HPLC Method Validation for the Analysis of ®-Carotene in Capsicum Oil

Laksana Charoenchai[1*], Apirada Sucontphunt[1], Tun Chusut[1], and Thaniya Wunnakup[1].

Drug and Herbal Product Research and Development Center, College of Pharmacy, Rangsit University, Pathum Thani, Thailand.
Corresponding author Email: laksana.c@rsu.ac.th

Abstract

Capsicum oil was obtained from dried chili using a screw- pressed, machine. Besides capsaicinoids like capsaicin, carotenoids such as ®-carotene were a major compounds in capsicum oil. ®-Carotene was commonly found in vegetables and fruits with red, orange, and yellow colors. It was a plant pigment and precursor of provitamin A. This study aimed to develop a method for the analysis of ®-carotene in capsicum oil using high-performance liquid chromatography (HPLC) with a photodiode array detector. The method was validated for accuracy, precision, linearity, specificity, the limit of detection, the limit of quantitation, and robustness. The results showed that the performance parameters were within the acceptance criteria. The linear range was assessed at a concentration of 3.125-200 ⌈g/mL and showed a linear correlation ($R^2 = 0.9992$). The percent recovery of the addition method was 82.75–92.14% for 25, 50, and 75 ⌈g/mL of standard ®-carotene solution. Changing column temperatures and flow rates did not significantly affect the peak area values. The method successfully quantified ®-carotene in capsicum oil and may applied to analyze other capsicum samples.

Keywords*: *®-Carotene, Capsicum Oil, HPLC, Validation

1. Introduction

Capsicum oil was prepared by a screw-pressed machine. Capsaicinoids and carotenoids were major compounds found in capsicum oil. Carotenoids caused interest because they were sources of natural color and nutritional characteristics. Carotenoids were able to be biosynthesized in plants, while animals were incapable of carotenoid biosynthesis; they were obtained from diets. Carotenoids are also attributed to antioxidant activity and protective effects against degenerative diseases (Hassan, 2019). The major characteristic structures of carotenoids were isoprene units with conjugated double bonds. Carotenoids that were found in chili and pepper were ®-carotene, lutein, capsanthin, capsorubin, ⟨-carotene, zeaxanthin, and ®-cryptoxanthin (Rodriguez-Rodriguez, 2020). Carotenoids, which contained only carbons and hydrogens, were called carotene, while those containing oxygen were termed xanthophyll. This study focused on ®-carotene, which was the most abundant carotenoid in capsicum oil.

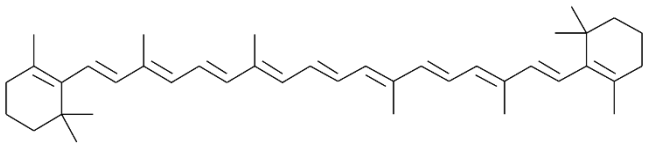

Figure 1 ®-carotene

The carotenoid compositions were affected by cultivars and other factors. The same carotenoids can be found in different varieties, either at low or high levels. Thus, the analytical method had to be adapted to the carotenoid compositions of each sample. According to AOAC and USP methods, sample preparation for analysis of ®-carotene in raw materials and supplements was soluble in either chloroform or tetrahydrofuran with a stabilizer (AOAC 2012; USP, 2014). Petroleum ether was suggested as an extraction solvent. However, petroleum ether and tetrahydrofuran need to be tested to remove peroxides and prevent carotenoid degradation. Although carotenoids were freely soluble in chloroform, dichloromethane was recommended for use instead due to its toxicity. Other water-miscible organic solvents, for example, acetone, methanol, and ethanol can also dissolve carotenoids. Capsicum oil was freely soluble in isopropyl alcohol, and soluble in methanol and ethanol. In this study, some organic solvents; for example, hexane, dichloromethane, ethyl acetate, diethyl ether, and cyclohexane were studied to extract ®-carotene from capsicum oil. Sample extraction and preparation were the preceding steps before the chromatographic analysis. Since capsicum oil exhibited oil characteristics, saponification process was required to remove interference lipids. In this study, the capsicum oil was saponified in mild conditions and extracted with organic solvent before the analysis.

Previous literature had reported carotenoid analysis using the spectrophotometric method and liquid chromatographic technique, both qualitative and quantitatively. Since carotenoids were chains and ®-carotene, which were bicyclic carotenes, they can be identified and quantified using high-performance liquid chromatography (HPLC). However, the retention time and the absorption spectra were not enough to identify different carotenoids because some carotenoids shared the same chromophore, resulting in the same retention time and absorption spectrum. Thus, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy were indispensable in the elucidation of carotenoid structures (Rodriguez-Amaya, 2001). In this study, the validated chromatographic system can identify mainly ®-carotene.

