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SIMULTANEOUS MICRODETERMINATION OF CAPSAICIN AND ITS FOUR ANALOGUES BY USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

An improved method is described for the simultaneous determination of capsaicin and its analogues at levels from nanograms to micrograms using high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry. This method consists of two steps: firstly, purification and determination of total capsaicinoid by HPLC, and secondly, the simultaneous determination of capsaicin and its analogues by mass chromatography (MC) or mass fragmentography (MF). Crude extracts of capsaicinoid were purified with a Zorbax SIL column. Total capsaicinoid was detected at 235 nm and measured automatically by a microcomputer. It was collected, evaporated, trimethylsilylated and subjected to MC or MF. After monitoring the molecular ions of trimethylsilyl derivatives of capsaicinoid and the internal standard, the absolute contents of each analogue were determined by computer. By using this method, capsaicin and all of its analogues can be determined simultaneously at levels from micrograms to nanograms without any interferences from other components.

INTRODUCTION

Capsicum fruit (hot pepper or red pepper) is one of the most widely used spices, together with pepper, paprika, nutmeg, cinnamon and ginger, and is used in foods such as curries, sauces and Chinese and Mexican dishes.

The pungent principle of capsicum fruit is a group of compounds called capsaicinoid, which is an acid amide of vanillylamine and C_9–C_{11} branched fatty acids. Five analogues of capsaicinoid have been reported: capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin. Of these, capsaicin and dihydrocapsaicin are the major components of most *Capsicum* species.

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There has been much interest in the capsaicinoid analogues from various viewpoints: for instance, are there any significant relationships between *Capsicum* species and analogue compositions?; how are they biosynthesized?; does the difference in the composition of analogues have any significant influence on the strength and/or qualities of pungency?; and how are they metabolized in animal bodies?

Although many papers dealing with capsaicinoid from the viewpoints of genetics[1], biogenesis[2–8], food chemistry[9] and physiology[10–12] have been reported, the data have been restricted to the total capsaicinoid level because most of the conventional methods for the analysis of capsaicinoid, such as colorimetry[13–17], gas chromatography (GC)[18–21], thin-layer chromatography (TLC)[22–26], nuclear magnetic resonance spectrometry[27] and paper chromatography[16], have inadequate sensitivity and/or give a poor resolution of analogues. Therefore, a new method is required in order to answer satisfactorily the above questions.

In our earlier work, a method for the simultaneous determination of capsaicin, dihydrocapsaicin and nordihydrocapsaicin by using mass fragmentography (MF) was proposed[28]. In that method, in order to purify crude capsaicinoid extract, oleoresin is first subjected to TLC on silica gel. However, when using a silica gel G plate, a large excess of interfering substances such as carotenes, if present, cannot be removed completely without following a time-consuming and complicated procedure. Moreover, the number of mass peaks that can be simultaneously monitored by MF is limited to only three, so the true simultaneous determination of five analogues can be accomplished only by another method. Therefore, we have developed an improved method for the true simultaneous determination of all five capsaicinoid analogues by using HPLC and mass chromatography (MC) instead of silica gel TLC and MF.

EXPERIMENTAL

Equipment and operating conditions

High-performance liquid chromatography. The chromatograph used for the purification and quantification of total capsaicinoid was a Shimadzu-DuPont Type LC-2 equipped with an SPD-1 spectrophotometer as a detector, and with a Shimadzu Chromatopac E-1A microcomputer for automatic quantification and peak detection. A Rheodyne 7120 syringe loading sample injector was used in place of a standard septum-type injector. A DuPont Zorbax SIL column (150 mm × 4.6 mm I.D.) was used for the purification and collection of total capsaicinoid.

Isopropanol–*n*-hexane–methanol (10:90:1) was used as the developing solvent system. The column pressure was kept at 50 kg/cm^2. As the absorption maximum of capsaicinoid was *ca.* 230–235 nm in the above solvent system, the wavelength of the SPD-1 was fixed at 235 nm. The column was run at room temperature. Monitoring of peak retention times and peak areas and calibration were performed automatically by the E-1A microcomputer.

