CHAPTER IV

RESULTS

1. Development of An Analytical Method for Capsaicin Content in Capsicum

In this study, the development of capsaicin analytical method was performed using the principle of the so-called "solid-phase extraction" combined with HPLC analysis. Based on this method, three major steps were first optimized. The first step was to select the suitable extraction method for exhaustive extraction of capsaicin from capsicum fruits. The second was to develop the solid-phase extraction for effective cleaning of the capsicum crude extract. The last step was to optimize the HPLC conditions for maximal separation of the extracted capsaicinoids.

1.1 Capsaicin Extraction from Capsicum fruits

The aim of this experiment was to compare the efficiency of three different extraction methods on the isolation of capsaicin from capsaicum fruits. These methods included soxhlet, reflux and sonication. The results obtained from this study would lead to the suitable extraction method that gave the highest yield of capsaicin and, therefore, the accuracy of capsaicin content present in the fruit samples.

In doing this, five different varieties of capsicum fruit were selected (Fig.18). Each sample was ground to powder and passed through No. 40 sieve before extraction. For Soxhlet extraction, a 5-gram amount of capsicum powder from each sample was packed in a timble and extracted with about 250 ml methanol for more than 10 hr. The extraction volume was adjusted to 250 ml before subjected to HPLC

analysis. For reflux and sonication methods, the powdered samples were weighed for 100 mg and refluxed with 10 ml methanol at 70°C for one hr or sonicated with 10 ml methanol at 45°C for one hr. The extracts obtained from these three extraction methods were then injected directly to HPLC and evaluated for their capsaicin content.

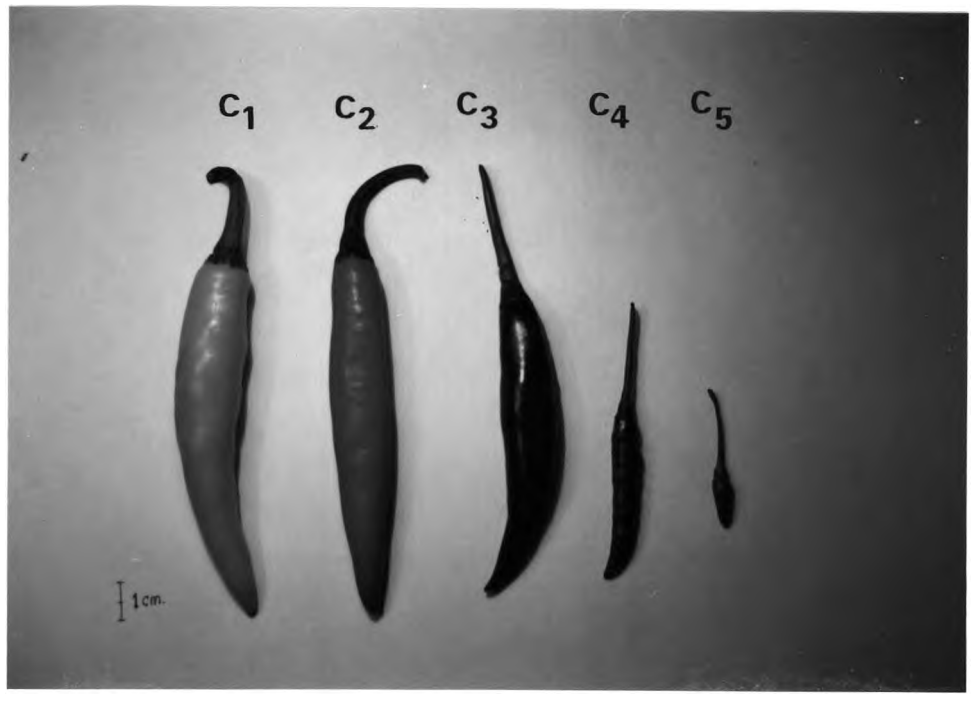

Fig. 18 Five varieties of *Capsicum* fruits used for studying the suitable method of capsaicinoid extraction. The fruit samples from left to right are C1 to C5 in which their local names are as follows, C1=พริกเหลือง, C2=พริกแดง, C3=พริกขี้ฟ้า, C4=พริกขี้หนู, C5=พริกขี้หนูสวน. All varieties were obtained from Mabunkrong fresh market.

The results showed that the HPLC chromatograms obtained from the three extraction methods were all very similar. A typical chromatogram of the crude extract is shown in Fig. 19. The retention time of capsaicin was approximately 12 minutes. It can be seen that there were a number of impurity peaks which were eluted before and after the peak capsaicin. Using the area under the capsaicin peak for calculation, the capsaicin content in each sample could be determined. As shown in Table. 4 and Fig. 20. The Soxhlet method gave the lowest content of capsaicin in all five varieties of

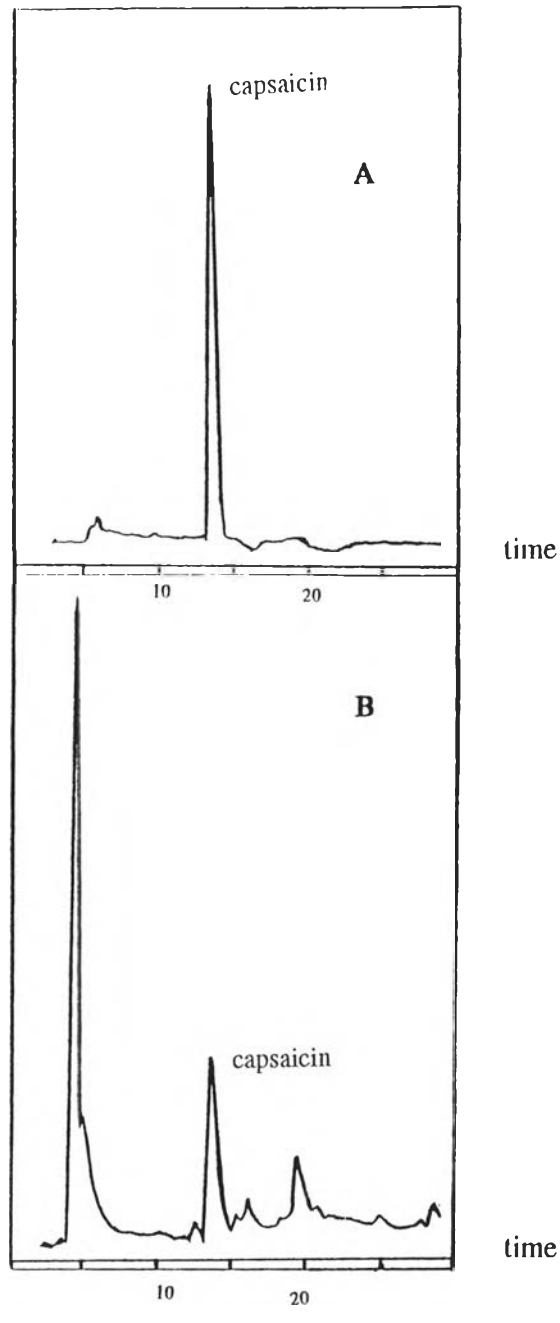

Fig. 19. HPLC chromatograms of standard capsaicin (A) and a crude extract (B) obtained from either Soxhlet, reflux or sonication.. The HPLC conditions were as followed : the HPLC column was a 125 mm x 4 mm. of reverse-phase octadecylsilane column with 5 μm particle size. The mobile-phase was 65% methanol in water, isocratic for 25 min., then the proportion of methanol was increased to 100% in 5 min. and maintained to the end at 45 min.. The flow rate of mobile-phase was adjusted at 0.75 ml/min, the injection volume for each sample was 20 μl, A.U.F.S. was 0.05 and the peak area of capsaicin was obtained from detection of ultraviolet absorption at 280 nm.

capsicum samples whereas reflux and sonication gave significantly higher content. Since the sonication method appeared to be the most effective on capsaicin extraction and most convenient in sample handling, it was the method of choice for the first step of capsaicin extraction.

Table. 4 Comparison among three different extraction methods used for the determination of capsaicin in five varieties of capsicum fruits, C1=พริกเหลือง, C2=พริกแดง, C3=พริกขี้ฟ้า, C4=พริกขี้หนู, C5=พริกขี้หนูสวน.

Capsicum cultivars	% capsaicin / dry weight		
	Soxhlet	Reflux	Sonicate
C1	0.063 ± 0.016	0.174 ± 0.020	0.180 ± 0.014
C2	0.095 ± 0.001	0.242 ± 0.031	0.256 ± 0.018
C3	0.233 ± 0.039	0.361 ± 0.041	0.404 ± 0.026
C4	0.278 ± 0.048	0.597 ± 0.016	0.613 ± 0.014
C5	0.532 ± 0.025	0.941 ± 0.045	0.982 ± 0.070

Each value represents the mean ± SD of six separate preparations.