2. Objectives

The objectives of the study were

1) to develop a sample preparation method for the analysis of ®-carotene in capsicum oil,
2) to validate the HPLC-PDA method for the analysis of beta carotene in capsicum oil according to ICH guidelines,
3) to determine the beta carotene in capsicum samples.

3. Materials and Methods

Beta carotene was purchased from Sigma Aldrich. Acetonitrile and acetone were HPLC grade solvents and purchased from Dunkans Pure Chemicals, Korea. Hexane, dichloromethane, ethyl acetate, isopropyl alcohol, methanol, and diethyl ether were ACS/AR grade and purchased from Fisher Scientific, UK. Potassium hydroxide was analytical grade. Deionized water was prepared by the Econos water purifier system.

Sample preparation and saponification

Capsicum oil (1 g) was soluble in isopropyl alcohol (50 mL). The saponification was prepared by adding 10% potassium hydroxide-methanol solution (Xu, 2023). The reaction was swirled and left for one hour. Then it was separated with organic solvents (diethyl ether, hexane, dichloromethane, ethyl acetate, and cyclohexane) three times. The solutions were washed with 50 mL of water until they obtained a neutral pH (3-5 times). Then the organic solvents were combined and removed under a vacuum using a rotary evaporator. The residue was reconstituted in acetone and injected into HPLC.

Standard solution and method validation procedure

®-Carotene standard solution was prepared at a concentration of 1,000 ⌠g/mL. Then it was diluted to prepare working solutions at a concentration of 3.125 – 200 ⌠g/mL. The analytical method was performed on a poroshell EC C_{18} (2.1 x 150 mm, 4 ⌠m) at 30 °C. The mobile phase was acetone (A) and water (B) in gradient elution of 0-5 min: 75%A; 5-10 min: 75-95%A; 10-17 min: 95%A; 17-22 min: 95-100%A; 22-27 min: 100-75%A and maintained for 3 minutes (Xu, 2023). The flow rate was 0.35 mL/minute. It was monitored at a wavelength of 450 nm. The injection volume was 10 ⌠L. The analytical method was validated for linearity, accuracy, precision, and specificity. Robustness was determined by changing column temperatures (28 and 32 °C) and flow rates (0.32 and 0.38 mL/min). The limit of detection (LOD) and limit of quantitation (LOQ) were also determined from (3.3 x slope)/SD and (10 x slope)/SD of the calibration curve. The results were compared using statistical analysis (ANOVA and t-test) for robustness using IBM SPSS version 22.

4. Results and Discussion

4.1 Results

The analytical method of ®-carotene in capsicum oil was properly validated to ensure the reliability of the analytical data and the good performance of the method. Table 1 summarizes the validated results. Peak areas of the standard ®-carotene solutions a showed linear response and were well correlated with a correlation coefficient greater than 0.995. The accuracy was determined using the standard addition method, and the percent recovery was in the range of 82.75 – 95.14 percent. The method was precise because the retention times and peak areas of the standard solutions showed the coefficients of variations (CV) were within the criteria on the same day. On three different days, this method was also reproducible. The method was specific and selective for ®-carotene. The ®-carotene peak showed a maximal absorption wavelength of 450-454 nm with the shoulder in the sample solution and the standard ®-carotene solution, respectively (Figure 2). The ®-carotene peak in the saponified sample was separate from other interferences (Figure 3). The ®-carotene peak in the sample solution was eluted at a retention time of 14.8-15.0 minutes, which corresponded to that of standard solution.

Table 1 Method validation results

Parameters					**Results**	**Criteria**
Linearity					y = 20.343x – 86.033	$R^2 > 0.995$
(3.125-200 ⌠g/mL)					$R^2 = 0.9992$	
Range					Y = 21.214x – 176.64	$R^2 > 0.995$
(25-75 ⌠g/mL)					$R^2 = 0.9999$	
Accuracy					%Recovery	80 – 110%
Conc. (⌠g/mL)						
25					82.75 ± 7.18	
50					92.30 ± 6.88	
75					95.14 ± 2.01	
Repeatability					%CV	%CV < 5.3 (Intraday)
Conc. (⌠g/mL)	Intraday	Day 1	2	3	Inter-day	%CV < 7.3 (Inter-day)
Retention time						
25		0.05	0.13	0.04	0.4	
50		0.10	0.27	0.08	0.3	
75		1.18	0.14	0.09	0.6	
Peak area						
25		0.86	3.86	1.04	5.0	
50		0.10	0.36	0.39	7.3	
75		0.64	0.98	0.16	7.3	
LOD (⌠g/mL)					5.41 ± 0.03	
LOQ (⌠g/mL)					9.00 ± 0.03	

