Direct Connection of Supercritical Fluid Extraction and Supercritical Fluid Chromatography as a Rapid Quantitative Method for Capsaicinoids in Placentas of *Capsicum*

Kyoko Sato,[†] Shiho Sakamoto Sasaki,[†] Yukihiro Goda,[†] Takashi Yamada,[†] Osamu Nunomura,[‡] Keiko Ishikawa,[§] and Tamio Maitani*[,†]

National Institute of Health Sciences, Kamiyoga 1-18-1, Setagaya, Tokyo 158-8501, Japan, Nihon Horticultural Production Institute, Kamishiki, Matsudo, Chiba 207-2221, Japan, and Faculty of Horticulture, Chiba University, Matsudo, Matsudo, Chiba 271-8510, Japan

The fruits of *Capsicum annuum* L. are used worldwide as chili peppers and in folk medicines. The pungent components of *C. annuum*, which are irritants, are called capsaicinoids (CAPS), and the most abundant components are capsaicin, dihydrocapsaicin, and nordihydrocapsaicin. To analyze CAPS in the placentas of *Capsicum* fruits rapidly and safely, we used a directly connected system of supercritical fluid extraction and supercritical fluid chromatography (SFE/SFC). As a column for SFE/SFC, only a silica-type column was found to be suitable. The CAPS contents in placentas of *C. annuum* cv. Jalapeno (hot type) and *C. annuum* cv. Shishitoh (less-hot type) determined by the SFE/SFC method agreed well with those in the range of 0−13.81 mg g^{-1} fr. wt determined by the usual extraction-HPLC method. The SFE/SFC method has the advantages of no need for pretreatment and no (or minimal) need for organic solvents. We conclude that this method is useful as a rapid (20 min) and safe screening test for the pungency of various *Capsicum* fruits.

Keywords: *Capsicum; capsaicinoid; supercritical fluid extraction (SFE); supercritical fluid chromatography (SFC); SFE/SFC*

INTRODUCTION

The fruits of varieties of *Capsicum* (family Solanaceae) vary widely in size, shape, flavor, color, and pungency. *Capsicum annuum* L. has been used worldwide as chili peppers, vegetables, in folk medicines, and also as a source of food additives. The pungent components in the fruits of *Capsicum* plants are called capsaicinoids (CAPS), and their structures are the vanillylamides of branched fatty acids, with 9−11 carbons. The most abundant components are capsaicin, dihydrocapsaicin, and nordihydrocapsaicin (Thomas et al., 1998).

CAPS are known to be biosynthesized and accumulated in the placentas of *Capsicum* fruits (Iwai et al., 1979; Fujiwake et al., 1982). However, it is not fully understood how the biosynthesis of CAPS is controlled. One of the impediments to this understanding may be the lack of a suitable rapid analytical method, because, for the study of the genetic control mechanisms of pungency, the analysis of CAPS in many fruits is essential. Although high-performance liquid chromatography (HPLC) is the usual analytical method, time-consuming pretreatments are required for it. In addition, since CAPS are irritants, the pretreatment must be performed cautiously. A rapid and safe method for estimating CAPS in fruits is thus desirable.

The direct connection of supercritical fluid extraction and supercritical fluid chromatography (SFE/SFC) has received much attention recently. This method has the advantages of not requiring a concentrating procedure or a cleanup procedure before analysis. Moreover, the critical temperature (31.1 °C) of carbon dioxide (CO_2), the most widely used supercritical fluid, is ideal for the extraction of thermally labile compounds. CO_2 is nontoxic, nonflammable, and environmentally preferable to organic solvents.

Many researchers have reported applications of SFE/SFC for various compounds from different matrixes, including caffeine extraction, tocopherol enrichment, and the analysis of pesticide residues (Sugiyama et al., 1985; Ryan et al., 1990; Daimon and Hirata, 1991 and 1994; Saito et al., 1989; Ong et al., 1991; Nam and King, 1994). However, there have been no reports of the application of SFE/SFC to CAPS, although several groups reported that CAPS were extracted or chromatographed by using supercritical CO_2. Namely, extraction efficiency for CAPS was compared between SFE and usual organic solvent extraction (Yao et al., 1994; Yasumoto et al., 1994; Peusch et al., 1997), and SFC was applied for capsicum as one of the foods tested (Knowles et al., 1988).