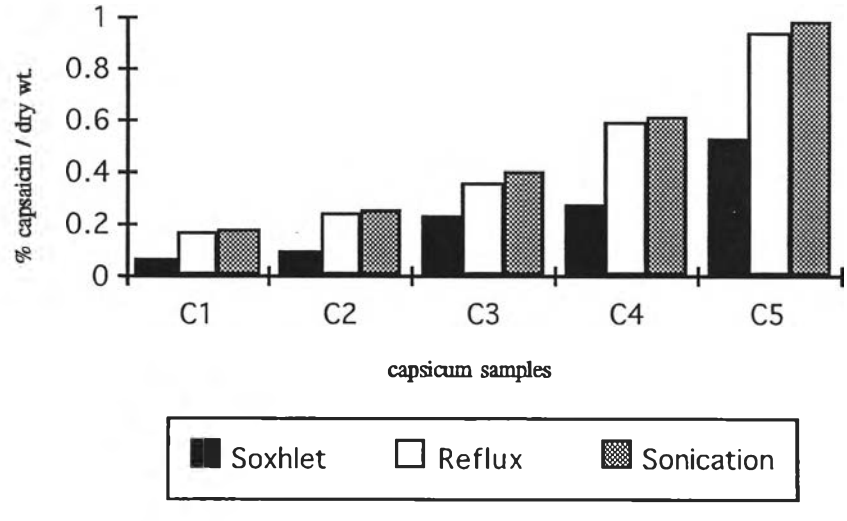

Fig. 20 Bar graph for the comparison among three different extraction methods used for the detemination of capsaicin contents in five diferent varieties of capsicum fruits. The values ploted were based on the data shown in Table. 4.

1.2 Cleaning of Capsaicin Crude Extracts by Solid-Phase Extraction.

1.2.1 Selection of the Sorbent Bed.

For removal of impurities from capsaicin extracts by using solid-phase extraction technique, a suitable absorbent bed was first investigated. In this study, a number of absorbents including silica gel, alumina oxide and octadecylsilane (ODS) were tried. Experimentally, the methanolic extracts of capsicum prepared by sonication were passed through each kind of the sorbent beds which was pre-equilibrated with methanol. The resulted eluates were then detected by HPLC. It was found that all the sorbent beds could not trap capsaicin on their beds under the conditions of methanol. By increasing the initial polarity of the crude extract, however, it was found that the sorbent bed of octadecylsilane (400 mg in 5 ml column) could effectively trap capsaicin when the crude methanolic extract was mixed with water in a 1:1 ratio to give the crude extract in 50% methanol. Under these conditions, no capsaicin was detected in the eluate of the same solvent (50% methanol). Therefore, octadecylsilane was chosen as sorbent bed for subsequent study.

1.2.2 Selection of Eluting Solvent.

In order to select a suitable organic solvent to elute capsaicin out of the octadecylsilane column, ten different mixed-solvents with different polarity were prepared. These solvents included :

1. methanol : acetonitrile, 50:50
2. methanol : acetonitrile, 60:40
3. methanol : acetonitrile, 70:30
4. methanol : acetonitrile, 80:20
5. methanol : acetonitrile, 90:10
6. methanol : acetonitrile, 100:0
7. methanol : water, 90:10

8. methanol : water, 80:20
9. methanol : water, 70:30
10. methanol : water, 60:40

In doing this experiment, one hundred milligrams of capsicum powder was extracted with 10 ml of methanol by sonication at 45^{o}C for 1 hr. The extract of each sample was centrifuged for 2 min and 1 ml of the clear solution was taken to mix with the equal volumn of distilled water in a test tube. In loading the sample, each octadecylsilane column was pre-equilibrated with 3 ml 50% methanol in water followed by loading 0.6 ml of the mixed-extract onto each column. After each extracts was passed through the sorbent bed by vaccumm suction, the column was washed with 3 ml 50% methanol in water in order to get rid of impurities. After the washing, 3 ml of each mixed-solvent described above (No. 1-10) was passed through each column and its eluate was collected under vaccuum. The eluates from all samples were then dried simutaneously in a Speed Vac centrifugation and redissolved with 2 ml methanol. For convenience in the evaluation of the elution efficiency of capsaicin from the column, the uv-spectra of each eluate was produced by using a spectrophotometer. The resulted spectra were then compared with the spectrum of standard capsaicin. The best solvent for capsaicin elution would give the absorption spectrum very similar to that of the standard capsaicin. As shown in Table. 5, the combination of methanol and acetonitrile (from 50:50 to 90:10) or pure methanol gave the eluates with yellow color and their absorption spectra were all of type A (Fig. 21A). On the other hand, the combination of methanol and water from 90:10 to 70:30 ratios gave colorless eluates and their absorption spectra were all of type B (Fig. 21B).

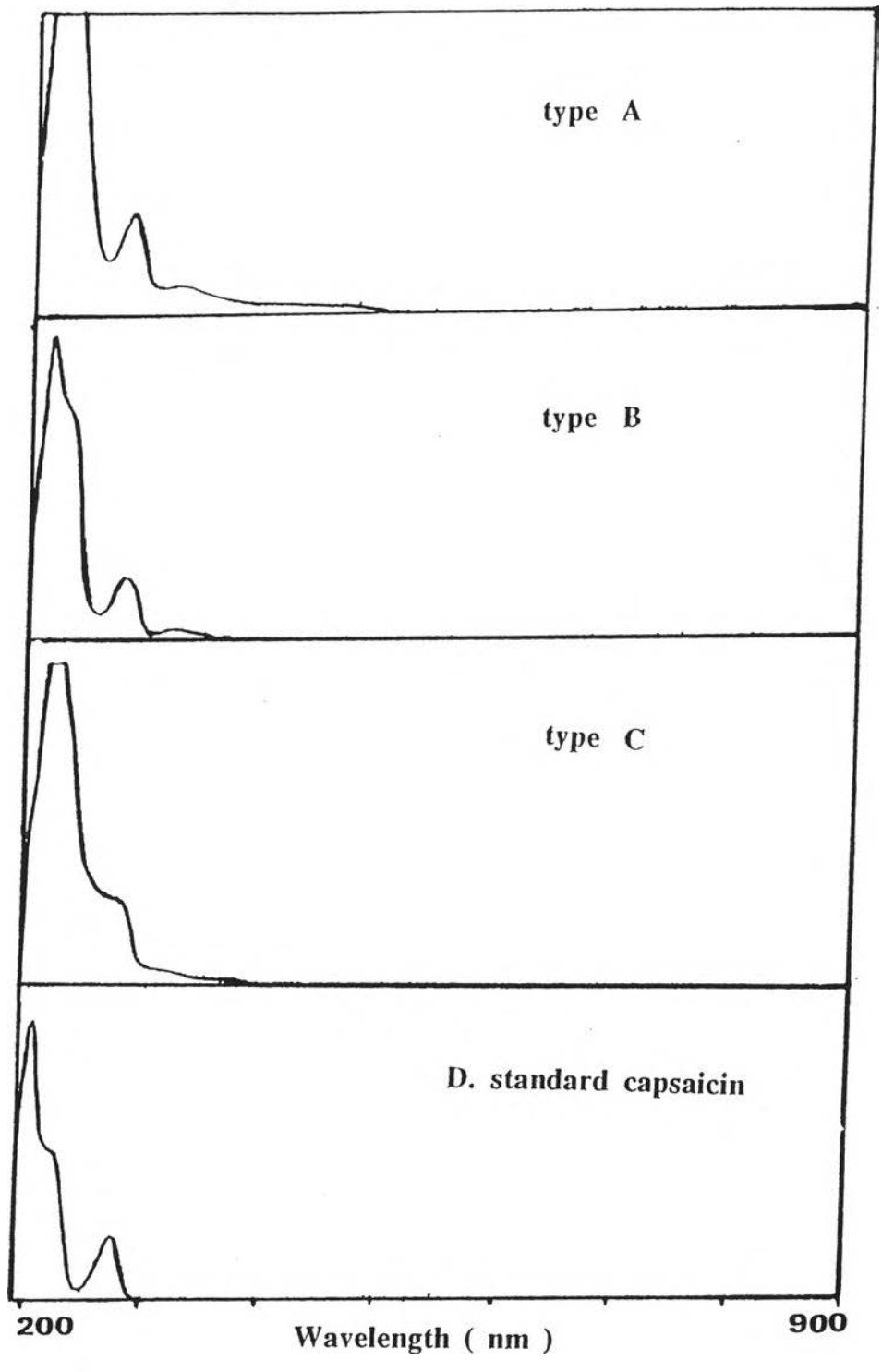

Fig. 21 The absorption spectra of different capsaicin eluates obtained from the use of different solvents to elute the trapped capsaicin onto the octadecylsilane column. The absorption spectrum type A (A) is obtained with the eluents of methanol and acetonitrile (50:50 to 90:10) or pure methanol. The absorption spectrum type B (B) is obtained with the eluants of methanol and water (90:10 to 70:30) and the type C (C) with methanol and water 60:40. D is the absortion spectrum of standard capsaicin.

Table. 5 The effect of different eluents on the elution of capsaicin out of the octadecylsilane column . The eluate obtained from each elution was observed for the presence of color and its type of absorption spectrum.

Eluent	Eluate color	Absorption spectrum
MeOH:CH_3CN = 50:50	yellow	type A (fig. 21A)
MeOH:CH_3CN = 60:40	yellow	type A
MeOH:CH_3CN = 70:30	yellow	type A
MeOH:CH_3CN = 80:20	yellow	type A
MeOH:CH_3CN = 90:10	yellow	type A
MeOH:CH_3CN = 100:0	yellow	type A
MeOH:H_2O = 90:10	colorless	type B (Fig. 21B)
MeOH:H_2O = 80:20	colorless	type B
MeOH:H_2O = 70:30	colorless	type B
MeOH:H_2O = 60:40	colorless	type C (Fig. 21C)

Further increase of water in methanol in 60:40 (methanol : water) however, resulted in a change of the absorption spectrum of the eluate to type C (Fig. 21C). When all spectra were compared with the absorption spectrum of standard capsaicin (Fig. 21D), it appeared that the spectrum of type B was the most similar to that of the standard. These results suggested that the solvent of methanol and water in a ratio from 90:10 to 70:30 could all be used to elute mainly capsaicin out of the octadecylsilane column. Since the 70:30 (methanol : water) is the most economy comparing with the other solvents, it was used through out in this study.

