CHAPTER III

MATERIALS AND METHODS

1. Quantitative Analysis of Capsaicin in *Capsicum* spp.

1.1 Plant Materials

Fruit samples of two *Capsicum* species, *C. frutescens* and *C. annuum* , were harvested fully ripened from forty three gardens in twenty six provinces covering almost all plantation regions in Thailand as follows :

Northern	:	Chiang Rai, Chiang Mai, Tak, Phrae, Nan
North easthern	:	Buri Ram, Ubon Ratchathani, Si Saket, Nakhon Ratchasima, Sakon Nakhon, Muk Dahan, Nongkhai, Phetchabun
Central	:	Ratchaburi, Nakhon Pathom, Kanchanaburi, Nonthaburi, Prachuap Khiri Khan, Suphan Buri, Nakhon Sawan, Lop Buri
Southern	:	Chumphon, Songkla
Eastern	:	Chon Buri, Prachin Buri, Chachoengsao

Each fruit sample, either fresh or dry, was dried in hot air oven under 50° C for 24 hr. before the determining for capsaicin and carotenoid contents.

1.2 Reagents, Standards and Apparatus

1.2.1 Methanol for extraction and sample preparation was GR grade (Merck, Damstadt, Germany)

1.2.2 Methanol for HPLC separation was gradient grade (Merck, Damstadt, Germany) and double distilled water was used. All solvents were passed through 0.45 mm nylon membrane filter before used.

1.2.3 Standard capsaicin (99% pure) and standard capsaicinoid (60% capsaicin pure) were obtained from Sigma^{R} (St. Louis, U.S.A.)

1.2.4 Minicolumn for solid-phase extraction contained 400 mg of octadecylsilane (ODS, C_{18}) which was a ready-packing in 5 ml. syringe-like shaped column (Adsorbex^{R}, Merck; Damstadt, Germany) (Fig. 16).

1.2.5 Sample preparation apparatus was the Adsorbex SPU^{R} (Merck, Damstadt, Germany) which comprised of twenty four collection tubes and connected with vaccuum pump (Fig. 17).

1.2.6 HPLC Apparatus

The HPLC was of a Varian Chromatrograph System (Varian^{R}, U.S.A) which consisted of a Varian model 9010 ternary solvent delivery system, connected to a Varian model 9050 variable uv-visible detector. The data were processed by mean of a Varian model 4400 integrator to evaluate the peak area. Samples were automatically injected by Varian model 9095 autosampler that can be injected 105 samples continuously.

1.2.7 HPLC analytical column was octadecylsilane (C_{18}) reverse phase column, 5 mm in particle size, 125 mm x 4 mm I.D. (Varian^{R}, U.S.A .)

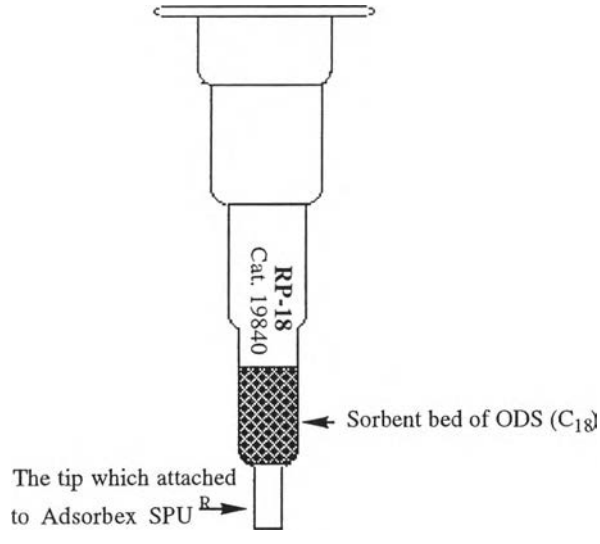

Fig. 16 The Minicolumn for Solid-Phase Extraction (Adsorbex^{R}).

