Quantitative Analysis of Capsaicinoid in Chili Pepper (*Capsicum* sp.) by High Performance Liquid Chromatograpy—Operating Condition, Sampling and Sample Preparation—

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Summary. The feature of chili pepper (*Capsicum* sp.) is its peculiar pungency, whose principle is called capsaicinoid. In the breeding of chili pepper, quantitative determination of capsaicinoid content is indispensable. To establish a quantitative analysis method of capsaicinoid using high performance liquid chromatography (HPLC), method of sample preparation and capsaicinoid extraction and the operating condition for the analysis were examined. Sampling method of fruits for the analysis was discussed in consideration of the variation among fruits. In genetically homogeneous variety such as commercial varieties, randomly collected bulk samples of 10 fruits is thought to be enough for the analysis with about 5 % of coefficient of variation. Metaxenia, the effect of pollen genotype on capsaicinoid content, was not observed, so it is concluded that open pollinated fruits can be used as the sample for quantitative determination of capsaicinoid.

Keywords: *Capsicum*, chili pepper, capsaicinoid, HPLC analysis, metaxenia

Introduction

Chili pepper (*Capsicum* sp.) is one of the most important spices and widely cultivated and used all over the world. The pungent principle of chili pepper is called capsaicinoid (CND), which is acid amides of vanillylamine and C_9-C_{11} branched fatty acids[1]. Fourteen analogues of CND have been reported[2]. As for the composition, capsaicin and dihydrocapsaicin occupy more than 80 %, and nordihydrocapsaicin is the remainder. The other derivatives exist very little[3)4)].

The feature of chili pepper is its peculiar pungency, which is an important agronomic character in the evaluation of chili pepper. Therefore, in the breeding of chili pepper, establishment of a quantitative analysis method of CND is indispensable. Several methods of quantitative analysis of CND have been already reported[1)4)5)6)]. However, research on quantitative analysis of CND made from breeding standpoint is few. Sampling and preparing methods of chili pepper with consideration to the growing condition of the plant[7)] and variation of CND content among fruits[6)8)] have not been examined enough.

Then, in this paper, we describe a quantitative analysis method of CND using high performance liquid chromatography (HPLC), including the methods of sample preparation and CND extraction and optimal operationg conditions for the analysis. Subsequently, sampling method of the fruits for the analysis is discussed in consideration of the variation of CND content among fruits. Metaxenia, the effect of pollen genotype on CND content, is also investigated, because outcrossing occurs 5 - 10 % and 7 - 36 % in *C. annuum* and *C. frutescens-chinense* complex, respectively[9)].

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Materials and Methods

HPLC equipment and operating condition

The HPLC equipment used is a full automatic gradient system, CCP&8010 series (TOSO Corp.), which consists of a pump (CCMP), a column oven (CO-8010), an online degasser (SD-8012), a UV

detector (UV-8010), and a system controller (SC-8010) for the automatic peak detection and quantitation.

According to Iwai (1979)[1] and Kawada (personal communication), the operating condition was set as follows ; a YMC-Pack FL-C_{18} (50 x 4.6 mm I. D., YMC) column was used and run at 30°C. Methanol (60 %) was used as mobile phase at a flow rate of 1.0 ml/min. The UV detector was set at 280 nm.

Authentic capsaicin (Wako Pure Chem.) was dissolved in methanol to prepare the standard solutions (20, 2000, 10000, and 20000 μg/20ml). They were injected and analyzed to make a calibration graph. Then, each standard solution was injected five times to evaluate the reproducibility of HPLC analysis with the operating condition described above. Five μl of standard solutions were injected via a sample loop.

Sample preparation and CND extraction

A Japanese commercial variety, Takanotsume (*C. annuum*) was grown with a conventional method at the Experimental Farm, Faculty of Agriculture, Shinshu University. Full ripe fruits were collected and used for the experiment.