1.3 Separation of Capsaicinoids in Capsicum Extract by HPLC.

The separation of capsaicinoids in capsicum extracts after being treated by solid phase extraction was performed by HPLC as described in Section 5.2 of Chapter III. The resulted HPLC chromatograms (Fig. 22) showed that there were only two major peaks present in various prepared capsicum extracts. The main peak with the retention time of 12 min was capsaicin whereas the smaller peak with the retention time of 15 min was thought to be dihydrocapsaicin. It should be noted that the HPLC chromatogram of capsicum extract after the step of solid-phase extraction was very similar to the chromatogram of 60% pure standard capsaicin (Fig. 22). This indicated that the step of solid phase extraction was very effective in getting rid of impurities in the crude extract out of the capsaicin fractions.

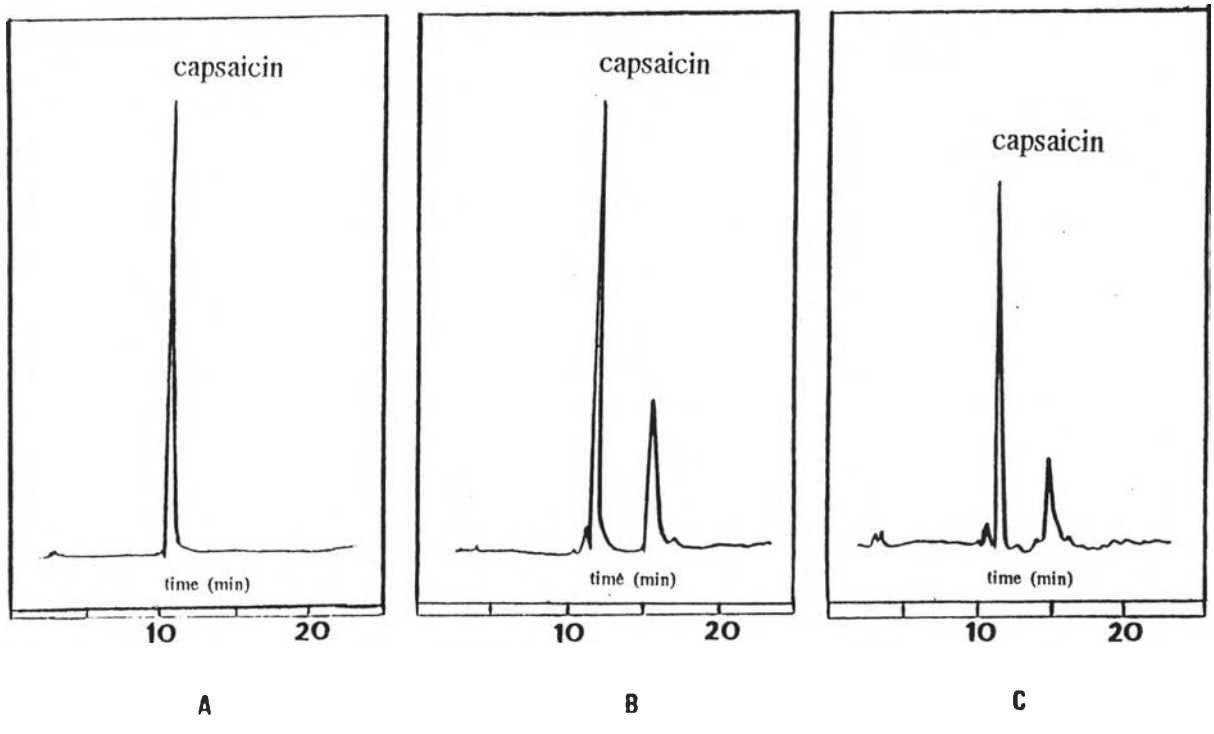

Fig. 22 HPLC chromatrograms of (A) standard capsaicin with 99% purity (Sigma^R No. M-2028); (B) standard capsaicin with 60% purity (Sigma^R No. M-3403) and (C) capsicum extract after subjected to solid-phase extraction.

1.4 The Efficiency of Sample Preparation by Solid-Phase Extraction.

The aim of this experiment was to check the recovery of capsaicin after the process of solid-phase extraction of capsicum extract. This was carried out by comparing the capsaicin content present in the crude capsicum extracts before and after the solid-phase extraction. Five varieties of capsicum that differ in capsaicin content were selected (Fig. 23). Each variety was extracted in methanol by sonication as described in Chapter III and the clear solution was devided into two parts. One part was injected directly to HPLC and the peak area obtained was calculated for capsaicin content before solid-phase extraction. The other part was subjected to solid-phase extraction followed by HPLC injection (Chapter III,.Section 5.2). The resulted chromatogram was used to calculated capsaicin content after solid-phase extraction. The HPLC separation conditions were followed by Section 1.1 of this Chapter. The comparison of capsaicin content is showed in Table 6 and Fig. 24. It can be seen that the recovery of capsaicin in the samples varied between 85-110% indicating that no significant loss of capsaicin occured during the process of solid-phase extraction. The efficiency of the solid-phase extraction could also be presented in Fig. 24 which showed linearity of the relationship between capsaicin content in capsaicinoid extracts obtained before and after solid-phase extraction process.

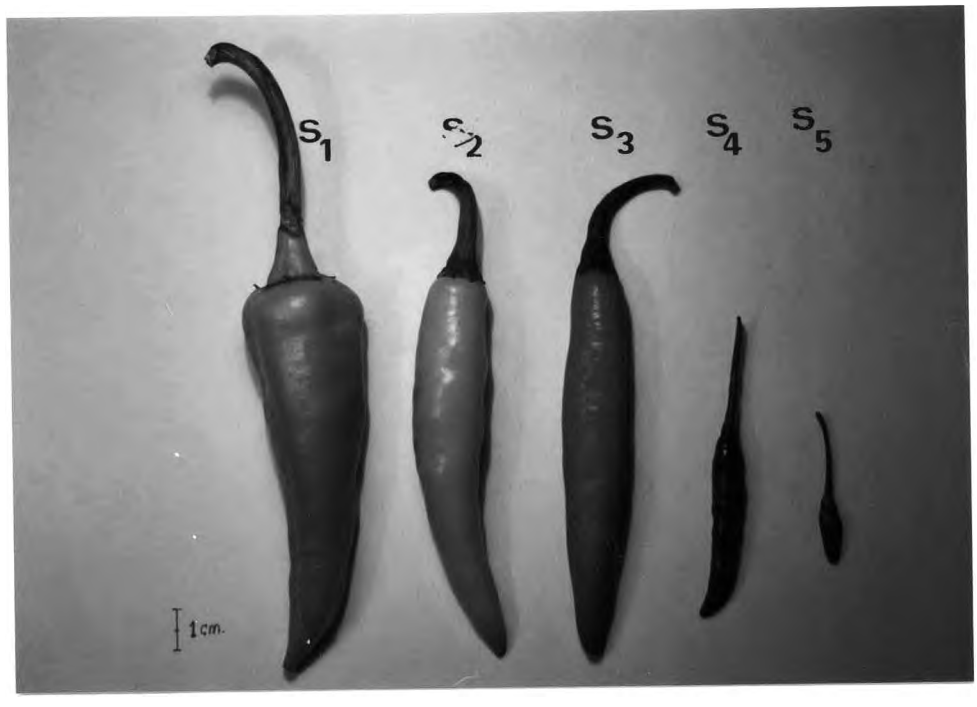

Fig. 23 The five varieties of *Capsicum* fruits that used for checking the recovery of solid-phase extraction; from left to right are S1 to S5 which local name as follows, S1=พริกหยวก, S2=พริกเหลือง, S3=พริกแดง, S4=พริกขี้หนู, S5=พริกขี้หนูสวน. All of varieties were from Mabunkrong fresh market.

Table. 6 The recovery of capsaicin in sample preparation process by solid-phase extraction technique of five different varieties of capsicum fruits, S1=พริกหยวก, S2=พริกเหลือง, S3=พริกแดง, S4=พริกขี้หนู, S5=พริกขี้หนูสวน.

Capsicum cultivars	% capsaicin (dry weight)		%recovery
	Before solid-phase extraction (sonicate only)	After solid-phase extraction (sonicate, sample prep)	
S1	0.078 ± 0.030	0.066 ± 0.012	84.62
S2	0.180 ± 0.014	0.199 ± 0.039	110.55
S3	0.256 ± 0.018	0.240 ± 0.032	93.75
S4	0.613 ± 0.014	0.593 ± 0.054	96.74
S5	0.982 ± 0.070	1.076 ± 0.018	109.57
Mean of % recovery = 99.05 ± 9.85		% CV = 9.93	

Each value represents the mean ± SD of six separate preparations.