Mass chromatography. MC for the simultaneous determination of capsaicinoid was carried out on a Shimadzu-LKB 9000 combined gas chromatograph–mass spectrometer equipped with an MS-PAC 300DGB computer data system. The separation column used (2 m × 3 mm I.D.) was packed with 3% SE-52 on Chromosorb W (60–80 mesh, acid washed and silanized). The column temperature was kept at 260°, the molecular separator temperature at 280° and the ion source temperature at 290°.

The carrier gas flow-rate was 30 ml/min. The ionization energy, acceleration voltage, trap current and slits were set at 20 eV, 3.5 kV, 60 μA and 0.1 mm, respectively. Mass spectra were recorded automatically at intervals of 10 sec with a scanning speed of 8. The off-set time and stop time were set at 2 and 10 min, respectively. The threshold level was set at 100, and mass spectra were recorded from *m/e* 30 to 450. For calibration purposes 0.1–1.0 μg of authentic capsaicinoid was injected together with 0.5 μg of 5-α-cholestane as the internal standard.

Mass fragmentography. MF for the simultaneous determination of three capsaicinoid analogues was carried out using the Shimadzu-LKB 9000 instrument and MID-PM 9060S multiple ion detector. As the number of channels capable of monitoring molecular ion peaks was limited to only three, capsaicin (*m/e* 377), dihydrocapsaicin (*m/e* 379) and nordihydrocapsaicin (*m/e* 365) were monitored first, then homocapsaicin (*m/e* 391) and homodihydrocapsaicin (*m/e* 393) later. 5-α-Cholestane was used as the internal standard. The conditions for GC–mass spectrometry (MS) were as described above, and the conditions for MF were as described in a previous paper[24].

Materials

Capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin were synthesized according to Rangoonwala and Seitz's procedure[29]. 5-α-Cholestane, used as the internal standard in MC and MF, and ethyl acetate (guaranteed reagent grade), used as a solvent and as the internal standard in HPLC, were obtained from Nakarai Chemicals Co. (Kyoto, Japan). The trimethylsilylation reagent, TMS-HT, was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). The other chemicals used were all of guaranteed reagent grade and were obtained from Nakarai Chemicals Co.

Dried fruits of *Capsicum annuum* var. *annuum* cv. Karayatsubusa, *C. annuum* var. *annuum* cv. Takanotsume (H) and *C. annuum* var. *annuum* cv. Chili were obtained from the National Vegetable Experimental Station at Kurume, Japan. Laha You (red pepper oil), a typical Chinese food additive, was prepared by mixing a crude extract of *C. annuum* var. *annuum* cv. Karayatsubusa with hot sesame oil to make a *ca.* 0.1% capsaicinoid solution.

Procedure

About 0.1–1.0 g of powdered dried capsicum fruits was mixed with 40 volumes of acetone and/or ethyl acetate and filtered. The filtrate was concentrated under reduced pressure, then evaporated to dryness under a stream of nitrogen. The crude capsaicinoid extract was dissolved in known volumes of ethyl acetate, which also acted as the internal standard. When insoluble materials remained, the residue was removed by filtration prior to injection. Ten microlitres of solution were generally injected.

The retention times, absolute volumes of ethyl acetate and weight of capsaicinoid were printed out by the E-1A microcomputer. Eluates corresponding to capsaicinoid were collected at the fractionation vent of the LC-2, then the solvent was evaporated under a stream of nitrogen and the residue was trimethylsilylated for MC or MF. About a 10–100-fold excess of TMS-HT reagent was added, and the mixture was kept overnight at room temperature in a desiccator or heated at 50–60° for 15–30 min. The known amount of 5-α-cholestane present was calculated manually from the peak

width at half-height, and the capsaicinoid contents were determined from previously prepared calibration graphs. Each experiment was repeated three or four times.

RESULTS AND DISCUSSION

High-performance liquid chromatography of capsaicinoid

As shown in Fig. 1, the absorption maximum of capsaicin in the solvent system used in HPLC was about 235 nm, and other capsaicinoid analogues also showed similar absorption maxima. The molar absorption coefficient of capsaicin was about 9300 and the other compounds also showed similar values.

