas for 10 sec and incubated for 60 min (at room temperature). After saponification process, 250 μ L of NaCl (25 mg/

L) was added and vortex-mixed for 20 sec. Followed by the addition of 750 μ L hexane/ethyl acetate mixture (9:1), which was vortex-mixed for 1 min and then centrifuged at 2,000 g for 3 min at 10°C, the organic layer was transferred into test tube, the extraction process was repeated 3 times and all of organic layers were combined. Collected organic layer extract was evaporated to dryness by centrifugal evaporator (40°C), and the residue was reconstituted with 200 μ L of mobile phase A. After filtered by 0.45 μ m membrane filter, 20 μ L of reconstituted solution was injected onto the column. The pretreatment procedures for other carotenoids quantitation were demonstrated according to the previous report.²⁰⁾ The remarkable differences were concentration of KOH for saponification which was changed to 13.5 M and the incubation time of saponification process which was decreased to 30 min.

Validation of the methods

The AX concentration was quantitated by standard addition method using AX supplements spiked with two different concentrations of AX (0, 16.6 and 50.6 μ g/mg oil of AX), triplicate measurements for each concentration were performed. The linear of regression line from standard addition curve was forced backward to meet the negative horizontal axis. The cross negative point was the concentration itself by eliminating negative mark. Calibration curves of other carotenoids were prepared using AX supplements spiked with known concentrations of carotenoids; triplicate measurements for each concentration were performed. The limits of detection (LOD) and quantification (LOQ) were calculated as the peak heights at a signal-

to-noise (S/N) ratio of 3 and 10, respectively. Intra- and inter-day assay precisions for peak height and recovery were assessed using AX supplement spiked with 0–50.6 $\mu\text{g}/\text{mg}$ oil of AX, 80–2,600 ng/mL oil of α -, β -cryptoxanthins and α -carotene, and 80–5,200 ng/mL oil of β -carotene; five replicate measurements for each concentration were performed. The recovery was calculated by comparing the peak height for direct injection of the carotenoids standard in mobile phase A with those obtained by AX supplement containing same amount of AX.

Application to AX supplements

Those methods were applied to four brands of AX supplement. The supplement was prepared according to the method described, followed by pre-treatment for each purpose, AX or four carotenoids quantitation. For AX, AX supplements were spiked by 0, 16.6 and 50.6 $\mu\text{g}/\text{mg}$ oil of AX standard

and pretreated as mentioned earlier. The AX contents of the supplement were calculated by standard addition method and the correlation to indicate label was calculated as AX content ratio between original data and the label. For four carotenoids, AX supplements were pretreated as mentioned earlier. The concentrations of α -, β -cryptoxanthins, α - and β -carotenes in AX supplement were calculated from the corresponding calibration curve.

Results and Discussion

Separation conditions of AX and other carotenoids

For AX separation, various mobile phases were assessed. Eventually, the ratio of acetonitrile and water (=99:1, v/v) for 9 min and followed by washing solution with ratio of acetonitrile, methanol and ethyl acetate (=68:5:27, v/v/v) for the last 11 min provided the optimal result with retention times of 5.2 min. No interferences to AX peaks from the AX supplement matrix was found (Fig. 2).