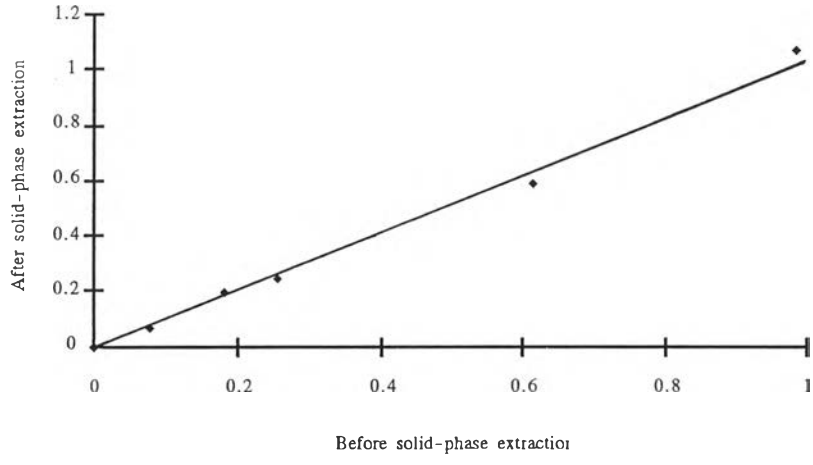

Fig. 24 The comparison of capsaicin contents in five varieties of capsicum fruits in term of linear relation between the percentage of capsaicin by HPLC of sample extracts only (before sample preparation) and sample extracts with solid-phase extraction (after sample preparation).

1.5 Calibration Curve of Standard Capsaicin by HPLC

The calibration curve of capsaicin was obtained by HPLC which conditions were followed on Chapter III, Section 1.4 (Fig. 25). The curve show linearity of the peak area-capsaicin contents relationship between the capsaicin content range of 0.12-5.86 μg. Result of the regression analysis and correlation coefficient (r) was found to be 0.9997 and the regression equation of standard capsaicin was $\mathbf{y} = 103191.3\,\mathbf{x} + 4626.09$ ($\mathbf{n} = 10$)

where **y** = peak area of capsaicin

x = capsaicin content (μg)

n = the number of standard capsaicin concentration levels

r = correlation coefficient

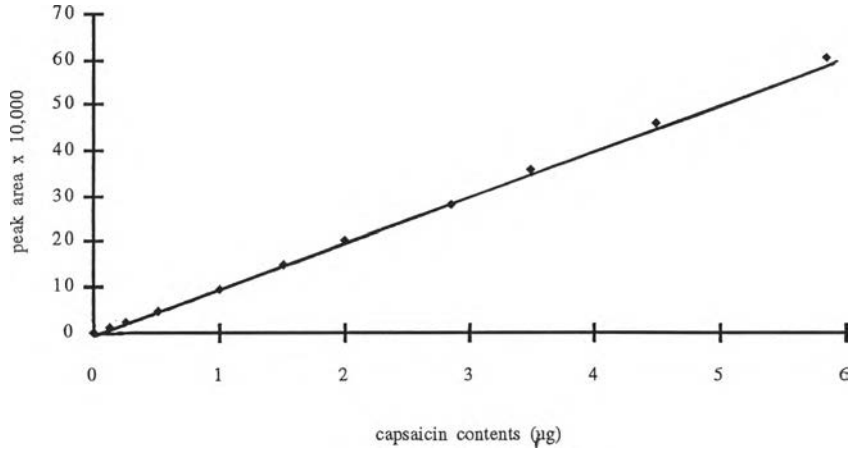

Fig. 25 Calibration Curve of Standard Capsaicin.

2. Capsaicin Content in *Capsicum* spp.

2.1 Variation of Capsaicin Content in Various Capsicum Cultivars.

Capsicum fruits of both *Capsicum frutescens* and *C. annuum* from more than forty cultivars were collected from many provinces of Thailand in order to study the variation of their capsaicin content. The collected mature capsicum fruits appeared to have high variation in their shape and size (Fig. 26). The shape of the fresh fruits can be described as slender, large, thick flesh, thin flesh, cylindrical shape, heart shape, blent end or taper end etc. The length of the fruits varied from the shortest of 1.18 cm to the longest of 11.65 cm and the weight was varied from the lightest of 53 mg to the heaviest of 2.04 gm of dry weight.

Fig. 26 The Variation in Shape and Size of Mature Capsicum Fruits Collected from many Provinces in Thailand.

When these samples were determined for their capsaicin content, it was found that the values were highly variable, both in terms of percent capsaicin and total capsaicin per fruit. As shown in Table.7, the capsicum fruits from 43 cultivars

Table. 7 The Source, Mean of Fruit Weights and Capsaicin Contents of Capsicum Cultivars in Thailand.

Code	*Capsicum* spp.	Source	Dry weight (gm)	% capsaicin • (dry wt.)	Total capsaicin (mg in one fruit)
c1	*Capsicum frutescens*	นครปฐม*	0.053 ± 0.018	0.544 ± 0.055	0.228
c2	*Capsicum frutescens*	จันทบุรี	0.070 ± 0.016	0.793 ± 0.068	0.555
c3	*Capsicum frutescens*	เทียงราย*	0.071 ± 0.025	0.687 ± 0.017	0.488
c4	*Capsicum frutescens*	ราชบุรี	0.072 ± 0.033	0.746 ± 0.022	0.537
c5	*Capsicum frutescens*	ประจวบศีรีขันธ์*	0.072 ± 0.021	0.519 ± 0.017	0.374
c6	*Capsicum frutescens*	นนทบุรี	0.075 ± 0.025	0.667 ± 0.018	0.500
c7	*Capsicum frutescens*	ชลบุรี	0.089 ± 0.023	0.489 ± 0.024	0.435
c8	*Capsicum frutescens*	ชุมพร	0.098 ± 0.033	0.478 ± 0.044	0.468
c9	*Capsicum annuum*	สงขลา*	0.136 ± 0.050	0.302 ± 0.017	0.411
c10	*Capsicum annuum*	ราชบุรี	0.142 ± 0.024	0.199 ± 0.014	0.283
c11	*Capsicum annuum*	หนองคาย	0.240 ± 0.041	0.312 ± 0.009	0.749
c12	*Capsicum annuum*	นครสวรรค์*	0.260 ± 0.080	0.348 ± 0.004	0.905
c13	*Capsicum annuum*	เพชรบูรณ์	0.291 ± 0.042	0.118 ± 0.003	0.343
c14	*Capsicum annuum*	นครสวรรค์*	0.297 ± 0.061	0.162 ± 0.014	0.481
c15	*Capsicum annuum*	สกลนคร	0.314 ± 0.047	0.324 ± 0.001	1.017
c16	*Capsicum annuum*	เทียงราย*	0.351 ± 0.060	0.323 ± 0.011	1.134
c17	*Capsicum annuum*	สงขลา*	0.354 ± 0.042	0.283 ± 0.002	1.002
c18	*Capsicum annuum*	ลพบุรี	0.360 ± 0.100	0.412 ± 0.007	1.483
c19	*Capsicum annuum*	ประจวบศีรีขันธ์*	0.370 ± 0.080	0.249 ± 0.006	0.921
c20	*Capsicum annuum*	ตาก*	0.380 ± 0.100	0.249 ± 0.004	0.946
c21	*Capsicum annuum*	ฉะเชิงเทรา	0.381 ± 0.050	0.341 ± 0.020	1.296
c22	*Capsicum annuum*	เทียงใหม่	0.395 ± 0.035	0.284 ± 0.005	1.122
c23	*Capsicum annuum*	ตาก *	0.421 ± 0.121	0.273 ± 0.010	1.147
c24	*Capsicum annuum*	เทียงราย*	0.422 ± 0.110	0.534 ± 0.023	2.243
c25	*Capsicum annuum*	กาญจนบุรี*	0.451 ± 0.082	0.269 ± 0.003	1.213
c26	*Capsicum annuum*	อุบลราชธานี*	0.456 ± 0.120	0.146 ± 0.002	0.666
c27	*Capsicum annuum*	ศรีสะเกษ	0.465 ± 0.050	0.231 ± 0.008	1.074
c28	*Capsicum annuum*	นครปฐม*	0.500 ± 0.092	0.220 ± 0.012	1.100
c29	*Capsicum annuum*	อุบลราชธานี*	0.528 ± 0.086	0.222 ± 0.021	1.172
c30	*Capsicum annuum*	บุรีรัมย์	0.577 ± 0.049	0.174 ± 0.011	1.004
c31	*Capsicum annuum*	นครราชสีมา*	0.647 ± 0.070	0.160 ± 0.002	1.035
c32	*Capsicum annuum*	นครสวรรค์*	1.081 ± 0.290	0.092 ± 0.008	0.994
c33	*Capsicum annuum*	ตาก*	1.112 ± 0.274	0.248 ± 0.005	2.753
c34	*Capsicum annuum*	สุพรรณบุรี*	1.140 ± 0.148	0.054 ± 0.001	0.062
c35	*Capsicum annuum*	นครราชสีมา*	1.221 ± 0.205	0.122 ± 0.005	1.490
c36	*Capsicum annuum*	สุพรรณบุรี*	1.249 ± 0.191	0.000	0.000
c37	*Capsicum annuum*	อุบลราชธานี*	1.284 ± 0.214	0.085 ± 0.004	1.091
c38	*Capsicum annuum*	สุพรรณบุรี*	1.388 ± 0.179	0.000	0.000
c39	*Capsicum annuum*	กาญจนบุรี*	1.390 ± 0.221	0.035 ± 0.001	0.487
c40	*Capsicum annuum*	นครปฐม*	1.621 ± 0.440	0.054 ± 0.001	0.875
c41	*Capsicum annuum*	บุรีรัมย์	1.679 ± 0.475	0.052 ± 0.002	0.873
c42	*Capsicum annuum*	สุพรรณบุรี*	1.900 ± 0.401	0.081 ± 0.007	1.539
c43	*Capsicum annuum*	นครสวรรค์*	2.041 ± 0.272	0.189 ± 0.014	3.856

• Each value represents the mean ± SD of six separate preparations.