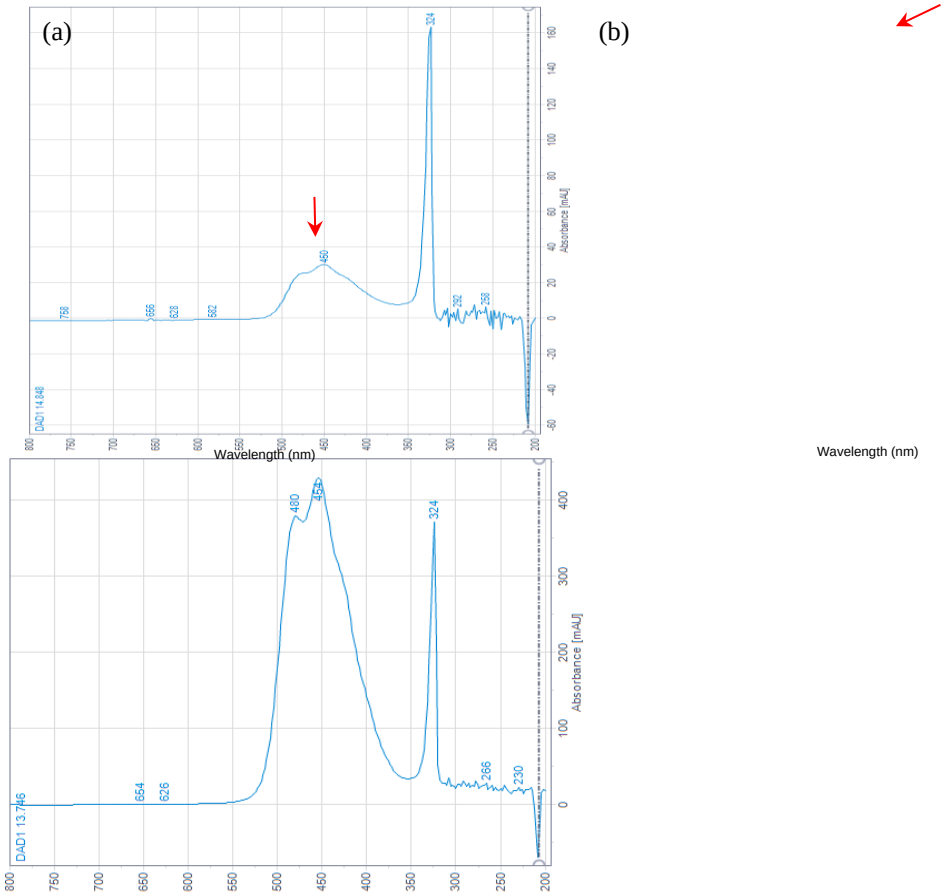

Figure 2 Spectrum of ®-carotene in the sample solution (a) and in the standard solution (b)

The method was robust because changing column temperatures and flow rates did not change their peak areas statistically significantly. However, increasing column temperature and flow rate resulted in faster elution of ®-carotene peak significantly. Two analysts analyzed ®-carotene in capsicum oil with the same procedure. The results showed comparable accuracy.

Table 2 Robustness of HPLC method

Parameters		**Results**		***p*-value**
Column temperature (°C)	28	30	32	
Retention time (minute)	15.17 ± 0.01	14.87 ± 0.00	14.69 ± 0.09	0.00
Peak area	290.82 ± 195.02	264.82 ± 167.18	303.48 ± 210.34	0.94
Flow rate (mL/min)	0.32	0.35	0.38	
Retention time (minute)	15.40 ± 0.08	14.94 ± 0.06	14.40 ± 0.10	0.00
Peak area	754.79 ± 530.51	682.69 ± 436.19	658.34 ± 462.87	0.94
	Analyst 1	Analyst 2		
Retention time (minute)	14.88 ± 0.02	14.80 ± 0.04		0.03
Peak area	752.33 ± 361.04	831.33 ± 424.15		0.68

In addition, the stock standard ®-carotene solution was prepared and kept in the refrigerator for 15 days. On the seventh and fifteenth it was diluted to prepared the working solutions (3.125 – 200 ⌈g/mL) and injected into HPLC to determine their peak areas compared with the first day. Figure 4 showed that the ®-

carotene remained higher than 50% after being stored for 7 days, and the compound was degraded and decreased to less than 50 % on the fifteenth day. Although the stock solution was protected from light and kept at a cool temperature (4-8 °C), the stock standard solution was stable for approximately 3 days or less than 7 days.