1) Drying Method ; Fifteen fruits were dried at 36°C and 43°C for 4 days, respectively, in a forced air flow oven (WFO-600ND, TOKYO RIKA). For the control, they were dried naturally at a room temperature for 30 days. Then several fruits were powdered together as a bulk sample and the CND content was determined by HPLC to investigate the effect of heat drying.

2) Extraction solvent and time ; Ethyl acetate and acetone were mixed in five different ratios as extraction solvents (Fig. 3). After removing peduncles, twenty naturally dried fruits were ground into powder for 20 seconds by an electric mill (MK-52M, National). About 0.4 g of the powder were put into 20 ml of solvent, stirred and left stand for 4 hours at room temperature. Then, the amount of CND extracted by the solvent was measured. To determine the time required for the CND extraction, change in the amount of CND extracted during the course of extraction was examined from 10 to 120 minutes.

Crude liquid extract was filtrated first by a circular quantitative filter paper (0.18 mm, 87 g/m^2, TOYO) and secondly by a syringe filter (0.45 μm, 25 mm, NALGENE), and then the filtrate was stored at −2°C as sample solution. Five μ l of the sample solution were injected for the analysis.

Variation of CND content

Full ripe fruits were collected separately from 15 plants of Takanotsume. To examine the source of variation of the CND content, the content of individual fruit was measured in one fruit each of 15 plants and in three fruits each of 5 plants. To estimate the variation among plants, bulk samples of three fruits of each of all plants were also analyzed. In addition, from a practical point of view, five fruits were taken randomly from the whole of fruits yielded and powdered together. Three such bulk samples were subjected to the analysis..

Cross pollination

To examine whether metaxenia is found or not in the CND content, artificial cross pollination was made among *C. annuum* varieties. Two sweet pepper lines, Shishitou and Fushimi-amanaga, and three pungent lines, Nikkou, Sapporo and Nepalese local strain No. 871292, were grown in a glass house. Flower buds were castrated and bagged the day before anthesis and were self-and cross-pollinated artificially on the day of anthesis. Crosses were made between sweet × sweet, sweet × pungent and pungent × pungent varieties (Table 2) and the CND contents of the fruit obtained were determined.

Results and Discussion

Reliability of HPLC analysis

The HPLC chromatogram of authentic capsaicin is shown in Fig. 1A. Capsaicin was eluted around 6 minutes after injection. A linear relationship was obtained between the capsaicin content and the detected peak area ($r=0.999$, $p<0.001$, Fig. 2).

Coefficients of variation (C.V.) of the peak area obtained by repeated injection of standard capsaicin solutions were less than 2 %. Therefore, the

result obtained by HPLC analysis with the operating condition used in the present study described above is reliable.

A chromatogram of the extract from Takanotsume obtained by the same method is shown in Fig. 1B. Three kinds of CND were detected and identified as nordihydrocapsaicin, capsaicin and dihydrocapsaicin by their retention time. No other capsaicinoid was detected in the extract. The calibration graph for capsaicin is said to be also applicable to dihydrocapsaicin and nordihydrocapsaicin[1].

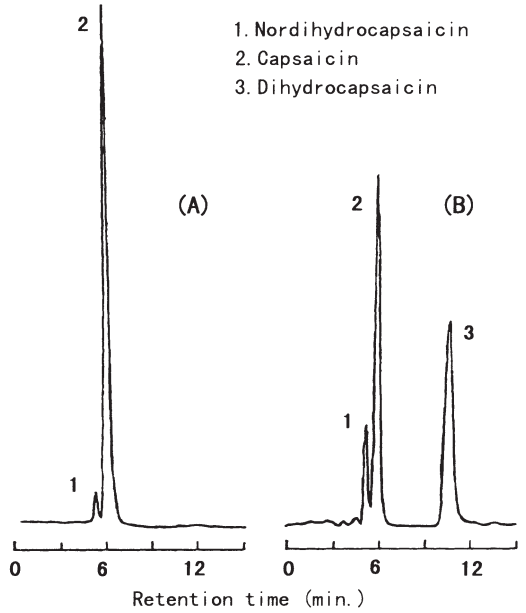

Fig. 1 Elution pattern of capsaicinoid.
(A) Authentic capsaicin
(B) Extract of Takanotsume (*C. annuum*) fruit

