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Analytical Methods Section

Effective separation and quantitative analysis of major heat principles in red pepper by capillary gas chromatography

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An effective analytical method for the separation and quantitative analysis of the major heat principles in red pepper has been established. Capsaicinoids were extracted with acetone, and undesirable components, such as pigments, lipoids, and lipids, were effectively removed by liquid–liquid fractionation to avoid interference of the chromatogram or contamination of the column. A moderately polar fused silica capillary column, bonded with cross-linked cyanopropylphenyldimethyl siloxane, separated capsaicinoids with good resolution, and hydrogen eluted the components within reasonable retention times.

INTRODUCTION

Capsaicin has been recognized for many years as the pungent principle of capsicum. In recent studies, it has become clear that other structurally similar components, capsaicinoids, also contribute to the pungency of red pepper (Bennett & Kirby, 1968). Among them, capsaicin and dihydrocapsaicin are the most abundant heat principles of capsicum (Iwai *et al.*, 1979; Chiang, 1986), and their heat pungencies are almost twice those of homocapsaicin, homodihydrocapsaicin, and nordihydrocapsaicin (Todd *et al.*, 1977).

In order to determine the pungency of capsicum, the sensory method devised by Scoville (1912) has been widely used (Govindarajan *et al.*, 1977; Rhyu *et al.*, 1978; Gillette *et al.*, 1984). With some modification, the method has now been adopted by the American Spice Trade Association (ASTA), International Organization for Standardization (ISO), and Essential Oil Association of USA (EOA).

The Scoville method, however, has been severely criticized by many researchers (Suzuki *et al.*, 1957; Govindarajan *et al.*, 1977; Todd *et al.*, 1977; Rhyu, 1978) for the method has some disadvantages. By their nature, these sensory methods are subjective and difficult to reproduce between independent panels as a result of the variation in sensitivity of individual panelists. Because of the inherent uncertainties with the Scoville method, more precise, objective, and reproducible chemical and/or instrumental methods to standardize the heat level of red pepper and red-pepper products are required. Approaches to the problem of heat measurement have ranged over the past few decades from colorimetry, spectrophotometry, paper chromatography (PC), and gas chromatography/mass spectrometry (GC/MS) to high-performance liquid chromatography (HPLC) (Shuster & Lockhart, 1954; Suzuki *et al.*, 1957; Rosebrook *et al.*, 1968; Masada *et al.*, 1971; Govindarajan & Ananthakrishna, 1974; Lee *et al.*, 1976; Mori *et al.*, 1976; Todd *et al.*, 1977; Jurenitsch *et al.*, 1978; Dicecco, 1979; Altinkurt, 1980; Saria *et al.*, 1981; Srinivasan *et al.*, 1981; Hoffman *et al.*, 1983; Kawada *et al.*, 1985; Chiang, 1986).

However, most of these procedures are not suitable for individual quantitative analysis of the capsaicinoids. Recently, with some advantages claimed for HPLC over chemical analysis, its application for the analysis of these components has frequently been proposed. Even though sample preparation for HPLC analysis may be simple and convenient, the interfering substances extracted with capsaicinoids interfere with the chromatogram and can easily be absorbed to the column-packing materials, increasing pressure in the system and shortening the column life. There are also reports on the determination of the individual capsaicinoids by GC (Todd *et al.*, 1977; Jurenitsch *et al.*, 1978; Dicecco, 1979), but the results are not very satisfactory because extremely poor resolution leads to poor quantitative analysis. Hence the authors adapted mass fragments for the quantitative analysis of the capsaicinoids (Masada *et al.*, 1971; Lee *et al.*, 1976). Although GC/MS methods offer good results, they still require specific sample preparation and complex, costly instrumentation.

This paper describes an effective sample-clean-up

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procedure based on liquid—liquid fractionation to reduce interfering substances and a new capillary GC technique that provides good resolution and quantitative analysis of individual major heat principles in capsicum.

MATERIALS AND METHODS

Materials

Standard capsaicin (98%), dihydrocapsaicin (90%), and squalane were purchased from Sigma Chemical Co., USA, and natural capsaicinoids from Fluka AG., Switzerland. A standard solution containing *c.* 5 mg ml^{-1} of each capsaicinoids in dichloromethane was prepared and kept in a refrigerator at −18°C. Squalane was also diluted with dichloromethane for use as an internal standard (IS) solution containing half the amount of capsaicinoids. All other chemicals were of analytical-reagent grade. Ten varieties of dried red pepper obtained from the Office of Rural Development, Korea, were finely ground to pass a 50-mesh standard screen with a scientific mill (A. H. Thomas Co., USA).