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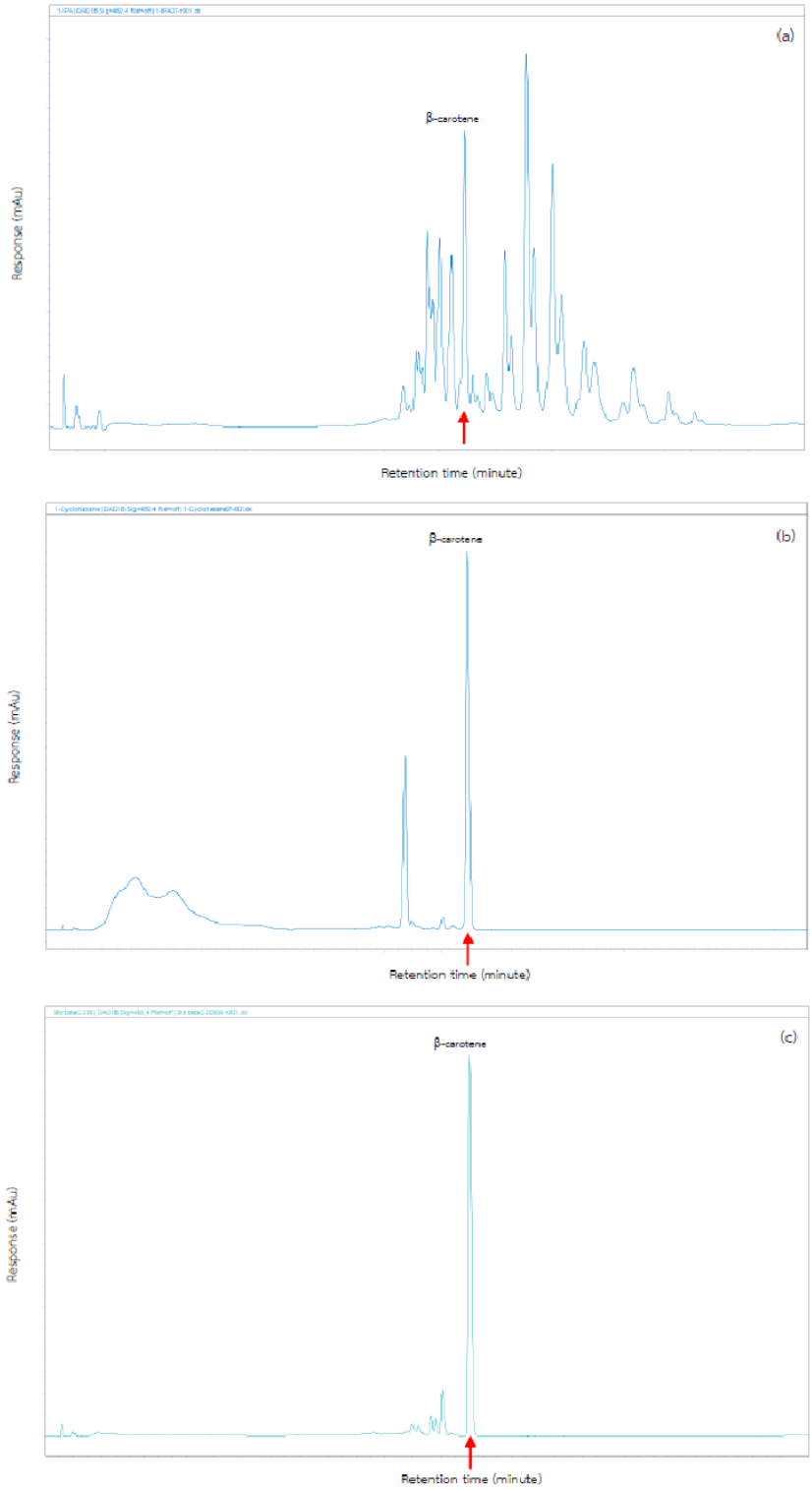

Figure 3 HPLC chromatogram of beta carotene in (a) sample solution in isopropyl alcohol (b) sample solution after saponification and (c) in the standard solution

4.2 Discussions

Organic solvents play an important role in removing carotenoids and transforming them into homogenous and representative samples. Diethyl ether was partitioned to the extracted solution after saponification and yielded the highest ®-carotene content (0.58±0.29% w/w) compared with dichloromethane, ethyl acetate, and hexane (0.37-0.40 %w/w), respectively. In this partitioning step in the separatory funnel, ®-carotene and some carotenoids may be lost due to an emulsion forming. During washing potassium hydroxide with water, ®-carotene may also be lost.