In the present study, we applied SFE/SFC to analyze CAPS in the placentas of *Capsicum* plants rapidly and safely. Fruits of *C. annuum* cv. Jalapeno and *C. annuum* cv. Shishitoh were used, because the former is a representative hot-type *Capsicum* and the latter is a representative less-hot type and is a typical cultivar in Japan.

MATERIALS AND METHODS

Plant Materials. The mature fruits of *C. annuum* cv. Jalapeno and *C. annuum* cv. Shishitoh were collected from

* To whom correspondence should be addressed (fax +81-3-3700-9403; e-mail maitani@nihs.go.jp).
[†] National Institute of Health Sciences.
[‡] Nihon Horticultural Production Institute.
[§] Chiba University.

10.1021/jf990488r CCC: $18.00
Published on Web 10/14/1999

pepper plants grown in a greenhouse of the Nihon Horticultural Production Institute (Matsudo).

Chemicals. CO_2 of over 99.95% purity (Nihon Sanso, Tokyo) was used for supercritical fluid. HPLC analysis-grade acetonitrile was purchased from Katayama Chemical Industries (Tokyo). Other chemicals were of reagent grade. Authentic samples of capsaicin and dihydrocapsaicin were purchased from Wako Pure Chemical Industries Ltd. (Osaka).

Sample Preparation. Placentas were cut out at sites one-half and one-third of the total fruit length relative to the top. Thus-removed placentas were separated into two parts by cutting lengthwise and used for the SFE/SFC analysis and HPLC analysis.

Column Selection. The columns tested were as follows: Superpak SIL separation column (silica gel, 250 × 4.6 mm i.d., JASCO, Tokyo), TSKgel Silica-60 (silica gel, 150 × 4.6 mm i.d., Tosoh, Tokyo), Superpak SIL C1 separation column (C1, 150 × 4.6 mm i.d., JASCO), Superpak Crest C18 separation column (ODS, 150 × 4.6 mm i.d., JASCO), or TSKgel ODS-80Ts (ODS, 250 × 4.6 mm i.d., Tosoh).

SFE/SFC Analysis. A placenta sample (2−80 mg) was put in the extraction vessel (1 mL) of an SFE/SFC system (Super-200 System 3, JASCO) with a 0.1-mL aliquot of methanol. The placenta was extracted with supercritical CO_2 for 5 min at a flow rate of 5 mL min^{-1} at 40 °C under a pressure of 200 kg cm^{-2}, and the extract was adsorbed to a trap column (Superpak SIL-TP trap column (silica gel, 50 × 4.6 mm i.d., JASCO)) under the same pressure. By switching the injector valve, the adsorbed compounds were transferred to the separation column (Superpak SIL separation column) with the supercritical CO_2 as the solvent (5 mL min^{-1}) and ethanol as a modifier solvent (0.3 mL min^{-1}), and the compounds were chromatographed. The temperatures of the extraction vessel and the columns were kept at 40 °C throughout the experiment. Detection (200−400 nm) was performed with a photodiode-array detector (MD-910, JASCO).

CAPS content was calculated as capsaicin from the peak area detected at 280 nm. The calibration curve was linear (R^2 = 0.999) from 0.5 to 200 μg in placenta samples.

Recovery Test for SFE/SFC. A 0.1-mL aliquot of the standard solution (0.5 mg capsaicin mL^{-1} in methanol) was added to 10 mg of placenta of bell peppers (*C. annuum* cv. Plutona), which had been confirmed beforehand to contain no detectable CAPS.

Solvent Extraction for HPLC. One-milliliter of acetone was added to the other half of the placenta sample for HPLC (4−100 mg). The placenta was mashed and then centrifuged at 3000 rpm for 5 min. The supernatant fraction was filtered through a 0.5-μm filter cartridge and used as the test solution for HPLC analysis.

HPLC Analysis. The HPLC systems used were a Tosoh SC-8010 system (Tosoh) with a Tosoh UV-8010 detector and a Shimadzu LC-10A system (Shimadzu, Kyoto) with a Shimadzu SPD-10AV photodiode-array detector. Ten-microliter aliquots of test solution was applied to the HPLC system equipped with an Inertsil ODS-2 column (150 × 4.6 mm i.d., GL Sciences, Tokyo), and the column was eluted with a mixture of acetonitrile/water/trifluoroacetic acid (500/500/1) at a flow rate of 1.0 mL min^{-1}. CAPS content was calculated from the peak area detected at 280 nm. The standard curve was linear (R^2 = 1.000) from 0.005 to 2 μg of capsaicin in 10-μL samples (from 0.5 to 200 μg in placenta samples).