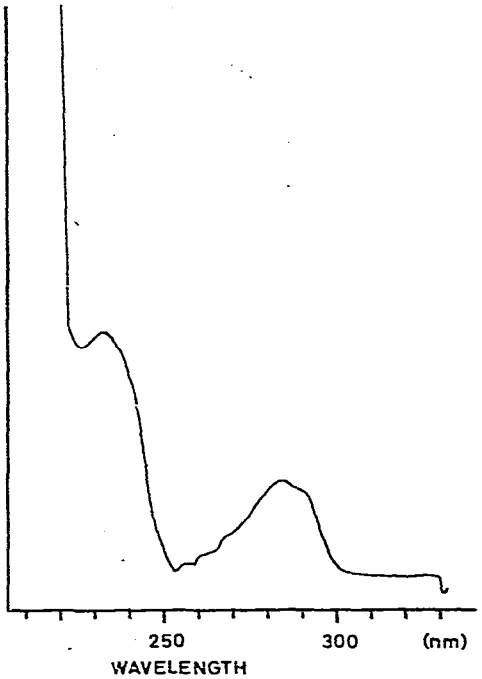

Fig. 1. Ultraviolet adsorption spectrum of capsaicin. Solvent: isopropanol–*n*-hexane–methanol (10:90:1). The spectrum was recorded with a Hitachi 124 spectrophotometer fitted with a Hitachi recorder.

Fig. 2a shows the elution pattern of capsaicinoid which was obtained by using a column which showed only slight adsorption of chlorophylls. When 5–10 μg of capsaicinoid mixture was injected, dihydrocapsaicin and capsaicin were separated from each other with a resolution of 0.3. Dihydrocapsaicin was eluted after *ca.* 6.8 min and capsaicin after *ca.* 7.1 min. Homodihydrocapsaicin was eluted at about the same position as dihydrocapsaicin, and nordihydrocapsaicin and homocapsaicin had retention times similar to that of capsaicin.

When the column was aged and contaminated by repeated injections of crude samples, no resolution between dihydrocapsaicin and capsaicin could be observed (Fig. 2b). However, it is not important whether dihydrocapsaicin and capsaicin were

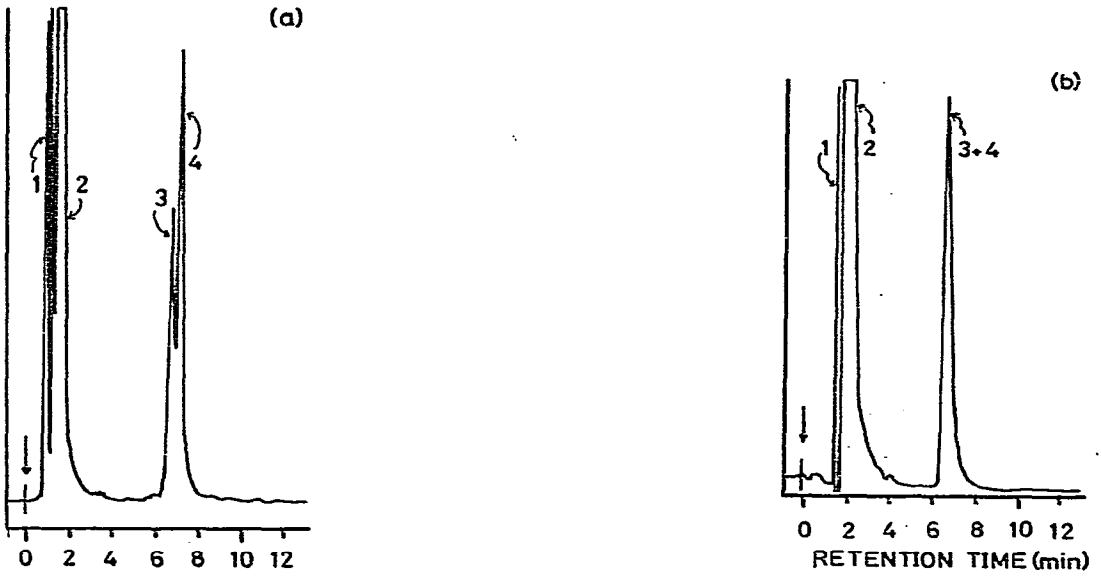

Fig. 2. Elution pattern of crude capsaicinoid extract from *Capsicum* fruits of *C. annuum* var. *annuum* cv. Karayatsubusa on the Zorbax SIL column. (150 × 4.6 mm I.D.). Isopropanol–*n*-hexane–methanol (10:90:1) was used as the developing solvent. Elution was carried out at room temperature, and eluates were detected at 235 nm. The arrows indicate the injection of the sample. (a) Elution pattern of crude capsaicinoid extract obtained from the column with only slight adsorption of chlorophylls. (b) Elution pattern of crude capsaicinoid extract obtained from the column which was considerably contaminated by large amounts of chlorophylls. Peaks: 1 = carotenes; 2 = ethyl acetate; 3 = dihydrocapsaicin; 4 = capsaicin and nordihydrocapsaicin.