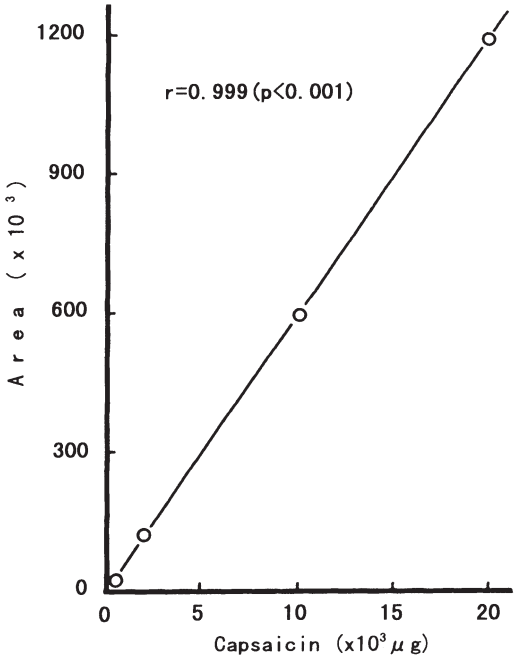

Fig. 2 Calibration graph for capsaicin.

Sample preparation and CND extraction

1) Drying temperature ; By the natural drying method, it takes about one month or more to dry up the chili fruits completely and even after such long drying period there still be a considerable variation of moisture content among dry fruits. In addition, the fruits often receive the mold damage. Therefore, heat drying method which doesn't affect CND content is necessary. The CND contents of the fruits dried at 43°C and 36°C were 46 % and 101 %, respectively, compared with those of natural air dried ones. This result indicates that drying at 36°C for 4 days using a forced air flow oven is available for the quantitative determination of CND (Table 1).

2) Extraction solvent and time ; Relationships of the amount of CND extracted with the composition of solvent and with the extraction time are shown in Fig. 3. The CND content was the highest in the extract obtained by the 1 : 1 mixture of ethyl acetate and acetone. As for the extraction time, extraction of CND was almost completed within 60 minutes and the CND content of the extract was the highest at 60 minutes.

From these results, the extraction by the 1 : 1 mixture of ethyl acetate and acetone for 60 minutes was adopted. Six measurements on the same powder sample by using this extraction method gave C. V. of 5.9 %. Therefore, this extraction method is reliable.

Variation of CND content and sampling method

The variation of CND content among fruits

Table 1. Effect of drying method on capsaicinoid content.

Drying method	Capsaicinoid content	
	(μ/gDW)	(%)[2]
4 days at 36°C[1]	3940	101
4 days at 43°C[1]	1817	46
30 days in a room	3918	100

1) Using forced air flow oven.
2) Percentage to the content of naturally dried fruits.

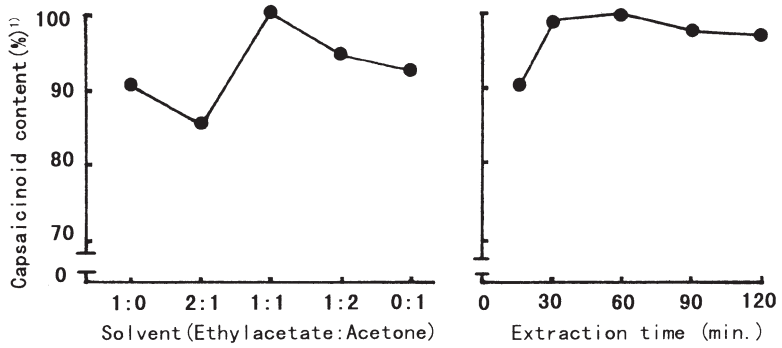

Fig.3 Effects of extraction solvent and extraction time on capsaicinoid content of dried fruit powder.
1) Percentage to the highest capsaicinoid content.