*The capsicum samples are from different garden in each same provinces

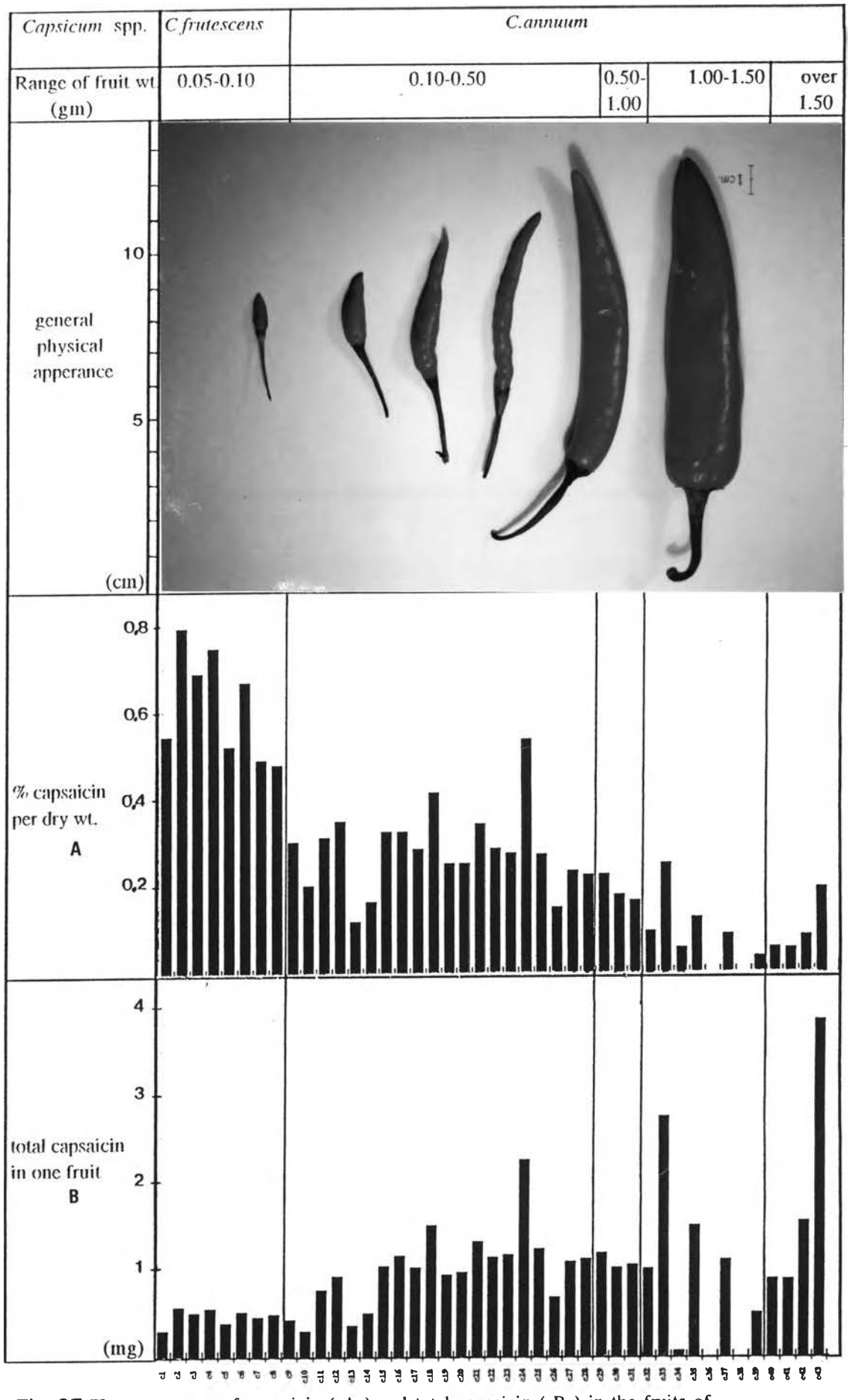

Fig. 27 The percentage of capsaicin (A) and total capsaicin (B) in the fruits of various *Capsicum* cultivars which were in an order from the lowest dry fruit weight at C_1 to the highlest dry fruit at C_{43}.

showed the variation from zero to 0.8 percent of capsaicin and from zero to 3.8 mg capsaicin in one fruit. It was clear from the bar graph (Fig. 27) that the small *C. frutescens* contained relatively high percent capsaicin (from approx. 0.5% to 0.8%) whereas the big *C. annuum* contained significantly lower values of the content (from zero to 0.5%) (. Fig. 27A). When the values were expressed using the unit of capsaicin per fruit, it was found that the *C. frutescens* cultivars mostly contained less than 0.5 mg of total capsaicin, whereas the *C. annuum* cultivars mostly contained more than 0.9 mg (Fig. 27B). It should be noted that there were two cultivars in *C. annuum* which contain no capsaicin. These two cultivars were C_{36} and C_{38} which both were harvested from Supanburi province with different local names. C_{36} is "Prik Bang Loang" and C_{38} is "Prik Fa Keaw".

To evaluate whether there is a correlation between the percentage of capsaicin content and the dry weight of capsicum fruit, a graph composed of the two factors was plotted as semi-logarithemic. As showed in Fig. 28, there was a linear relation between the value of ln(%capsaicin x 1000) and their dry fruit weights.

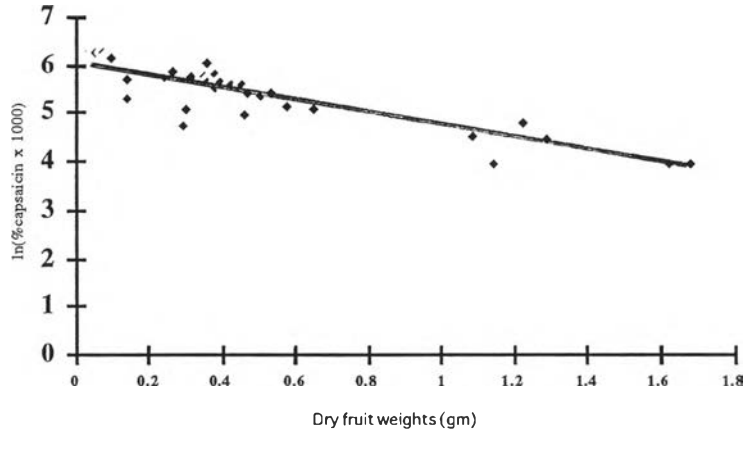

Fig. 28 The Linear Relation between the Percentage of Capsaicin in Capsicum Fruits and their Dry Weight in Semi-logarithemic Plotted Graph.

2.2 Capsicum Fruits Containing High Capsaicin Content.

2.2.1 Capsicum Fruits with High Percentage of Capsaicin.

When the percentage of capsaicin content (from 0 to 0.8) was devided into eight narrow ranges in a stepwise manner (starting from 0.00-0.10, 0.11-0.20 until 0.71-0.80) and the frequency of capsicum samples with different capsaicin content was put in each range, a distribution graph could be obtained (Fig. 29). It can be seen that the percentage of capsaicin in *C. annuum* fruits was mostly distributed between the range of 0.00 to 0.40%. Only two out of 35 samples of *C. annuum* were found to contain more than 0.40% of capsaicin. These were C_{24} from Chiang Rai and C_{19} from Lop Buri (Table. 8). *C. frutescens* also showed relatively even distribution between the range of 0.40 to 0.80% with the frequency of two samples in each one percent range. However, the top two *C. frutescens* containing the highest capsaicin content were found to be C_3 of Chantaburi and C_4 of Ratchaburi (Table. 9)

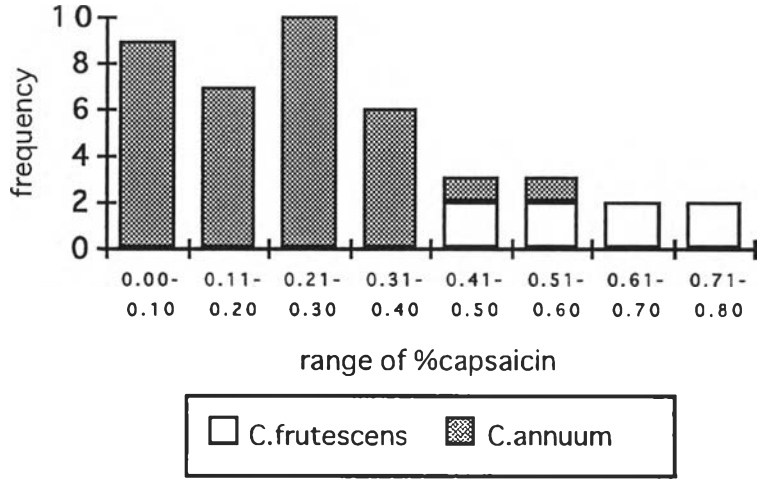

Fig. 29 The Distribution Graph of Percentage of Capsaicin in Capsicum Fruits.