®-Carotene was identified and quantified at a moderate level in the mobile phase system. Other carotenoids were found at a small level (Xu, 2023); however, they did not show up in the HPLC chromatogram. It may be that the organic solvents that were used in the partition step cannot be extracted them or lost during the saponification step. Although 10% potassium hydroxide in methanol saponified the capsicum oil for one hour, the duration of the reaction was not long enough compared to overnight, as mentioned in the literature (Rodriguez-Amaya, 2001). In addition, the combined extracted solvents were removed using a rotary evaporator. The temperature of the water bath was 45 °C. Heat may cause the loss of some carotenoids. Capsanthin, capsorubin, and zeaxanthin which were found to small amount in capsicum (Xu, 2023), were soluble in petroleum ether.

The absorption spectrum of carotenoids showed specific characteristics in different solvents. During the elution in the HPLC mobile phase system, which was either isocratic or gradient, there was a mixture of at least two organic solvents. Therefore, there might be a hypsochromic shift or a bathochromic shift. ®-Carotene was absorbed maximally at three wavelengths, resulting in two peak spectra; λ_{max} = 454, 480 nm in acetone (Figure 2 (b)). The absorption spectra of the sample showed a similar pattern, with a small extent due to the low level of ®-carotene compared with that of the standard. The λ_{max} values were higher by 2-6 nm in acetone relative to petroleum ether.

Light, heat, and oxygen can cause the oxidation, isomerization, and cyclization of carotenoids. Carotenoids may decompose in acids, but most carotenoids are stable towards alkali. The stock standard ®-carotene solution, which was prepared in acetone and kept in a refrigerator. The solution was not flush with nitrogen, so it degraded dramatically within 7 days in a refrigerator.

Natural carotenoids were present in the t*rans* configuration. To separate cis/trans-isomers, the C_{30} stationary phase was recommended. In this study, a poroshell EC C_{18} (2.1 x 150 mm, 4 ⌈m) HPLC column was used. Therefore, other carotenoids were not identified in this chromatographic mobile phase system. The chromatographic condition was adopted from the condition reported by Xu, in 2023. In that study, Spherisorb ODS-2 C_{18} (4.6 x 250 mm, 5 ⌈m) was used, and the retention time of ®-carotene was slightly later.

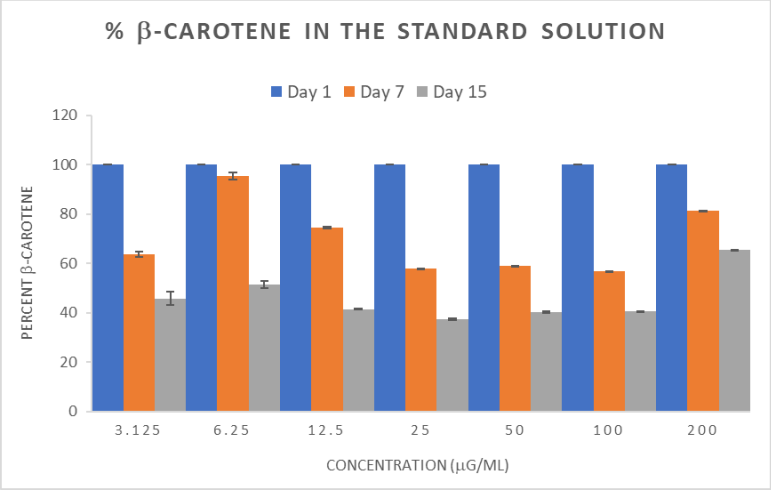

Figure 4 Percent remain of ®-carotene in the working standard solution

5. Conclusion

The analytical method for ®-carotene in capsicum oil was validated. Diethyl ether, dichloromethane, and hexane were the optimal solvents for the extraction after saponification. Acetone was used as a reconstituted solvent before injection into HPLC. The validated analytical method for ®-carotene was accurate, precise, and selective. The method was reliable for the analysis of ®-carotene in capsicum oil and can be applied to analyze capsicum powder.

6. Acknowledgements

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