Sample preparation

From ground red peppers (2–3 g), the capsaicinoids were extracted with acetone for 2 h in a Soxhlet apparatus. The extract was then evaporated to dryness, and the residue was dissolved with 80% methanol solution (50 ml). Non-polar components were removed by extracting the methanol layer twice with *n*-hexane (2 × 50 ml) and the combined *n*-hexane layers were counter-extracted with a second addition of methanol solution (50 ml).

The capsaicinoids should now be in methanol layers. An excess of water was added to the pooled methanol layer to remove more polar components, and the capsaicinoids were extracted with dichloromethane (3 × 50 ml). After dehydration with anhydrous Na_2SO_4, the solvent was removed by using a vacuum rotary evaporator and IS solution (2 ml) was added prior to quantitation of the capsaicinoids by injection of sample solution (0·2 μl) into the GC.

Gas chromatograph

A Varian Vista 6000 GC, equipped with a flame ionization detector, capillary inlet system, and GC Protector (J.&W. Sci. Inc., USA), was used to throughout the analysis. The chromatograph was controlled and interfaced to a VISTA 402 Chromatography Data System for quantitative analysis and report formatting. The separation was carried out with an 0·32 mm × 25 m fused-silica capillary column bonded with cross-linked cyanopropylphenyl-dimethyl siloxane (BP-10) made by Scientific Glass Engineering Pty. Ltd, Australia. Hydrogen was used as the carrier gas. Sample solution (*ca.* 0·2 μl) was loaded into the injection port at 280°C with a split ratio of 1 : 50. The inlet pressure of the carrier gas was controlled at 12 psi (82·7 kPa) and the linear velocity was 33 cm s^{-1} at the initial column-oven temperature of 220°C. The initial oven temperature was held for 1 min and then programmed up to 250°C at 3°C min^{-1} and held for 4 min at the final temperature. The eluent was detected with a flame ionization detector at 300°C.

Identification of capsaicinoids separated by GC was achieved by comparing mass spectra of the eluted peaks from a standard and samples by using a Concept II double-focusing mass spectrometer made by Kratos, UK. The capsaicinoids, separated by Hewlett-Packard GC with almost similar conditions to Varian except that the carrier gas was substituted by helium, were introduced to the mass spectrometer. The ion-source temperature was held at 300°C during the runs, and the mass spectra were obtained in the electron-impact-ionization mode with 70 eV of electron energy.

RESULTS AND DISCUSSION

Capillary-column GC separation has been achieved with intermediate and non-polar liquid phases. The natural mixture of capsaicinoids is separated into two major peaks and several trace peaks as shown in Fig. 1.

In this study, a moderately polar liquid phase such as cyanopropylphenyl-dimethyl siloxane was found to be suitable as well as a non-polar phase, such as methyl-silicone. Another interesting fact was that the elution of IS was faster than that of capsaicinoids in the intermediate phase, whereas it was slower in the non-polar phases. These results showed that the influence of polarity of the phase on the retention temperature was very significant in the GC of capsaicinoids and IS. An explanation of this observation must include a large affinity of capsaicinoids to an intermediate phase.

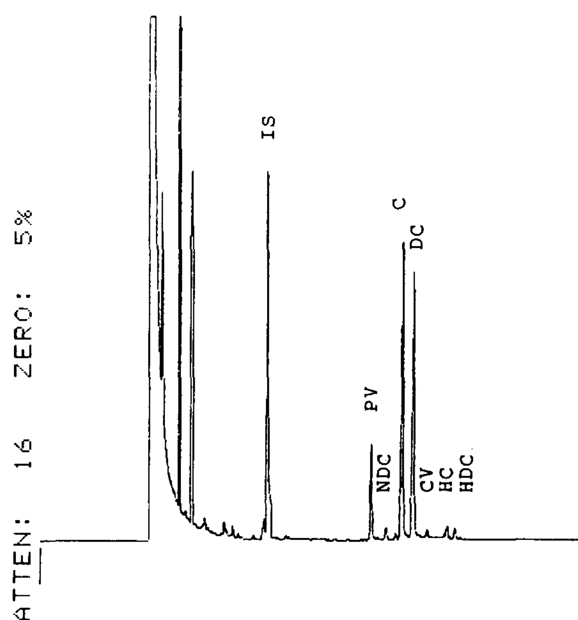

Fig. 1. Chromatogram of capsaicinoids in dried red pepper.