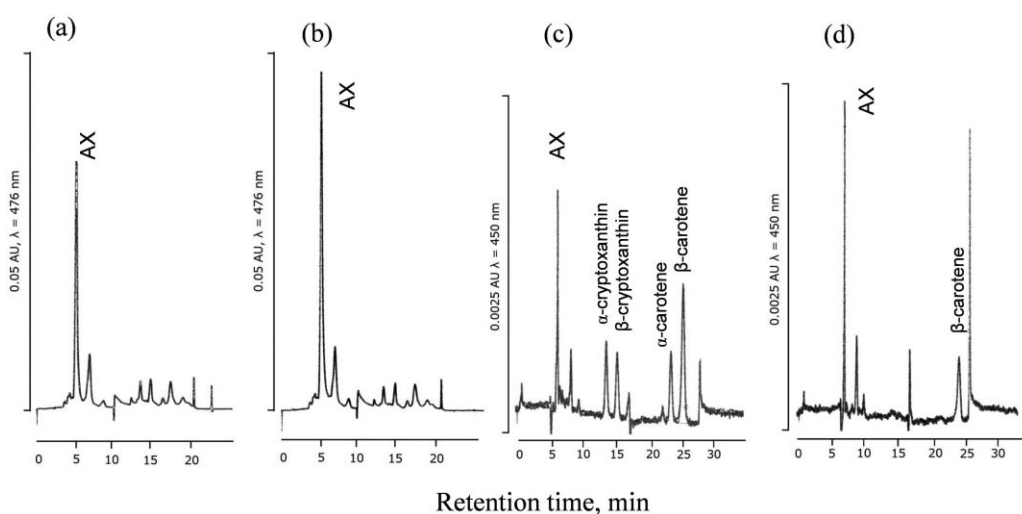


Fig. 2 Chromatograms of AX supplement.

Sample: AX supplement A (a) and (c), and those spiked with AX standard (16.6 mg/mg oil) (b), and α -, β -cryptoxanthin, α -carotenoid (163 ng/mg oil) and β -carotene (326 ng/mg oil) standards (d). (a) and (b) were isocratically eluted; (c) and (d) were eluted with the gradient program.

Optimization of saponification conditions of AX

The simple way to analyze AX content in the sample was by calculating the free form AX instead of calculating each fragment of AX including monoester and diester. Saponification procedure was needed to quantitate total AX. In this study, several concentrations of KOH were examined to hydrolyze AX ester (0.4, 0.7, 0.8, 0.9, 1, 1.3 M). The result showed that 0.9 M KOH gave the highest peak of AX (Fig. 3), this concentration was then used for saponification procedure of AX. Although some carotenoids were stable to alkaline solutions,¹⁸⁾ our result indicated that AX was not stable to high concentration of alkaline solution. AX is a labile-alkaline compound that higher concentration of KOH gave lower peak height than that on 0.9 M KOH. It might imply to

degradation of AX. Another condition such as saponification temperature or time should also be considered. Saponification with heating provides short incubation time, but the cold method does not affect the thermolabile carotenoids. In previous report, it is reported that a higher temperature (e.g. 40°C) favored the hydrolysis of AX esters, but the degradation of AX was also promoted at the same time compared with the ambient temperature saponification.¹⁹⁾ Therefore, it is suggested that high temperature should be avoided to the hydrolysis of AX esters. Next, the time for saponification process were examined (30, 60, 75, 90, 120 min). The highest peak height was given with 60 min of saponification, since longer incubation time also promoted degradation of AX (Fig. 3).

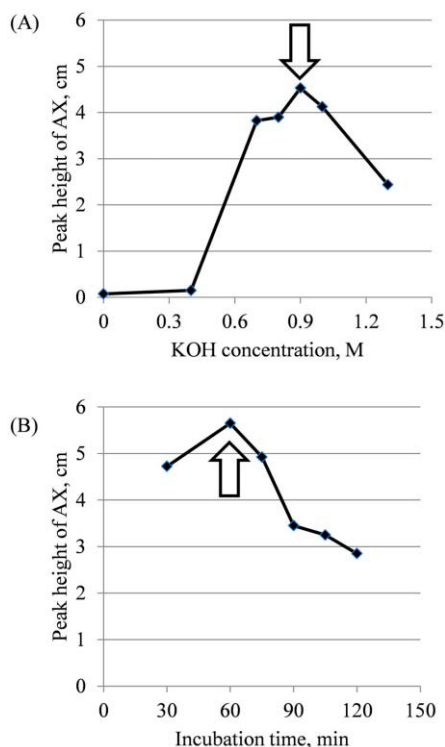


Fig. 3 Effects of KOH concentration (A) and saponification time (B) on peak height of AX