resolved or not in this instance because these analogues can be identified and quantified by subsequent MC. Carotenes, one of the major interfering compounds, was eluted between ethyl acetate and capsaicinoid. When a large excess of chlorophylls (generally more than 100 μg of chlorophylls per 10 μl) are loaded, the column should be reconditioned by elution of chloroform or methylene chloride in order to remove the adsorbed chlorophylls.

In Fig. 3, calibration graphs for capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin and homodihydrocapsaicin prepared by microcomputer are shown. Linear relationships were found for amounts between 0.1 and 15 μg for each analogue.

Data for Laha You obtained from injections repeated four times are presented in Table I. Although 10 μl of sample solution were taken and injected, the volume actually injected was lower in each case: the mean value of the real injected volume was 8.72 $\pm$ 0.6 μl (standard deviation) (6.8% relative standard deviation). The mean value of the total capsaicinoid weight was 1.59 $\pm$ 0.13 μg (standard deviation) (8% relative standard deviation). When the weight of capsaicinoid in each injection was corrected to the weight in 10 μl, the mean value was 1.82 $\pm$ 0.02 μg (1.1% relative standard deviation). Therefore, the real injected volume should be checked and the data corrected by use of the internal standard

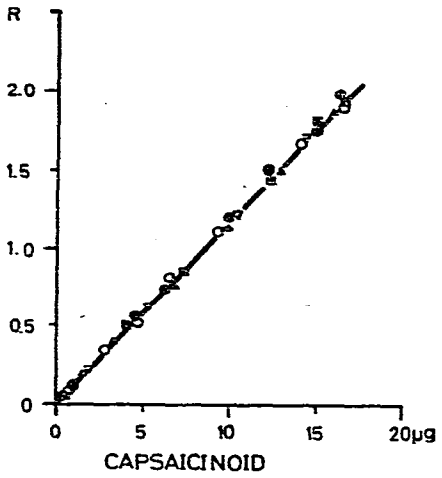

Fig. 3. Calibration graphs for five capsaicinoid analogues on a Zorbax SIL column. R = ratio of weight of capsaicinoid analogue to the volume of the internal standard (ethyl acetate). ●, Capsaicin; ■, dihydrocapsaicin; ▲, nordihydrocapsaicin; ○, homocapsaicin; ▽, homodihydrocapsaicin.

TABLE I

REPRODUCIBILITIES OF INJECTED VOLUMES AND AMOUNTS OF CAPSAICINOID ELUTED IN HPLC

Ten microlitres of capsaicinoid extract of Laha You (red pepper oil) in ethyl acetate solution were used for each injection, and injected on to a Zorbax SIL column (150 mm × 4.6 mm I.D.). Volumes actually injected and amount of total capsaicinoid eluted were detected at 235 nm, and calibrated automatically by the E-1A microcomputer.

Sample volume taken (μl)	*Volume actually injected (μl)*	*Amount of capsaicinoid eluted (μg)*	*Corrected amount of capsaicinoid (μg per 10 ml)*
10	8.4	1.49	1.79
10	9.7	1.79	1.85
10	8.3	1.51	1.82
10	8.4	1.52	1.81
10	8.8	1.62	1.83
Mean ± S.D.	8.7 ± 0.6	1.59 ± 0.13	1.82 ± 0.02