collected one each from 15 plants showed 17.9 % C. V.. This variation consists of genetic and environmental variation among plants and physiological variation among fruits within a plant. Blooming of chili pepper lasts continuously for a long time, so mature stage and CND content of fruits vary even in a plant[6)8)]. The average of C. V. among fruits within a plant was 18.1 %, which might be mainly due to the physiological variation. Therefore, variation of CND content among fruits was almost ascribed to the physiological variation.

As for the genetic variation, Takanotsume is a commercial variety and thought to be genetically homogeneous. In fact, when bulk samples of three fruits each were analyzed, the C. V. among plants was 7.7 %. This indicates that bulk sample of several fruits from one plant is available for the quantitative analysis if the environmental variation is ignored, although when the environmental variation is taken into consideration, random sampling in a variety is better. Bulk samples of five fruits each in a variety showed 8.1 % of C. V. Then, C. V. around 5 % can be expected by the analysis on bulk samples of 10 fruits collected randomly. This type of sampling may be enough for the estimation of CND content of the samples of genetically homogeneous varieties such as Takanotsume. However, for the genetically heterogeneous samples such as those of local varieties and *C. frutescens-chinense* complex, sampling of two or more fruits from each plant is required.

Metaxenia does not exist in CND content

Table 2 shows the CND content of self- and cross-pollinated fruits. The difference in CND content was not observed between the self- and cross-pollinated fruits in all varieties tested.. Capsaicinoid is produced at the placenta of mater-

Table 2. Comparison of capsaicinoid content[1)] between self- and cross-pollinated fruits in *C. annuum*.

Self pollination	Cross pollination (♀×♂)[2)]			
	S×S	S×P	P×S	P×P
Shishitou	Shi×Fu	Shi×No.871292		
0	0	0		
Fushimi		Fu×Ni		
0		37		
Nikkou			Ni×Fu	
2649			3520	
Sapporo				Sa×No.871292
3016				2212
No.871292			No.871292×Shi	No.871292×Sa
4129			4407	3885

1) μg/g dry weight 2) S: Sweet, P: Pungent

nal plant[8] and then the CND content of fruits is dependent on the maternal genotype. In fact, the result of cross pollination was well consistent with this logic as the previous report[10] and no occurrence of metaxenia was found.

From this result, it is concluded that open pollinated fruits can be used as the sample for quantitative determination of CND without concern about outcrossing.

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トウガラシの辛味成分のＨＰＬＣ分析法と試料調製法

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要　約

香辛料作物としてのトウガラシ（*Capsicum* sp.）の特徴はその独特な辛味成分にある。したがってトウガラシの育種のためには辛味成分であるカプサイシノイド（CND）の定量法を確立することが不可欠である。そこで簡便で汎用的な高速液体クロマトグラフィー（ＨＰＬＣ）を用いたCNDの定量法を検討し，確立した。まずＨＰＬＣの分析条件を検討し，変動係数２％以下の分析条件を確立した。次いで分析用試料の調製法を検討し，36℃で４日間通風乾燥した果実を20秒間粉砕後，酢酸エチルとアセトンの等量混合液で60分間抽出する方法を決定した。さらに分析用果実の採取法を検討するためにCND含量の果実間変異を明らかにした。その結果，遺伝的変異の少ない育成品種では，個体ごとに収穫する必要はなく，収穫した全果実から無作為に選んだ10果実程度をまとめて分析すればよいと考えられた。なお，トウガラシでは完全な自家受粉ではなく，一部他家受粉が生じていることから，人為交配を行いメタキセニアの有無を調査したところ，メタキセニアは認められず，放任受粉により得た果実を定量分析に使用できることを確認した。

キーワード：*Capsicum*，トウガラシ，辛味成分，HPLC分析，メタキセニア