Method validation

The standard addition curve of AX using AX supplement spiked with 0, 16.6 and 50.6 $\mu\text{g}/\text{mg}$ oil of standard was prepared. The LOD and LOQ at S/N ratio of 3 and 10 were 1.05 and 3.50 $\mu\text{g}/\text{mg}$ oil, respectively. Intra- and inter-day precisions of the proposed method were evaluated by five replicate measurements of AX supplement spiked with known concentrations of AX (0, 16.6 and 50.6 $\mu\text{g}/\text{mg}$ oil). The intra- and inter-day assay precisions of peak height (relative standard deviation, RSDs) were less than 9.1% and 11.0. The recoveries were ranged from $57.0 \pm 5.6\%$ to $68.6 \pm 3.0\%$. The low recovery might be caused by simultaneously reaction of hydrolysis of AX ester which was converted to AX and degradation of AX. Saponification by KOH is carried out to hydrolyze AX ester to become AX, but in the same time KOH may act as catalyst of degradation process of AX. The calibration curve in AX supplement that spiked with α -, β -cryptoxanthins, α - and β -carotenes was obtained by plotting the peak height of each carotenoid versus the concentration in ng/mg oil, with replicate measurements. The calibration curve was linear in the range of 80–2,600 ng/mg oil for α -, β -

cryptoxanthins and α -carotene, and 80–5,200 ng/mg oil for β -carotene with a correlation coefficient of 0.999. The LODs at S/N ratio of 3 were 19.5, 20.2, 22.2 and 20.8 ng/mg oil and LOQs at S/N ratio of 10 were 64.4, 66.7, 73.3 and 68.8 ng/mg oil, respectively. Intra- and inter-day precisions, recovery and accuracy for 4 carotenoids were acceptable (Suppl. Table 1)

AX and β -carotene content in AX supplement

The proposed method was applied on AX supplement to investigate content of carotenoids in AX supplement. The quantitation results are summaries in Table 1. Sample A has 19.5 $\mu\text{g}/\text{mg}$ oil as stated in the label. The proposed method could quantitate 19.8 ± 0.52 $\mu\text{g}/\text{mg}$ oil, it means the percentage to the claim was $101.3 \pm 2.7\%$. The percentages to the claim for Sample B, C, D were $104.4 \pm 3.8\%$, $100.5 \pm 5.2\%$ and $98.1 \pm 3.2\%$, respectively. Furthermore, the proposed method of four carotenoids quantitation was applied to the supplements (Table 1). Only β -carotene could be quantitated in AX supplements used, whereas α -, β -cryptoxanthins and α -carotene were less than LOD of the method. The β -carotene content in AX supplements were

Table 1 Characteristics of AX supplements and their AX and β -carotene amounts

		Supplement			
		A	B	C	D
AX, $\mu\text{g}/\text{mg}$ oil	claim	19.5	46.7	31.9	15.5
	measured	19.8 ± 0.5	48.7 ± 1.8	32.1 ± 1.7	15.2 ± 0.5
	Percentage to claim %	101.3 ± 2.7	104.4 ± 3.8	100.5 ± 5.2	98.1 ± 3.2
β -carotene, ng/mg oil	claim	— ^a	— ^a	— ^a	3,627
	measured	127 ± 5	355 ± 35	229 ± 21	$3,556 \pm 89$
	Percentage to claim %	—	—	—	98.0 ± 2.5

a. No information on the package.

found 127 ± 4.6 ng/mg oil, 355 ± 34.7 ng/mg oil, 229 ± 21.3 ng/mg oil and $3,556 \pm 89.3$ ng/mg oil for sample A, B, C and D, respectively. The result of this analysis indicated that source of AX in those supplements also contain β -carotene even though in small amount. There is no information about other carotenoids in the sample except for sample D. The β -carotene content on the nutrition fact was 3,627 ng/mg oil, and agreed to our result ($98.0 \pm 2.5\%$ percentage to claim). The result of Nobre et al. concluded that beside esterified AX as main carotenoid of *H. pluvialis*, there were other minor carotenoids namely lutein, free AX, β -carotene and canthaxanthin.⁸⁾ However, β -carotene content in sample D was very high compared with other samples. There were two considered possibilities, whether the company which produced sample D, could extract high amount of β -carotene from *H. pluvialis* and/or it was added to the supplement.

