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Note

Effective separation of capsaicin and its analogues by reversed-phase high-performance thin-layer chromatography*

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The pungent principles of hot pepper fruits are capsaicinoids, the structures of which are acid amides of C_9–C_{11} branched-chain fatty acids and vanillylamine[1]. Five analogues occurring in nature have been reported[1–4]: capsaicin [N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-*trans*-6-enamide)], dihydrocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-8-methylnonamide], nordihydrocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-7-methyloctamide], homodihydrocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-9-methyldecamide] and homocapsaicin [N-(4-hydroxy-3-methoxybenzyl)-9-methyldec-*trans*-7-enamide]. Recently, novel but minor analogues having *ante-iso* branched-chain fatty acyl moieties or shorter straight-chain fatty acyl moieties have also been reported[5]. With respect to capsaicinoid analysis, many methods using spectrophotometry[6,7], paper chromatography[8], thin-layer chromatography[9,10], gas chromatography[11–13] and gas chromatography–mass spectrometry[14,15] have been reported. However, the traditional methods appear to have some disadvantages relating to the reliability of the data, separation ability, running time and cost. In a previous paper we reported a method for the ultramicro and simultaneous analysis of capsaicin and its four analogues by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry[16]. The method appears to be excellent, but it is not universally popular because of the expensive apparatus.

However, HPLC offers a new approach in this field[17,18]. Recently, Johnson *et al.*[19] proposed an interesting method for the analysis of naturally occurring capsaicinoids by HPLC using a reversed-phase partition column developed with methanol containing 0.05 *M* silver nitrate for the separation of five capsaicin analogues. However, their method also is not popular because of the expensive apparatus and corrosion of the tubing.

On the other hand, high-performance thin-layer chromatography (HPTLC) also offers a new approach which greatly simplifies sample treatment and saves time and operating costs. Stimulated by Johnson *et al.*'s report, we attempted to separate

* Formation and metabolism of pungent principle of *Capsicum* fruits, Part VI.

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the naturally occuring capsaicinoids by HPTLC with similar solvents to those used by Johnson *et al.*, and obtained excellent results for the qualitative and quantitative analyses of capsaicinoids and their analogues.

EXPERIMENTAL

Materials

Reversed-phase thin-layer plates (RP-8) were purchased from E. Merck (Darmstadt, G.F.R.). Authentic capsaicin was purchased from Sigma (St. Louis, MO, U.S.A.). *cis*-Capsaicin, dihydrocapsaicin, nordihydrocapsaicin, *cis*-homocapsaicin and homodihydrocapsaicin were synthesized according to the method as described by Rangoonwala and Seitz[20]. Naturally occurring capsaicinoids were obtained from red chilli fruit (*Capsicum annuum* var. *annuum* cv. Red Chilli) by extraction with ethyl acetate[21]. Silver nitrate, boric acid, methanol and 2,6-dichloroquinone 4-chloroimide were guaranteed reagent-grade materials purchased from Nakarai Chemical (Kyoto, Japan).

One-dimensional HPTLC

A 5–200-nl volume of an 0.01 % ethyl acetate solution of individual capsaicinoids or a mixture were applied to the RP-8 plate (10 × 10 cm) with the aid of a Nanoapplicator (Atto Instruments, Tokyo, Japan). After the samples had been spotted, the plate was developed in a filter-paper lined chamber with 0.05 *M* each of silver nitrate and boric acid in 85% methanol for 5 cm without pre-conditioning. After development, the plate was dried at room temperature. Detection was accomplished by locating the blue spots after spraying with 0.1 % 2,6-dichloroquinone 4-chloroimide in methanol and subsequent exposure to ammonia vapour.