Identification Methods for the Unknown Peak. The CAPS-containing fractions obtained from several SFE/SFC experiments were pooled and then applied to the HPLC system under the conditions described above, and the fraction containing an unknown peak was collected. About 0.5 mg of unknown peak compound was thereby obtained. This compound was analyzed by liquid chromatography−mass spectrometry (LC/MS) (QP1100 EX, Shimadzu) equipped with an ODS column (CHEMCOBOND5-ODS−HI (150 × 4.6 mm i.d., Shimadzu)). The mobile phase was a mixture of acetonitrile/0.1 M ammonium acetate (40:60, v/v). Ionization was performed by thermospray method.

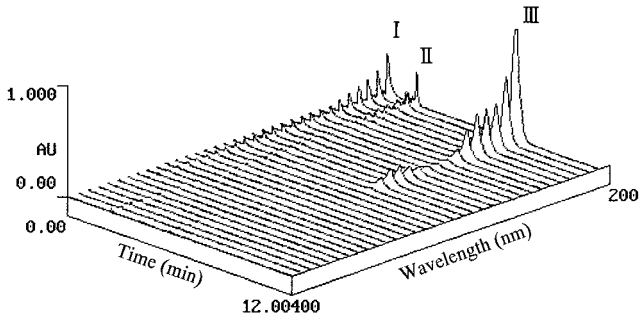

Figure 1. Three-dimensional SFE/SFC chromatogram of placenta of *C. annuum* cv. Jalapeno detected with photodiode-array detector at 200−400 nm.

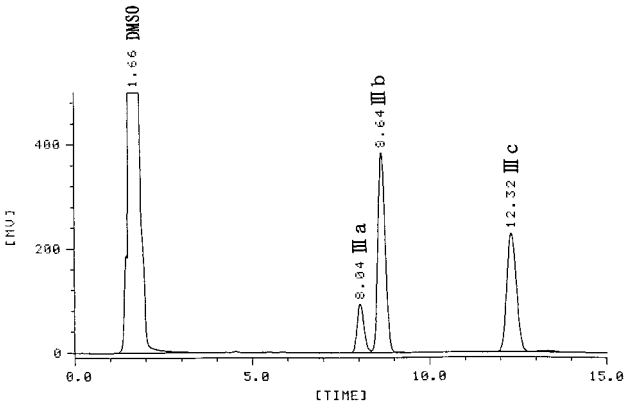

Figure 2. HPLC chromatogram of fraction III extracted and fractionated by the SFE/SFC method from placenta of *C. annuum* cv. Jalapeno.

RESULTS

Column Selection. Five columns of three types, i.e., the silica, C1, and ODS types, were tested for use in the SFE/SFC study. The C1 and ODS columns did not retain the applied CAPS in the SFC experiment. The silica-type column retained CAPS and showed adequate retention times when ethanol was added as a modifier solvent. Therefore, we selected a silica-type column to analyze CAPS, although the column showed no separation of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin under any conditions tested here.

SFE/SFC Analysis of CAPS in the Placenta of *C. annuum*. Figure 1 shows a three-dimensional SFE/SFC chromatogram of the placenta of Jalapeno extracted and chromatographed by SFE/SFC. Three peaks were detected. The UV spectrum of peak III (eluted at 7.9 min) obtained with the photodiode-array detector matched that of authentic CAPS (capsaicin and dihydrocapsaicin), and peak III was therefore considered to be CAPS. Peaks I and II remain unidentified. We obtained almost the same chromatogram from Shishitoh (data not shown).