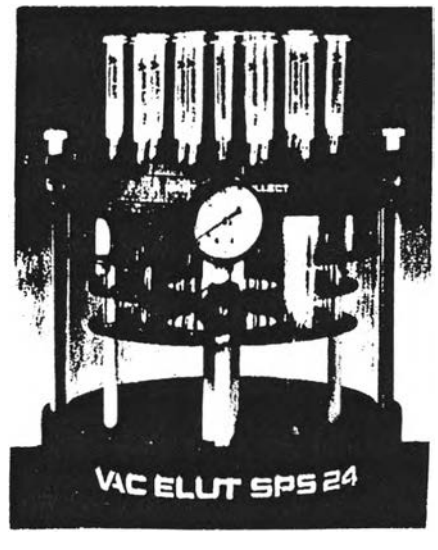

Fig. 17 The Sample Preparation Apparatus (Adsorbex SPU^{R}).

1.3 Sample Preparation by Solid-Phase Extraction

1.3.1 Extraction of Capsicum Capsaicinoids

Each sample of capsicum fruit was ground and passed through the sieve No. 40 before extraction. The capsicum powder (100 mg) was accurately weighed and tranferred to a 12 cm. x 1 cm. I.D. scewed-cap glass tube. Ten milliliters of methanol were then added and the tube was sonicated for one hour at 45^{O}C in ultrasonic bath. After being centrifuged for 2 min., one milliliter aliquote of the clear solution of the extract was mixed with an equal volume of water in a test tube. The extract solution was then subjected to solid-phase extraction as described below.

1.3.2 Preparation of Minicolumn for Solid-phase Extraction

The Adsorbex^{R} solid-phase extraction column that was holded on the Adsorbex SPU^{R} sample preparation apparatus was prepared prior to use for cleaning the extract by using two steps. In the first step, 3 ml methanol was passed through the sorbent bed of Adsorbex^{R} column in order to get rid of some impurity originally trapped column. In the second step, the Adsorbex^{R} column was equilibrated with 3 ml 50% methanol in order to preconditioning the column.

1.3.3 Solid-Phase Extraction of Capsaicinoids Crude Extract

The mixture of capsaicinoid extract and water (0.6 ml) prepared as described in Section 1.3.1 was loaded into the equilibrated Adsorbex^{R} column and let the extract be concentrated in the sorbent bed by using vaccuum pump. The column was washed with 3 ml 50% methanol to get rid of impurities. The trapped capsaicinoid was the eluted from the Adsorbex^{R} column by passing 3 ml 70% methanol into the column and the eluate was collected in a test tube of Adsorbex SPU^{R}. The eluate was dried by speed vaccuum (SAVENT, Formingdale, New York) at 45^{O}C and the

residue was dissolved with 0.3 ml methanol prior to injection to HPLC for capsaicin analysis.

1.4 HPLC Conditions for Capsaicinoid Separation

Column	:	SP-C_{18} reverse phase ;125 cm x 4 mn. I.D., 5 µm particle size
Mobile phase	:	isocratic 65% methanol in water
End time	:	25 minute per sample
Flow rate	:	0.75 ml./min
UV detection	:	280 nm
Injection volume	:	20 µl
Chart speed	:	0.25 cm./min
A.U.F.S.	:	0.05 units

2. Quantitative Analysis of Total Capsicum Carotenoids.

2.1 Plant Materials

Plant Material is as described in Materials and Methods, Section 1.1.

2.2 Reagents, Standards and Apparatus.

2.2.1 Acetone for carotenoid extraction and for spectrophotometry was GR grade (Merck, Damstadt, Germany).

2.2.2 Standard β-carotene was obtained from Sigma^{R} (St. Louis, U.S.A.)

2.2.3 UV-Vis spectrophotometer.

The visible spectrophotometry was processed by the Lambda 3B double-beam UV-Vis spectrophotometer (Perkin-Elmer, Connecticut, U.S.A.) connected with the 80286 series microcomputer to calculated the data by PECSS software.

2.3 Sample Preparation

Each sample of capsicum fruit was grounded and passed through sieve No. 40 before carotenoid extraction. The capsicum powder (10 mg) was accurately weighed and transferred to a 12 cm x 1 cm I.D. scewed-cap glass tube. Ten milliliters of acetone was then added and the tube was shaken for 3 min. The tube was then kept in the dark for 16 hr. The tube was then shaked again and let the capsicum powder to precipitate. The clear solution was used for total carotenoid detection by visible spectrophotometry.

2.4 Visible Spectrophotometric Analysis.

The clear supernatant of each sample was detected by visible spectrophotometry at 460 nm by using the β-carotene as standard and the total carotenoid content was expressed based on β-carotene equivalent value.