Table. 8 The Top Two High Percentage of Capsaicin in *Capsicum annuum.*

Capsicum cultivars	% capsaicin / dry weight
1. Chiang Rai (C24) (เชียงราย)	0.534 ± 0.023
2. Lop Buri (C18) (ลพบุรี)	0.412 ± 0.007

Table. 9 The Top Two High Percentage of Capsaicin in *Capsicum frutescens.*

Capsicum cultivars	% capsaicin / dry weight
1. Chanthaburi (C2) (จันทบุรี)	0.793 ± 0.018
2. Ratchaburi (C4) (ราชบุรี)	0.746 ± 0.022

2.2.2 Capsicum Fruits with High Total Capsaicin Content

When the total capsaicin content per fruit was devided into night ranges in a stepwise manner (starting from 0.0-0.3 until 1.2-1.5 and then 1.5-2.1 unitl 3.3-3.9) and the frequency of capsicum samples with different total capsaicin content was put in each range, a distribution graph could be obtained (Fig. 30).

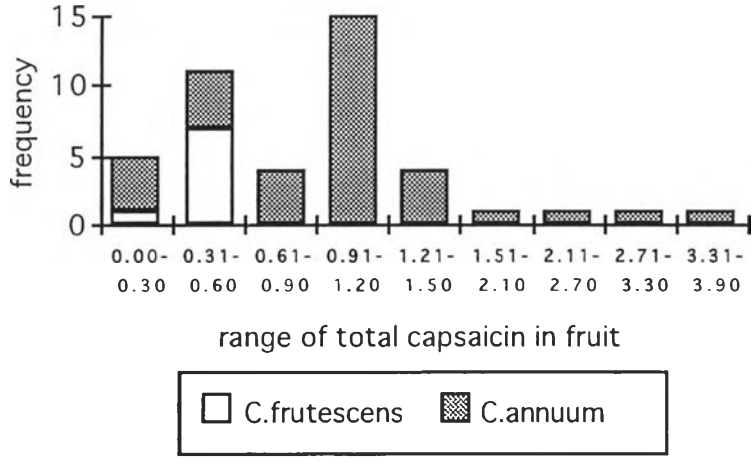

Fig. 30 The Distribution Graph of Total Capsaicin in one Fruit of Capsicum Fruits.

It can be seen that the content of capsaicin in one fruit of *C. frutescens* samples was mostly distributed between the range of 0.31-0.60 mg/fruit. Only one out of eight samples of *C. frutescens* was found to contain less than 0.30 mg capsaicin in one fruit. This was C_1 from Nakhon Pathom. The top two *C. frutescens* containing highest capsaicin content were found to be C_2 from Chantaburi and C_4 from Ratchaburi (Table. 10). *C. annuum* also showed relatively even distribution between the range of zero to 3.9 mg capsaicin. It was found that, the top two *C. annuum* containing the highlight capsaicin content were found to be C_{43} of Nakhon Sawan and C_{33} of Tak (Table. 11).

Table. 10 The Top Two High Total Capsaicin in one Fruit of *Capsicum frutescens.*

Capsicum cultivars	Weight of fruits (gm/fruit)	Capsaicin contents (mg/fruit)
1. Chanthaburi (C2) (จันทบุรี)	0.070 ± 0.016	0.555
2. Ratchaburi (C4) (ราชบุรี)	0.072 ± 0.033	0.537

Table. 11 The Top Two High Total Capsaicin in one Fruit of *Capsicum annuum.*

Capsicum cultivars	Weight of fruits (gm/fruit)	Capsaicin contents (mg/fruit)
1. Nakhon Sawan (C43) (นครสวรรค์)	2.041 ± 0.272	3.856
2. Tak (C33) (ตาก)	1.112 ± 0.274	2.753

3. Carotenoids in *Capsicum* spp.

3.1 HPLC Separation of Carotenoid in Capsicum Fruits

In order to obtain information on the carotenoid components in capsicum fruits, selected capsicum samples were extracted with acetone followed by HPLC analysis. Figure 31 shows a typical HPLC chromatogram of the extracted carotenoid. It can be seen that the extract was composed of several carotenoid components with β-carotene being a major components (Rt = 38.06 min.). Because of the chemical complication, the HPLC method was not appropriate for the determination of each carotenoid component. This was due to the difficulty of the optimization of HPLC conditions which allowed complete separation of each carotenoid component. Furthermore, the more meaningful data would be the value of "total carotenoid content" which was more related to the quality of coloring agent. Therefore, the spectrophotometric method described previously Kenneth (1990) was adapted.

3.2 Calibration Curve of Standard β-Carotene by Visible Spectrophotometry

The calibration curve of β-carotene was obtained by spectrophotometric method which conditions were followed on Chapter III, Section 2.4 (Fig. 32). The curve show linearity of the peak area-β-carotene concentration relationship between the β-carotene concentration range of 0.17-22 μg/ml. Result of the regression analysis and correlation coefficient (r) was found to be 0.9992 and the regression equation of standard β-carotene was $\mathbf{y} = 0.098896\ \mathbf{x} + 0.021764$ ($\mathbf{n} = 10$)

where **y** = absorbance of β-carotene

x = β-carotene concentration (μg/ml)

n = the number of standard β-carotene concentration levels

r = correlation coefficient

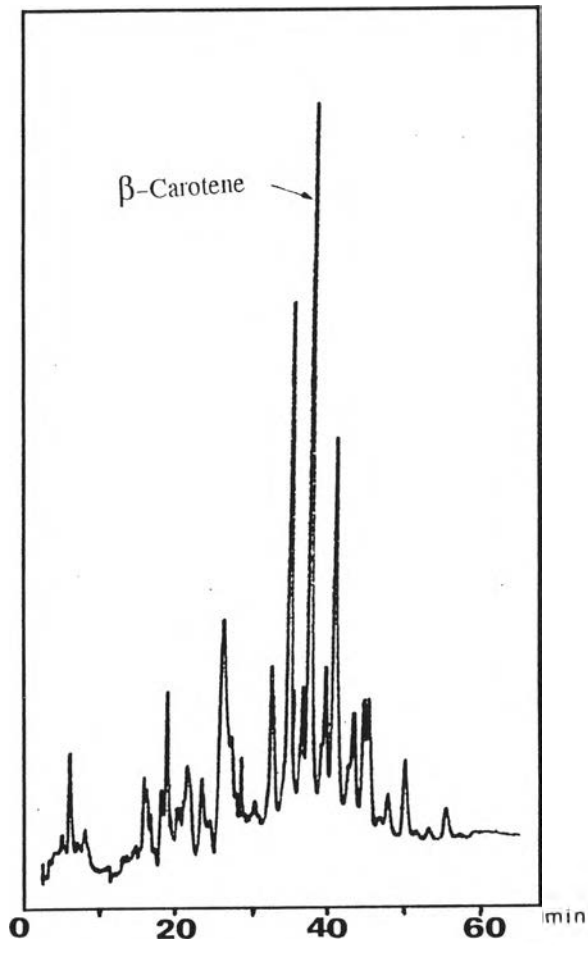

Fig. 31 HPLC separation of carotenoids which condition as followed : column : ODS 5 μm particle size ; 12.5 cm x 4 mm. ID ; mobilephase : methanol : water = 90:10 isocratic for 5 min. after that the proportion was change to methanol 100% in 5 min. and hold for 10 min before gradient to methanol : acetone, 60:40 at 55 min and hold to the end at 65 min; detect at : 460 nm and sample volume : 20 μl of acetone extract.

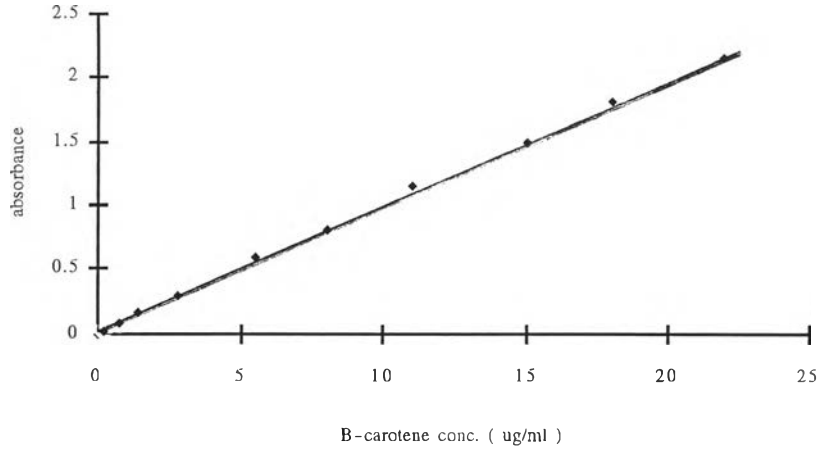

Fig. 32 Calibration Curve of Standard β-carotene.

3.3 Variation of Carotenoid Content in Various Capsicum Cultivars.

The carotenoid content in capsicum components was determined by a spectrophotometric method as described in the Materials and Methods (Section. 2 ,Chapter. III). Various samples used for this carotenoid determination were the same samples used for capsaicin determination. By appearence, the riped fruits of capsicum which were cultivated in many provinces of Thailand showed variation in their color, from orange to dark red. The spectrophotometric analysis based on β-carotene content, also confirmed this high variation of carotenoid content (Fig. 33 and Table. 12). The lowest carotenoid content was 0.065% and the highest was 0.556% dry weight.