Identification of Peak III Compounds. To identify the constituents of peak III, it was fractionated and reanalyzed by HPLC. The HPLC chromatogram showed three peaks, at 8.0 (peak IIIa), 8.6 (peak IIIb), and 12.3 min (peak IIIc) (Figure 2). Peaks IIIb and IIIc were assigned to capsaicin and dihydrocapsaicin, respectively, by comparing the retention times and the UV spectra with those of the authentic samples. Although the UV spectrum of peak IIIa was similar to the spectra of capsaicin and dihydrocapsaicin and peak IIIa thus also seemed to be a CAPS homologue, it could not be

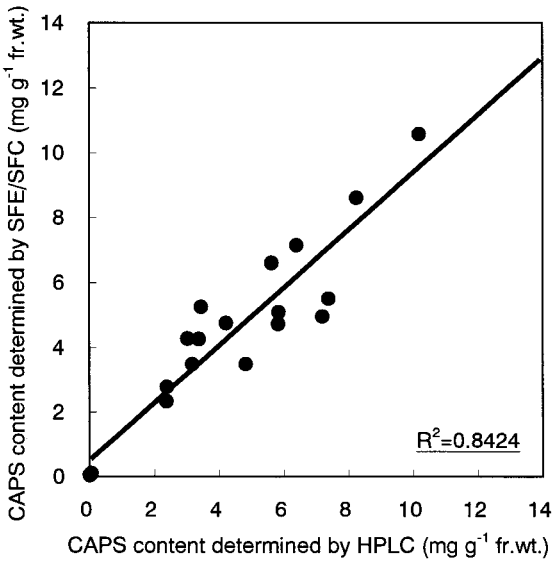

Figure 3. Correlation of CAPS contents in *C. annuum* cv. Jalapeno as determined by SFE/SFC and HPLC.

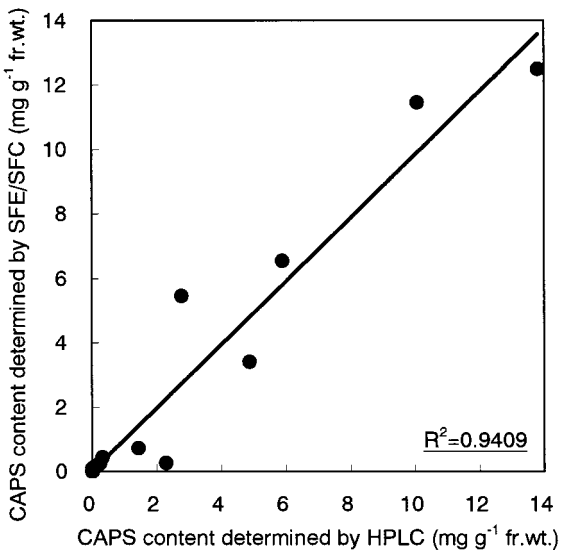

Figure 4. Correlation of CAPS contents in *C. annuum* cv. Shishitoh as determined by SFE/SFC and HPLC.

identified at this stage, because we could not obtain any other authentic samples of CAPS homologues.

To identify peak IIIa, the fraction was collected from SFE/SFC and HPLC experiments several times, pooled, and analyzed by LC-MS. The LC-MS spectrum displayed a pseudo molecular ion peak at *m/z* 294, and the mass chromatogram at *m/z* 294 showed one major peak (data not shown). There are three already known capsaicinoids which exhibit *m/z* 294, namely, nordihydrocapsaicin, pseudocapsaicin, and nordihydrocapsaicin II. However, it was reported that principal capsaicinoids in *C. annuum* are capsaicin, dihydrocapsaicin, and nordihydrocapsaicin (Thomas et al., 1998). Therefore, it was considered that the unknown peak was nordihydrocapsaicin. Moreover, the retention time was in accord with the data of Attuquayefio and Buckle (1987). From these results, peak IIIa was identified as nordihydrocapsaicin. Thus, the peak observed at 7.9 min in the SFE/SFC chromatogram contained three compounds, namely, capsaicin, dihydrocapsaicin, and nordihydrocapsaicin.

Recovery and Detection Limit of Capsaicin in SFE/SFC. To determine the recovery rate of capsaicin, individual placentas (10 mg) of not-pungent bell pepper were spiked with 50 μg of capsaicin, and the capsaicin content was analyzed by SFE/SFC. The recovery rate and CV were 92.1% and 7.9% ($n = 5$), respectively, and the detection limit of capsaicin was 0.5 μg under the experimental conditions.

Correlation of Data Obtained by SFE/SFC and HPLC. The CAPS contents determined by SFE/SFC and HPLC were compared. Jalapeno (18 fruits) and Shishitoh (25 fruits) were used as hot and less-hot *Capsicum* fruits, respectively, to analyze a wide range of CAPS concentrations.