Mass chromatography and mass fragmentography

Calibration of the molecular ion peaks of trimethylsilylated derivatives of nordihydrocapsaicin, capsaicin, dihydrocapsaicin, homocapsaicin and homodihydrocapsaicin in MC was carried out automatically with the MS-PAC 300 DGB computer data system using 5-α-cholestane as the internal standard. The equations for the calibration of each analogue were as follows: $y = -0.13596x^2 + 1.2647x + 0.025939$ for capsaicin, $y = -0.033649x^2 + 0.63294x + 0.013145$ for dihydrocapsaicin, $y = 0.9x$ for nordihydrocapsaicin, $y = -0.15441x^2 + 1.3343x + 0.026233$ for homocapsaicin and $y = -0.070138x^2 + 0.84805x + 0.033528$ for homodihydrocapsaicin. where y is the ratio of the weight of sample to that of 5-α-cholestane, ranging from 0.1 to 1.0 μg, and x is the ratio of the peak areas of the molecular ions of each analogue to that of 5-α-cholestane (m/e 372).

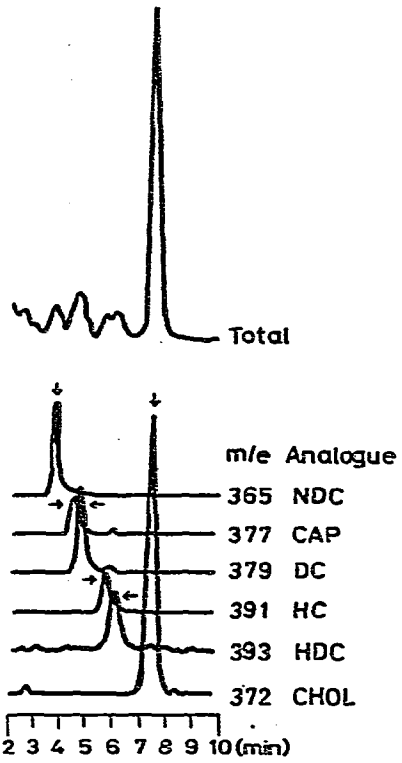

Fig. 4. Mass chromatogram of five capsaicinoid analogues. MC was carried out on a Shimadzu-LKB 9000 instrument equipped with an MS-PAC 300DGB computer system using a 3% SE-52 on Chromosorb column (2 m × 3 mm I.D.). Total = chromatogram obtained by monitoring total ion with total ion collector; NDC = O-TMS derivative of nordihydrocapsaicin; CAP = O-TMS derivative of capsaicin; DC = O-TMS derivative of dihydrocapsaicin; HC = O-TMS derivative of homocapsaicin; HDC = O-TMS derivative of homodihydrocapsaicin; CHOL = 5-α-cholestane (internal standard). The arrows indicate the peak of each capsaicinoid analogue and the internal standard.

TABLE II

PEAK RETENTION TIMES, PEAK AREAS, RELATIVE PEAK AREAS AND ABSOLUTE CONTENTS OF FIVE CAPSAICINOID ANALOGUES DETERMINED BY MASS CHROMATOGRAPHY

Capsaicinoid analogue	*M⁺ (m/e)*	*t_R (min)*	*Area**	*Relative peak area*	*Absolute capsaicinoid content (μg)*	*Real capsaicinoid content (μg)* *
Nordihydrocapsaicin	365	3.83	4778	8120	0.07	0.08
Capsaicin	377	4.50	2949	5012	0.11	0.10
Dihydrocapsaicin	379	4.67	5096	8661	0.09	0.10
Homocapsaicin	391	5.67	3043	5171	0.12	0.10
Homodihydrocapsaicin	393	6.00	3882	6596	0.10	0.10

* Area means integrated total ion intensities of each molecular ion (M^+) which were converted to digital values by the analogue–digital converter in the MS-PAC 300DGB system.

Fig. 4 is an example of the mass chromatogram of a mixture of the five authentic analogues, *viz.*, 0.08 μg of nordihydrocapsaicin (*m/e* 365), 0.1 μg of capsaicin (*m/e* 377), 0.1 μg of dihydrocapsaicin (*m/e* 379), 0.1 μg of homocapsaicin (*m/e* 391) and 0.1 μg of homodihydrocapsaicin (*m/e* 393). Retention times, peak areas, relative peak areas and absolute contents of the five analogues are presented in Table II. The values determined by MC virtually coincided with the real values.