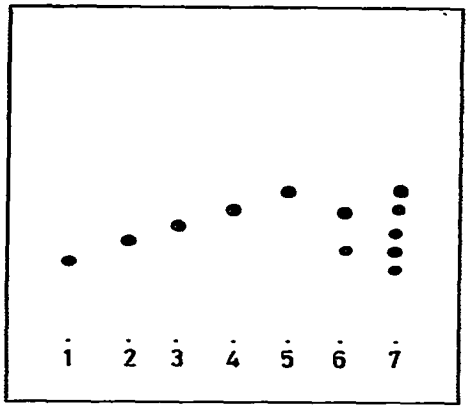
For the calibration of capsaicin and its analogues, the absorption of the located spots was measured with a Shimadzu Type CS-910 dual-wavelength thin-layer chromatoscanner, (Shimadzu Seisaku-sho, Kyoto, Japan). The absorption of the capsaicinoids was measured at 610 nm for the sample side and 710 nm for the reference side by the "zig-zag" mode scanning. The slit width and height were 1.25 mm. Measurement was carried out within 30 min after the location of the spots.

Two-dimensional HPTLC

The RP-8 plate loaded with the capsaicinoid mixture was developed first with 85% methanol, then air-dried prior to the second development with 0.05 *M* each of silver nitrate and boric acid in 85% methanol. The solvents were developed 7 cm in both directions. Detection was accomplished with the same reagents as used in one-dimensional HPTLC. The separations were repeated at least three times in each experiment.

RESULTS AND DISCUSSION

R_F values of capsaicin and its analogues are presented in Table I. When the chromatograms were developed with methanol only, the separation between *trans*-capsaicin and nordihydrocapsaicin was unacceptable. However, good separations of capsaicin and its analogues could be obtained by the addition silver nitrate and boric acid (Fig. 1).



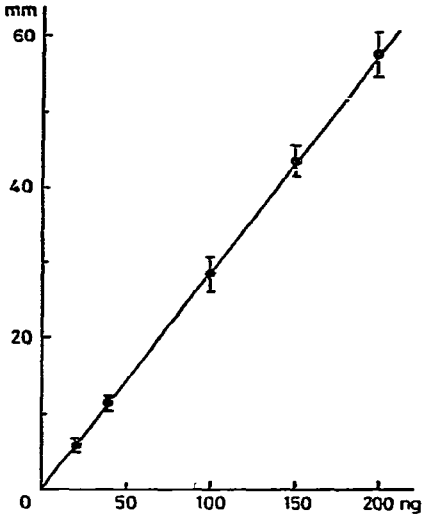


Fig. 1. Reversed-phase HPTLC of naturally occurring and synthetic capsaicin and its analogues on RP-8 plate developed with 0.05 *M* $AgNO_3$ and 0.05 *M* H_3BO_3 in 85% methanol. Spots were located by spraying with 0.1% 2,6-dichloroquinone 4-chloroimide and subsequent exposure to ammonia vapour. 1 = Homodihydrocapsaicin; 2 = synthetic dihydrocapsaicin; 3 = synthetic nordihydrocapsaicin; 4 = synthetic *cis*-homocapsaicin; 5 = synthetic *cis*-capsaicin; 6 = Sigma capsaicin, which was a mixture of capsaicin and dihydrocapsaicin; 7 = mixture of the samples 1–5.

Fig. 2. Calibration graph for capsaicin and its analogues, prepared by measuring the integration heights at 610 nm on a Shimadzu CS-910 dual-wavelength chromatoscanner. The integration heights are average values obtained from seven analyses. Linearity is obtained up to 200 ng ($y = 0.284x + 0.872$, where y is the integration height, and x ng the amount of capsaicin analogue). The calibration graph for *cis*-capsaicin and nordihydrocapsaicin is presented here, but other samples gave essentially the same results.

trans-Capsaicin and *cis*-homocapsaicin had the same R_F values, but this is not important because the latter does not occur in nature. All naturally occurring capsaicins and homocapsaicins have a *trans* configuration. The R_F value of *trans*-homocapsaicin is not presented here. However, as the R_F value of the synthesized *cis*-capsaicin was higher than that of *trans*-capsaicin, it would be located below *cis*-homocapsaicin. Even if the R_F value of *trans*-homocapsaicin were the same as that of *cis*-homocapsaicin, it would not matter in practice because *trans*-homocapsaicin has been detected in only trace amounts in most *Capsicum* species. Linearity was found at least from 20 to 200 ng (Fig. 2).