The sensitivity of the proposed method was comparable to those of our previous method for carotenes.²⁰⁾ However, the method was less sensitive for cryptoxanthins, because eluent for cryptoxanthins decrease the sensitivity. It was difficult to compare the sensitivity with other methods, because the numbers of analytes and matrices are different. Nevertheless, the sensitivity was comparable with previous HPLC-UV methods,^{11, 13)} and 20 times less sensitive than that of LC-MS method.¹⁵⁾ Only a few reports have focused on AX quantitation in AX supplement. Other methods for AX quantitation are in marine bacteria, green algae mostly in *H. pluvialis*, crustacean, fish and birds.^{9, 16, 19)} To the best of our knowledge, only one method studied on quantification of

total AX in health food.²¹⁾ It was reported that optimum reaction condition was at 37°C, for 120 min using enzyme cholesterol esterase from *Pseudomonas fluorescens* to conduct the hydrolysis process. Our proposed method only needed 60 min for incubation time using conventional base, KOH, and it was not necessary to prepare expensive enzyme reagent. While, the article did not compare the result of analysis to indicated on label, our proposed method showed the reliability of the method for analyzing total AX content in the AX supplement which will be useful for quality control of AX supplement. In addition, another proposed method also can quantitate β -carotene which is beneficial for obtaining information about other carotenoids in the supplements.

Conclusions

The quantitative methods for quantitation of carotenoids in AX supplement have been developed using HPLC-UV/Vis. The first proposed method was for quantitation of total AX in AX supplement. An optimal saponification procedure for hydrolysis of AX ester from AX supplement was obtained. It would considerably reduce the time required for sample preparation. Furthermore, the proposed method was precise and accurate, confirming its utility to quantitate AX in AX supplement. The second proposed method, for quantitation of four carotenoids was also considerably precise and accurate. Moreover, β -carotene in supplements could also be confirmed. Both of methods are useful for routine analysis and quality control of AX supplement.