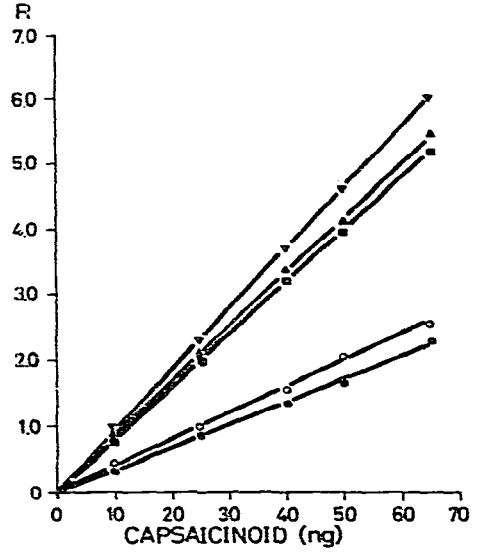

Fig. 5. Calibration graphs for five capsaicinoid analogues in mass fragmentography. R = ratio of molecular ion peak areas of capsaicinoid analogues to those of the internal standard (5-α-cholestane). In the preparation of the calibration graphs, 7 ng of the internal standard was used. ●, Capsaicin; ■, dihydrocapsaicin; ▲, nordihydrocapsaicin; ○, homocapsaicin; ▽, homodihydrocapsaicin.

TABLE III

TOTAL CAPSAICINOID CONTENTS AND COMPOSITIONS OF ANALOGUES IN VARIOUS *CAPSICUM* FRUITS AND LAHA YOU (RED PEPPER OIL), DETERMINED BY HPLC AND MASS CHROMATOGRAPHY OR MASS FRAGMENTOGRAPHY

Sample	*Total capsaicinoid content**			*Composition of analogues*** (%)*					*Method*
	μg per 10 μl	*mg per g of sample*	*%, w/w*	*NDC*	*CAP*	*DC*	*HC*	*HDC*	
Karayatsubusa*** (I)	12.2	6.08	0.61	7.61	36.33	56.06	ND	<0.01	MC
Takanotsume***	5.1	2.56	0.26	14.23	32.84	52.94	ND	ND	MC
Red chili***	7.9	3.97	0.40	5.99	48.97	43.72	0.34	0.98	MC
Karayatsubusa*** (II)	0.1	10.80	1.08	4.90	41.60	53.40	ND	0.10	MF
Laha You	1.8	0.90	0.09	0.10	68.00	31.90	ND	ND	MC

* Values in the first column are weight of total capsaicinoid recovered from HPLC column, and values in the second and third columns are total capsaicinoid content expressed as milligrams per gram of dry fruit and weight percent. In the case of Laha You, 0.9 mg of total capsaicinoid is contained in 1 g of the sample.

** NDC = nordihydrocapsaicin; CAP = capsaicin; DC = dihydrocapsaicin; HC = homocapsaicin; HDC = homodihydrocapsaicin. ND = not detected.

*** Karayatsubusa = *C. annuum* var. *annuum* cv. Karayatsubusa; Takanotsume = *C. annuum* var. *annuum* cv. Takanotsume (H); Red chili = *C. annuum* var. *annuum* cv. Red Chili. Karayatsubusa (I) and (II) were harvested at different seasons.

Calibration graphs for the five different analogues obtained by MF are shown in Fig. 5. Linearity between 5 and 60 ng was confirmed.

Table III gives results of examples of applications in which several *Capsicum* fruits and Laha You were purified and their total capsaicinoid contents quantified using a Zorbax SIL column, followed by determination of the composition of the capsaicinoid analogues by MC or MF. The results are mean values obtained by repeating the experiments three times.

Although the sensitivity of MF when using the MID-PM 9060S is much higher than that of MC, it is limited in the capacity of mass numbers monitored in a single measurement, the mass range and the stability of the equipment. Consequently, for convenience and time saving in routine analysis, microgram samples of capsaicinoid should be analysed by MC, and nanogram samples should be subjected to MF.

By this method, the simultaneous determination of all five capsaicinoid analogues at levels from micrograms to nanograms, which could not be achieved by conventional methods, can be carried out within 1–2 days. We believe that the method proposed here is the most accurate and convenient presently available.

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* *Note by Editor:* The paper by O. Sticher, F. Soldati and R. K. Joshi [*J. Chromatogr.*, 166 (1978) 211] had not appeared when this paper was received.