If complete separation is necessary, it is accomplished by two-dimensional reversed-phase (RP) HPTLC. Fig. 3 shows a thin-layer chromatogram of naturally occurring and synthetic capsaicin and its analogues accomplished by two-dimensional RP HPTLC. Complete separation between each analogue was achieved.

Fig. 4 shows a chromatogram of capsaicinoids extracted from red chilli. About 500 ng of capsaicinoids were applied on the RP-8 plate. Dominant spots of *trans*-capsaicin and dihydrocapsaicin were observed, together with small spots of nordihydrocapsaicin and homodihydrocapsaicin. Two-dimensional RP HPTLC can be accomplished in 3 h. One-dimensional RP HPTLC appears to be adequate for routine analysis.

TABLE I

R_F VALUES OF CAPSAICIN AND ITS ANALOGUES ON RP-8 HPTLC PLATES DEVELOPED WITH VARIOUS SOLVENT SYSTEMS

Solvent system	*cis-CAP*[a]	*trans-CAP*[a]	*cis-HC*[a]	*NDC*[a]	*DC*[a]	*HDC*[a]	*Development time (min)*
0.05 *M* $AgNO_3$ in 90% MeOH	0.59	0.52	0.53	0.47	0.43	0.37	120
0.05 *M* $AgNO_3$ in 80% MeOH	0.42	–	0.36	0.28	0.23	0.17	150
0.05 *M* $AgNO_3$ + 0.05 *M* H_3BO_3 in 80% MeOH	0.51	0.44	–	0.38	0.32	0.26	90
0.05 *M* $AgNO_3$ + 0.05 *M* H_3BO_3 in 85% MeOH	0.50	0.45	0.45	0.37	0.29	0.24	90
85% MeOH	0.36	0.36	0.29	0.37	0.29	0.23	40
90% MeOH	0.67	0.67	0.62	0.67	0.62	0.57	40

[a] *cis*-CAP = *cis*-capsaicin (synthetic); *trans*-CAP = *trans*-capsaicin; *cis*-HC = *cis*-homocapsaicin (synhtetic); NDC = nordihydrocapsaicin (synthetic); DC = dihydrocapsaicin (synthetic); HDC = homodihydrocapsaicin (synthetic). Each R_F value is the average value obtained from eight analyses and the error for each R_F value was less than 1.5%.

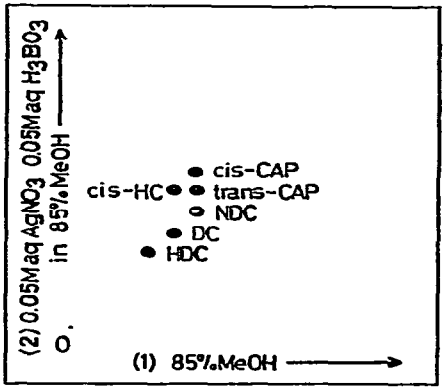


Fig. 3. Two-dimensional RP HPTLC of capsaicin and its analogues. The sample mixture consisting of *trans*-capsaicin (*trans*-CAP), *cis*-capsaicin (*cis*-CAP), dihydrocapsaicin (DC), nordihydrocapsaicin (NDC), homodihydrocapsaicin (HDC) and *cis*-homocapsaicin (*cis*-HC) was applied at the starting point (0). The success of the separation with 85% methanol in the first direction, and 0.05 *M* each of $AgNO_3$ and H_3BO_3 in 85% methanol in the second can be seen from the chromatogram.