Unlike the capsaicin content, the percentage of carotenoids in various capsicum fruits appeared to have no direct relationship with the fruit weights. As shown in Fig. 34A, the fruit carotenoids of *C. frutescens* which had low weight was found to have high percentage of carotenoids (0.21-0.50%) as compared with the percentage found in the heavier *C. annuum* fruits. Among various *C. annuum* cultivars, a. high variation of the carotenoid content (from 0.06 to 0.5%) was observed.

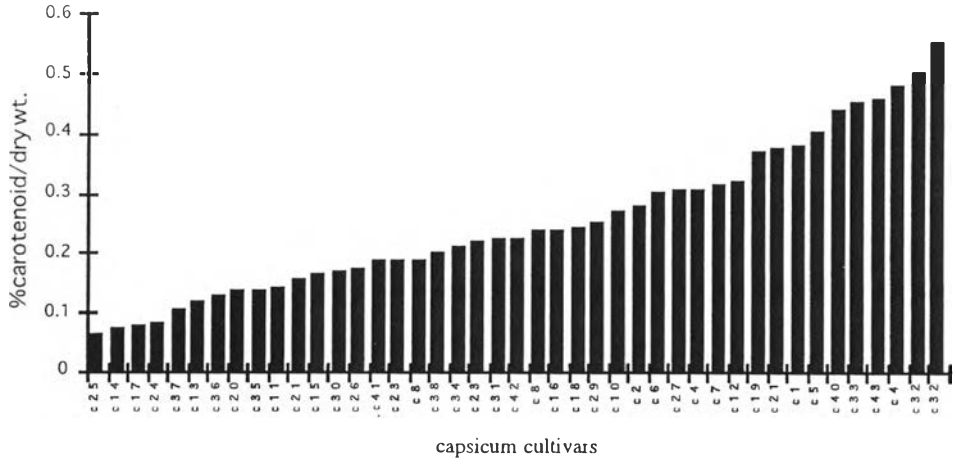

Fig. 33 The Variation of Carotenoids Contents (percent/dry weight) in Capsicum Cultivar. in Thailand.

In term of total carotenoid content however, the fruit dry weight appeared to be a major factor contributed to the total carotenoids. As shown in Fig. 34B, the total carotenoid content was essentially in a sequence of the dry fruit weight.

Table. 12 The Source, Mean of Fruit Weights and Carotenoids Contents of Capsicum Cultivars in Thailand.

Code	*Capsicum* spp.	Source	Dry weight (gm)	% carotenoids • (dry wt.)	Total carotenoids (mg in one fruit)
c1	*Capsicum frutescens*	นครปฐม ⋆	0.053 ± 0.018	0.379 ± 0.020	0.201
c2	*Capsicum frutescens*	จันทบุรี	0.070 ± 0.016	0.278 ± 0.005	0.195
c3	*Capsicum frutescens*	เชียงราย	0.071 ± 0.025	0.309 ± 0.027	0.219
c4	*Capsicum frutescens*	ราชบุรี	0.072 ± 0.033	0.484 ± 0.012	0.348
c5	*Capsicum frutescens*	ประจวบคีรีขันธ์ ⋆	0.072 ± 0.021	0.404 ± 0.027	0.291
c6	*Capsicum frutescens*	นนทบุรี	0.075 ± 0.025	0.305 ± 0.015	0.229
c7	*Capsicum frutescens*	ชลบุรี	0.089 ± 0.023	0.318 ± 0.019	0.283
c8	*Capsicum frutescens*	ชุมพร	0.098 ± 0.033	0.237 ± 0.017	0.232
c9	*Capsicum annuum*	สงขลา ⋆	0.136 ± 0.050	0.190 ± 0.012	0.258
c10	*Capsicum annuum*	ราชบุรี	0.142 ± 0.024	0.269 ± 0.007	0.382
c11	*Capsicum annuum*	หนองคาย	0.240 ± 0.041	0.143 ± 0.004	0.343
c12	*Capsicum annuum*	นครสวรรค์ ⋆	0.260 ± 0.080	0.323 ± 0.013	0.840
c13	*Capsicum annuum*	เพชรบูรณ์	0.291 ± 0.042	0.120 ± 0.008	0.349
c14	*Capsicum annuum*	นครสวรรค์ ⋆	0.297 ± 0.061	0.075 ± 0.005	0.223
c15	*Capsicum annuum*	สกลนคร	0.314 ± 0.047	0.165 ± 0.007	0.518
c16	*Capsicum annuum*	เชียงราย ⋆	0.351 ± 0.060	0.238 ± 0.001	0.835
c17	*Capsicum annuum*	สงขลา ⋆	0.354 ± 0.042	0.079 ± 0.001	0.280
c18	*Capsicum annuum*	ลพบุรี	0.360 ± 0.100	0.244 ± 0.003	0.878
c19	*Capsicum annuum*	ประจวบคีรีขันธ์ ⋆	0.370 ± 0.080	0.371 ± 0.017	1.373
c20	*Capsicum annuum*	ตาก ⋆	0.380 ± 0.100	0.135 ± 0.004	0.513
c21	*Capsicum annuum*	ฉะเชิงเทรา	0.381 ± 0.050	0.157 ± 0.009	0.597
c22	*Capsicum annuum*	เชียงใหม่	0.395 ± 0.035	0.220 ± 0.008	0.869
c23	*Capsicum annuum*	ตาก ⋆	0.421 ± 0.121	0.189 ± 0.004	0.794
c24	*Capsicum annuum*	เชียงราย ⋆	0.422 ± 0.110	0.083 ± 0.003	0.349
c25	*Capsicum annuum*	กาญจนบุรี ⋆	0.451 ± 0.082	0.065 ± 0.003	0.293
c26	*Capsicum annuum*	อุบลราชธานี ⋆	0.456 ± 0.120	0.174 ± 0.007	0.793
c27	*Capsicum annuum*	ศรีสะเกษ	0.465 ± 0.050	0.306 ± 0.011	1.423
c28	*Capsicum annuum*	นครปฐม ⋆	0.500 ± 0.092	0.375 ± 0.006	1.875
c29	*Capsicum annuum*	อุบลราชธานี ⋆	0.528 ± 0.086	0.252 ± 0.015	1.331
c30	*Capsicum annuum*	บุรีรัมย์	0.577 ± 0.049	0.168 ± 0.004	0.969
c31	*Capsicum annuum*	นครราชสีมา ⋆	0.647 ± 0.070	0.225 ± 0.012	1.456
c32	*Capsicum annuum*	นครสวรรค์ ⋆	1.081 ± 0.290	0.505 ± 0.011	5.454
c33	*Capsicum annuum*	ตาก ⋆	1.112 ± 0.274	0.454 ± 0.007	5.039
c34	*Capsicum annuum*	สุพรรณบุรี ⋆	1.140 ± 0.148	0.209 ± 0.002	0.238
c35	*Capsicum annuum*	นครราชสีมา ⋆	1.221 ± 0.205	0.139 ± 0.007	1.697
c36	*Capsicum annuum*	สุพรรณบุรี ⋆	1.249 ± 0.191	0.127 ± 0.001	1.586
c37	*Capsicum annuum*	อุบลราชธานี ⋆	1.284 ± 0.214	0.103 ± 0.005	1.323
c38	*Capsicum annuum*	สุพรรณบุรี ⋆	1.388 ± 0.179	0.201 ± 0.006	2.790
c39	*Capsicum annuum*	กาญจนบุรี ⋆	1.390 ± 0.221	0.556 ± 0.005	7.728
c40	*Capsicum annuum*	นครปฐม ⋆	1.621 ± 0.440	0.442 ± 0.005	7.160
c41	*Capsicum annuum*	บุรีรัมย์	1.679 ± 0.475	0.186 ± 0.009	3.123
c42	*Capsicum annuum*	สุพรรณบุรี ⋆	1.900 ± 0.401	0.225 ± 0.005	4.275
c43	*Capsicum annuum*	นครสวรรค์ ⋆	2.041 ± 0.272	0.459 ± 0.018	9.364

• Each value represents the mean ± SD of six separate preparations.