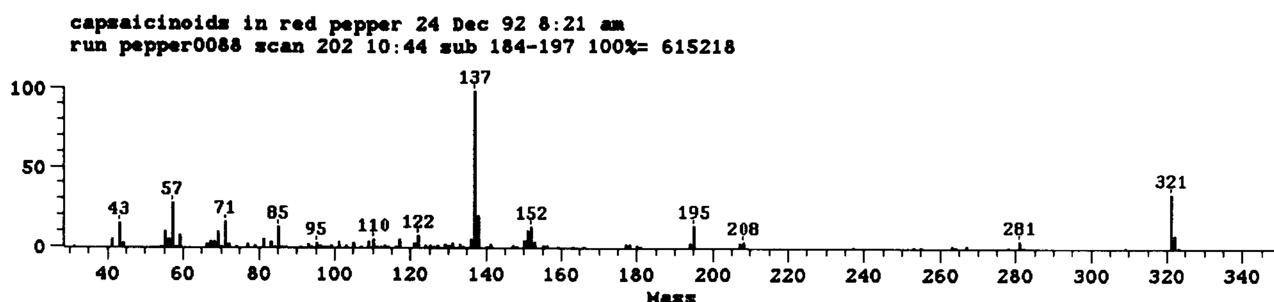

Fig. 2. The mass spectrum of homodihydrocapsaicin.

To achieve lower detection limits and to obtain satisfactory peak resolution and sharp symmetrical peak patterns, the electrometer sensitivity was turned up as far as practical, until the baseline noise and baseline drift became significant and the sample size was kept as small as possible.

To elute the peaks of capsaicinoids with satisfactory resolution based on their retention times, a gas velocity of less than 35 cm s^{-1} was found to be desirable. The carrier gas, hydrogen, gave complete separation of capsaicin and dihydrocapsaicin (α = 2·22), whereas nitrogen and helium gave lower resolution and could not elute the components in reasonable retention time under the same conditions.

The efficiency of extraction-solvent systems was not studied in this research, since a previous report (Mori *et al.*, 1976) indicated that acetone was the most effective solvent for the extraction of capsaicinoids. The extracting time, however, was determined with additional capsaicinoids of red pepper that had been analyzed previously. From this result, it was found that more than 99% of capsaicinoids could be extracted in an hour. This solvent, however, is not suitable for HPLC analysis because it also carries some undesirable components, which adversely affect the resolution of the individual components in HPLC analysis (Hoffman *et al.*, 1983).

After extracting the capsaicinoids with acetone, the solution was then evaporated to dryness and the residue was divided into two parts by adding *n*-hexane and methanol solution. The non-polar fraction could be removed by discarding *n*-hexane, and the capsaicinoids migrated into dichloromethane from the methanol layer in which the higher polar fraction would remain. To check any loss of capsaicinoids during the sample preparation through the discarded solutions, these solutions were concentrated and analyzed.

As a result of the clean-up of the sample solution, a number of intensive peaks in the direct extracts with acetone were removed by liquid fractionation. This meant that the sample prepared after fractionation contained less material that interferes with either separation or quantitative analysis, without any loss of the analyte.

Mass spectra were taken every one-and-a-half seconds over the *m/e* range of 35–350 with an ionization voltage of 70 eV. When an impure spectrum was suspected, selective ion chromatograms were obtained. If possible, a scan was taken at another point, and/or a neighbouring spectrum was subtracted to obtain a mass spectrum of a single compound.

From the data of mass-fragment ions, the peaks in the GC chromatogram of the soluble solution of red pepper were identified as pelargonic acid vanillylamide, nordihydrocapsaicin, capsaicin, dihydrocapsaicin, capric acid vanillylamide, and homocapsaicin and homodihydrocapsaicin. The relative retention times of the peaks in the total ion intensity of GC/MS agreed with those of the peaks in the GC chromatogram.