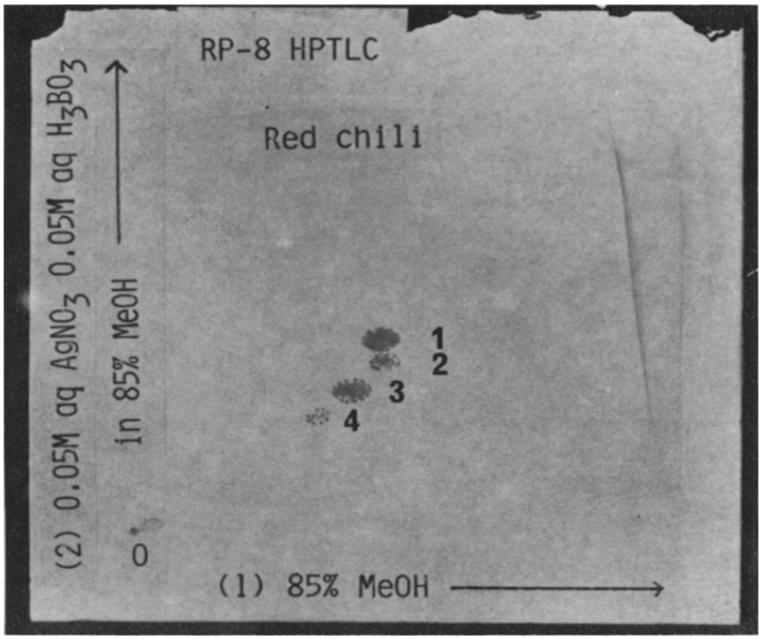


Fig. 4. Two-dimensional RP HPTLC of capsaicin and its analogues extracted from the fruits of red chilli. About 500 ng of capsaicinoids were applied at the starting point (0). 1 = *trans*-Capsaicin; 2 = nordihydrocapsaicin; 3 = dihydrocapsaicin; 4 = homodihydrocapsaicin.

Capsaicinoid analysis using RP HPTLC is sensitive, and much more convenient than former methods. Application of one-dimensional RP HPTLC to the dermination of the content and composition of capsaicinoids in some *Capsicum* species is shown in Table II. This method is excellent for the study of capsaicinoid biosynthesis in combination with radioautography. An example using this system will be described elsewhere.

TABLE II

APPLICATION OF HPTLC ON RP-8 PLATES TO THE DETERMINATION OF CONTENT AND COMPOSITION OF CAPSAICINOID IN SOME HOT PEPPER SPECIES PURCHASED FROM DIFFERENT AREAS

ND = not detected; tr = trace (<0.01 mg/dry fruit). The analysis was performed by one-dimensional HPTLC. The non-existence of *trans*-homocapsaicin (*trans*-HC) was confirmed by mass chromatography[16].

Hot pepper	*Harvested area*	*Capsaicinoid [mg/dry fruit] (%)*				
		NDC	*DC*	*trans-CAP*	*HDC*	*trans-HC*
C. annuum var. *annuum**	Guizhou (China)	0.18 (8.6)	0.59 (28.2)	1.23 (58.9)	0.09 (4.3)	ND (ND)
C. annuum var. *annuum**	Hainan (China)	0.03 (5.9)	0.15 (29.4)	0.32 (62.7)	0.01 (2.0)	ND (ND)
C. annuum var. *annuum**	Guangxi (China)	0.04 (5.1)	0.22 (27.8)	0.53 (67.1)	tr*[1] (tr)	ND (ND)
C. annuum var. *annuum* cv. Red chilli	Mexico	0.13 (7.3)	0.78 (44.1)	0.84 (47.5)	0.02 (1.1)	ND (ND)
C. pubescens cv. Roccoto	Bolivia	1.01 (13.7)	2.79 (37.8)	3.59 (48.6)	ND (ND)	ND (ND)
C. annuum var. *annuum* cv. Karayatsubusa**	Kyoto (Japan)	0.32 (12.0)	1.34 (50.2)	0.96 (36.0)	0.05 (1.9)	ND (ND)
C. annuum var. *annuum* cv. Karayatsubusa***	Kyoto (Japan)	0.20 (7.5)	1.50 (56.2)	0.97 (36.3)	tr (tr)	ND (ND)

* Although the name of cultivar was uncertain, the *Capsicum* fruits purchased from three different areas in People's Republic of China originated from the same species. In spite of different capsaicinoid content, the compositions resembled each other.

** *C. annuum* var. *annuum* cv. Karayatsubusa was used to compare the reliability of the RP-8 HPTLC plates with mass chromatography[16].

*** Data obtained by mass chromatography. Approximately the same values were obtained for ** and *** except for NDC and HDC. The higher values for ** for NDC and HDC should be attributed to the tailing of excess amounts of DC and CAP. For detailed experimental conditions for the capsaicinoid extraction and HPTLC, see text.

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