References

- 1) Rodriquez-Bernaldo de Quiros, A., Costa, H. S. (2006) "Analysis of carotenoids in vegetable and plasma: A review" *Journal of Food Composition and Analysis* 19(2-3), PP.97-111.
- 2) Tanaka, T., Makita, H., Ohnishi, M., Mori, H., Satoh K., Hara A. (1995) "Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin" *Cancer Research* 55(18), PP.4059-4064.
- 3) Hussein, G., Sankawa, U., Goto, H., Matsu-moto K., Watanabe H. (2006) "Astaxanthin, a carotenoid with potential in human health and nutrition" *Journal of Natural Products* 69(3), PP.443-449.
- 4) Guerin, M., Huntley, M. E., Olaizola, M. (2003) "Haematococcus astaxanthin: Applications for human health and nutrition" *Trends in Biotechnology* 21(5), PP.210-216.
- 5) Augusti, P. R., Conterato, G. M. M, Somacal, S., Sobieski, R., Spohr, P. R., Torres, J. V., Charao, M. F., Moro, A. M., Rocha, M. P., Garcia, S. C., Emanuelli, T. (2008) "Effect of astaxanthin on kidney function impairment and oxidative stress induced by mercuric chloride in rats" *Food and Chemical Toxicology* 46(1), PP.212-219.
- 6) Okada, Y., Ishikura, M., Maoka, T. (2009) "Bioavailability of astaxanthin in Haematococcus algal extract: The effects of timing of diet and smoking habit" *Bioscience Biotechnology and Biochemistry* 73(9), PP.1928-1932.
- 7) Machmudah, S., Shotipruk, A., Goto, M., Sasaki, M., Hirose, T. (2006) "Extraction of astaxanthin from Haematococcus pluvialis using supercritical CO₂ and ethanol as entrainer" *Journal of Industrial and Engineering Chemistry* 45 (10), PP.3652-3657.
- 8) Nobre, B., Marcelo, F., Passos, R., Beirão, L., Palavra, A., Gouveia, L., Mendes, R. (2006) "Supercritical carbon dioxide extraction of astaxanthin and other carotenoids from the microalga Haematococcus pluvialis" *European Food Research and Technology* 223(6), PP.787-790.
- 9) Yuan, J. P., Chen, F. (1998) "Chromatographic separation and purification of trans-astaxanthin from the extracts of Haematococcus pluvialis" *Journal of Agricultural and Food Chemistry* 46(8), PP.3371-3375.
- 10) Miao, F. P., Lu, D. Y., Li, Y. G., Zeng, M. T. (2006) "Characterization of astaxanthin esters in Haematococcus pluvialis by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry" *Analytical Biochemistry* 352(2), PP.176-181.
- 11) Taylor, K. L., Brackenridge, A. E., Vivier, M. A., Oberholster, A. (2006) "High-performance liquid chromatography profiling of the major carotenoids in Arabidopsis thaliana leaf tissue" *Journal of Chromatography A* 1121(1), PP.83-91.
- 12) Melendez-Martinez, A. J., Britton, G., Vicario, I. M., Heredia, F. J. (2006) "HPLC analysis of geometrical isomers of lutein epoxide isolated from dandelion (Taraxacum officinale F. Weber ex Wiggers)" *Phytochemistry* 67(8), PP.771-777.
- 13) Inbaraj, B. S., Chien, J. T., Chen, B. H. (2006) "Improved high performance liquid chromatographic method for determination of carotenoids in the microalga Chlorella pyrenoidosa" *Journal of Chromatography A* 1102(1-2), PP.193-199.
- 14) Li, L., Qin, J., Yin, S., Tang, G. (2007) "Effects of mobile phase ratios on the separation of plant carotenoids by HPLC with a C₃₀ column" *Chromatographia* 65, PP.91-94.
- 15) Breithaupt, D. E. (2004) "Identification and quantification of astaxanthin esters in shrimp (Pandalus borealis) and in microalga (Haematococcus pluvialis) by liquid chromatography-mass spectrometry using negative ion atmospheric pressure chemical ionization" *Journal of Agricultural and Food Chemistry* 52(12), PP.3870-3875.
- 16) Holtin, K., Kuhnle, M., Rehbein, J., Schuler, P., Nicholson, G., Albert, K. (2009) "Determination of astaxanthin and astaxanthin esters in the microalgae Haematococcus pluvialis by LC-(APCI) MS and characterization of predominant carotenoid isomers by NMR spectroscopy" *Analytical and Bioanalytical Chemistry* 395(6), PP.1613-1622.
- 17) Ittah, Y., Kanner, J., Granit, R. (1993)

- “Hydrolysis study of carotenoid pigments of paprika by HPLC/photodiode array detection” *Journal of Agricultural and Food Chemistry* 41(6), PP.899–901.
- 18) Minquez-Mosquera, M. I., Jaren-Galan, M. (1995) “Kinetics of the colouring of carotenoids pigments” *Journal Science of Food Agriculture* 67 (2), PP.153–161.
- 19) Yuan, J. P., Chen, F. (1999) “Hydrolysis kinetic of astaxanthin esters and stability of astaxanthin of *Haematococcus pluvialis* during Saponification” *Journal of Agricultural and Food Chemistry* 47(1), PP.31–35.
- 20) Wada, M., Fujimoto, K., Nishigaki, T., Febriyanti, E., Ikeda, R., Nakashima, K. (2013) “Determination of α - and β -cryptoxanthins, and α - and β -carotenes in Buah Merah oil by HPLC–UV detection” *Journal of Agro-Based Industry* 30(1), PP.1–8.
- 21) Ogawara, M., Ino, Y., Yoshida, M., Hishiyama, T., Igarashi, T. (2010) “High performance liquid chromatography quantification of total astaxanthin in health food” *Nippon Shokuhin Kagaku Kogaku Kaishi* 57(5), PP.205–214.