A typical mass spectrum of homodihydrocapsaicin is presented in Fig. 2. No great difference in fragmentation patterns appeared between capsaicinoids except molecular ion peak. Generally, all spectra showed simple patterns and a higher intensity of molecular peaks. However, the intensities of the other fragments are quite low. Each capsaicinoid can be easily identified according to the molecular ion peak. The molecular ion peaks are 293, 293, 305, 307, 307, 319 and 321 for pelargonic acid vanillylamide, nordihydrocapsaicin, capsaicin, dihydrocapsaicin, capric acid vanillylamide, homocapsaicin and homodihydrocapsaicin, respectively. The molecular ion of capsaicin is less abundant than that of dihydrocapsaicin. Because the relative intensities of the molecular ions reflect the bond strengths of the compounds, this indicates that dihydrocapsaicin is slightly more stable than capsaicin. Owing to the lower relative intensity of the molecular ion, the major fragmentation products of capsaicin were more abundant than the corresponding dihydrocapsaicin-fragment ions. However, the formation of ions is clearly favoured with capsaicin rather than with dihydrocapsaicin.

The recovery experiments were carried out by analyzing the contents of capsaicinoids in ground red pepper fortified with standards except nordihydrocapsaicin, which was unavailable as a standard. The natural level of capsaicin was 0·51 mg and that of dihydrocapsaicin 0·25 mg. This sample was fortified with standard compounds amounting to 2·31 mg of capsaicin and 1·08 mg of dihydrocapsaicin, respectively. Extraction, the clean-up procedure, and quantitative analysis were performed as previously described, and recoveries were calculated as indicated in Table 1.

The capsaicinoids in the sample were indicated by the relative retention time to IS and by comparing the mass spectra between standards and samples. Besides the major peaks, a few peaks eluted earlier or later and

Table 1. Recoveries of capsaicinoids (mg)

Capsaicinoids	Original	Added	Analyzed*	Recovery** (%)
Capsaicin	0·51	2·31	2·84 ± 0·12	94–105
Dihydrocapsaicin	0·25	1·08	1·34 ± 0·08	93–107
Total	0·76	3·39	4·18 ± 0·20	94–106

* Values are mean ± standard deviation of five determinations.
** Recovery: (analyzed–original) × 100/added.

were thought to be capsaicinoids. But these other capsaicinoids were not analysed by this method for they were found present in only trace quantities and standards were not available. This confirms the earlier GC and HPLC reports, which concluded that homocapsaicin and homodihydrocapsaicin were present only at low levels and norcapsaicin was not reported (Todd *et al.*, 1977; Hoffman *et al.*, 1983; Chiang, 1986).

Peak areas were determined by integration. Correlation coefficients between the capillary-GC data and concentration of these two capsaicinoids were all greater than 0·99, as is shown in Table 2.

The results of the analysis of ten varieties of dried pepper grown in Korea analysed in quadruplicate are reported in Table 3. The published capsaicinoid contents in red pepper are generally higher than those found here. This may be caused by differences between varieties and/or isolated interfering substances extracted simultaneously with capsaicinoids. In most varieties, the content of capsaicin was higher than that of dihydrocapsaicin except for three samples and significantly higher contents were found in two varieties than in the others in the levels of total capsaicinoids.

The work reported in this paper describes an effective sample preparation and a new capillary-GC analysis that provides good resolution and quantitative analysis of the major heat principles in capsicum. No particular problems were encountered with the sample preparation and instrumentation for this method. Retention-time shifts were negligible throughout the study, and resolution values remained stable. In addition to improvements in accuracy and precision, this work demonstrated another advantage; the sample clean-up procedure may be adapted for analysis of capsaicinoids by HPLC. For this purpose, the final extraction solvent, dichloromethane, must be substituted with solvents such as ethanol.

Table 2. Correlation coefficient, relative retention time, and linear range of capsaicinoids*

Components	Correlation coefficient	Relative retention time	Linear range, μg
Squalane (IS)	0·998	1·000	0–140
Norihydrocapsaicin	not analyzed	1·442	not analyzed
Capsaicin	0·995	1·567	0–70
Dihydrocapsaicin	0·994	1·614	0–60

* Values are means of five determinations.

Table 3. The contents of capsaicinoids in dried red pepper

Capsaicinoids	Range, mg per 100 g
Capsaicin	41·7–72·5
Dihydrocapsaicin	27·5–58·3
Total	67·2–130·8

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