⋆The capsicum samples are from different garden in each same provinces

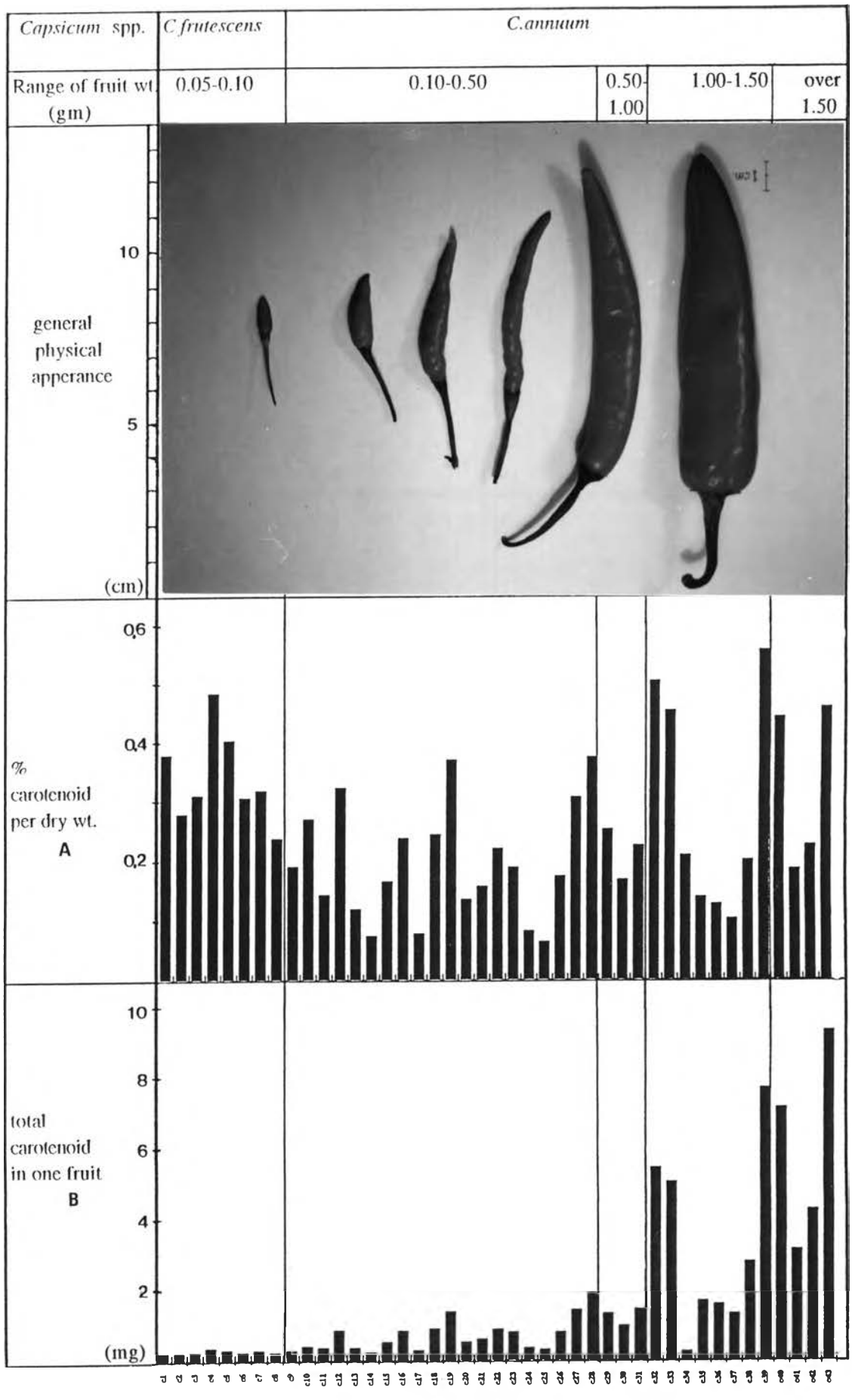

Fig. 34 The percentage of carotenoids (A) and total carotenoids (B) in the fruits of various *Capsicum* cultivars which were in an order from the lowest dry fruit weight at C_1 to the highlest dry fruit at C_{43}.

3.3 Capsicum Fruit Containing High Carotenoids Content.

3.3.1 Capsicum Fruit with High Percentage of Carotenoids.

Similar to the capsicin content in capsicum fruits, when the carotenoid content was divided into various ranges from 0.0-0.10, 0.11-0.20 until 0.51-0.60, a distribution graph of capsicum samples could be obtained. As shown in Fig. 35, the percentage of carotenoids in *C. annuum* fruits was widely distributed from the lowest (0.075%) to the highest (0.556%) with the majority (22 out of 35 samples) falling between 0.1 to 0.3%. Among the *C. annuum* samples, the top three high carotenoid containing fruits were found to be C_{39} of Kanjanaburi, C_{32} and C_{43} of Nakhon Sawan (Table. 13).

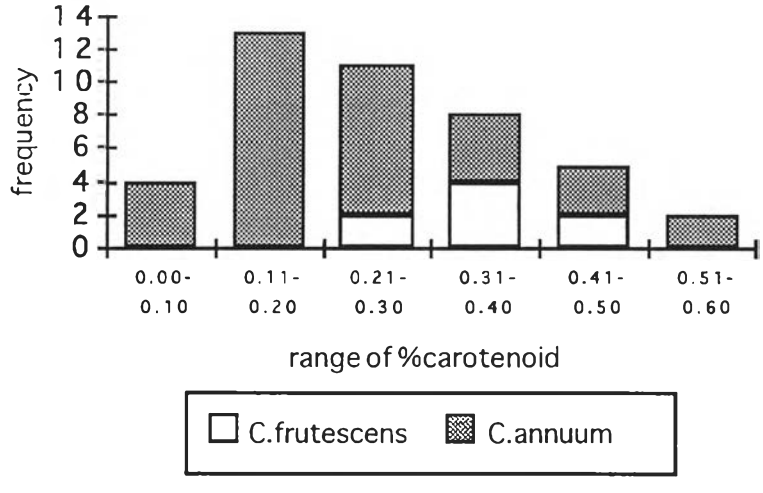

Fig. 35 The Distribution Graph of Percentage of Carotenoid in Capsicum Fruits.

Table. 13 The Top Three High Percentage of Carotenoids in *Capsicum annuum.*

Capsicum cultivars	% carotenoids / dry weight
1. Kanchanaburi (C39) (กาญจนบุรี)	0.556 ± 0.004
2. Nakhon Sawan (Pichai LeK) (C32) (นครสวรรค์ พันธุ์พิชัยเล็ก)	0.505 ± 0.011
3. Nakhon Sawan (Tak Far) (C43) (นครสวรรค์ พันธุ์ตากฟ้า)	0.459 ± 0.018

For *C. frutescens*, distribution of their percentage of carotenoids was appeared to be in the range between 0.20 to 0.50%. Four out of the eight samples contained 0.30 to 0.40% of carotenoid and the two high carotenoid containing fruits of *C. frutescens* were C_4 of Ratchaburi and C_5 of Prachuap Kiri Kan (Table 14).

Table. 14 The Top Two High Percentage of Carotenoids in *Capsicum frutescens.*

Capsicum cultivars	% carotenoids / dry weight
1. Ratchaburi (C4) (ราชบุรี)	0.484 ± 0.012
2. Prachuap Kiri Khan (C5) (ประจวบคีรีขันธ์)	0.404 ± 0.027

3.3.2 Capsicum Fruit with High Total Carotenoid Content

When the values of total carotenoid content in one fruit was devided into ten narrow ranges in a stepwise manner (starting from 0.00-0.20 until 0.81-1.00 ;1.01-2.00 and then 2.01-4.00 unitl 8.01-10.00) and the frequency of capsicum samples with different total carotenoid content was put in each range, the distribution graph could be obtained (Fig. 36).

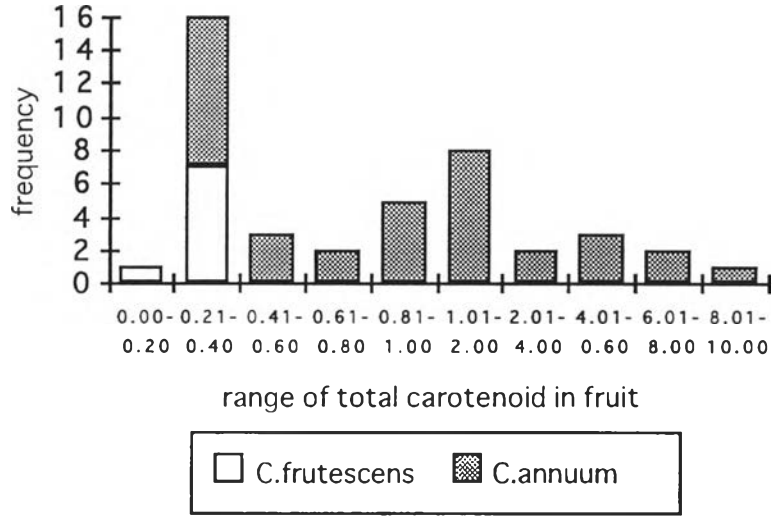

Fig. 36 The Distribution Graph of Total Carotenoid Content in Capsicum Fruits.

It can be seen that the total carotenoid content in one fruit in *C. frutescens* fruits was mostly distributed between the range of 0.2–0.4 mg. Only one out of eight sample of *C. frutescens* was found to contain less than 0.20 mg. These was C_2 of Chanthaburi. However, the top two *C. frutescens* containing highest carotenoid content were found to be C_4 of Ratchaburi and C_5 of Prajub Kiri Kan (Table. 15).

Table. 15 The Top Two High Total Carotenoids in one Fruit of *Capsicum frutescens*

Capsicum cultivars	**Weight of fruits** (gm/fruit)	**Carotenoids contents** (mg/fruit)
1. Ratchaburi (C4) (ราชบุรี)	0.072 ± 0.033	0.348
2. Prajub Kiri Kan (C5) (ประจวบศีรีขันธ์)	0.072 ± 0.021	0.291

Similar to *C. frutescens*, *C. annuum* also showed the mostly distribution in the range of 0.2-0.4 mg of total carotenoid in one fruit (9 out of 35 samples). It was found that the top three *C. annuum* containing the highest carotenoid content were found to be C_{43} of Nakhon Sawan, C_{39} of Kanjanaburi and C_{40} of Nakhon Pathom. (Table. 16)

Table. 16 The Top Three High Total Carotenoids Content of *Capsicum annuum*.

Capsicum cultivars	Weight of fruits (gm/fruit)	Carotenoids contents (mg/fruit)
1. Nakhon Sawan (C43) (นครสวรรค์)	2.041 ± 0.271	9.364
2. Kanchannaburi (C39) (กาญจนบุรี)	1.390 ± 0.221	7.728
3. Nakhon Pathom (C40) (นครปฐม)	1.621 ± 0.440	7.160