In the SFE/SFC analysis, CAPS homologues were detected as only one peak. Therefore, the CAPS content was calculated based on the peak area of standard capsaicin. In HPLC, the sum of the contents of capsaicin, dihydrocapsaicin, and nordihydrocapsaicin was used as the CAPS content. The calibration curve for capsaicin was also used for dihydrocapsaicin and nordihydrocapsaicin.

Figure 3 shows the relationships of the CAPS contents of the placentas of Jalapeno obtained by the SFE/SFC and HPLC methods. The CAPS contents determined by the HPLC method ranged from 0.01 to 10.18 mg g^{-1} fr. wt. The contents determined by the SFE/SFC method were well correlated with those determined by the HPLC method ($R^2 = 0.8424$), and the slope was approximately 1 (0.8838).

In Shishitoh, the CAPS contents determined by the HPLC method ranged from 0 to 13.81 mg g^{-1} fr. wt. and were well correlated with the CAPS contents determined by the SFE/SFC method ($R^2 = 0.9409$), and the slope was very close to 1 (0.9834) (Figure 4).

DISCUSSION

Although only the silica-type column could retain CAPS among the three types of columns in the SFE/SFC study, it could not separate capsaicin, dihydrocapsaicin, and nordihydrocapsaicin under any conditions tested. The structural differences of these homologues are very small; i.e., only a small difference in the numbers of carbon atoms in the branched fatty acids and/or the presence or absence of a double bond in the branched fatty acids. It may therefore be difficult to separate such similar compounds by SFC.

Knowles et al. (1988) reported that CAPS could be analyzed using a capillary SFC column of the dimethylpolysiloxane type. In that study, some peaks corresponding to CAPS homologues were detected, although the separation was not very good. Since capillary columns could not be used in our system, we were not able to separate capsaicin, dihydrocapsaicin, and nordihydrocapsaicin. However, it seems advantageous for our purposes to detect all CAPS homologues as one peak, because our objective is to quantify the total CAPS content rapidly and safely. Since capsaicin and dihydrocapsaicin are two principal constituents (Thomas et al., 1998) and their hotness is almost equal (Todd, 1977), the CAPS content estimated from the single peak seems a good index for the pungency of *Capsicum*.

Since CAPS are mucous membrane irritants, the SFE/SFC method, which involved fewer manual operations than the extraction-HPLC method, seemed preferable from the experimental standpoint. Moreover, since the SFE/SFC method used in this study needed only about 20 min for one run, the method was less time consuming

than the usual extraction (pretreatment)-HPLC method. Therefore, it is concluded that the SFE/SFC is a rapid and safe method.

The HPLC data showed that the average CAPS content in the placentas of Jalapeno (4.65 mg g^{-1} fr. wt.) was higher than that in the placentas of Shishitoh (1.68 mg g^{-1} fr. wt.). Since Jalapeno is a hot-type fruit, it is reasonable that Jalapeno fruits should contain more CAPS than Shishitoh fruits. However, 4 out of 25 fruits of Shishitoh, which is a typical less-hot type, showed CAPS contents higher than the average content of Jalapeno fruits. This may be due, at least in part, to measurement only a small part of the placenta. Measuring larger portions of the peppers might help decrease some of the variability.

The present report is the first to describe the application of SFE/SFC for the quantitation of CAPS contents in *Capsicum* fruits. This new method seems likely to prove very useful as a rapid screening test for the pungency of various *Capsicum* fruits.

ACKNOWLEDGMENT

We thank Ms. H. Ise for technical assistance at the initial stage of this study.

LITERATURE CITED

Attuquayefio, V. K.; Buckle, K. A. Rapid sample preparation method for HPLC analysis of capsaicinoids in capsicum fruits and oleoresins. *J. Agric. Food Chem.* **1987**, *35*, 777−779.

Daimon, H.; Hirata, Y. Directly coupled supercritical-fluid extraction/capillary supercritical-fluid chromatography of polymer additives. *Chromatographia* **1991**, *32* (11/12), 549−554.

Daimon, H.; Hirata, Y. Direct coupling of capillary supercritical fluid chromatography with supercritical fluid extraction using modified carbon dioxide. *J. High Resolut. Chromatogr.* **1994**, *17*, 809−813.

Fujiwake, H.; Suzuki, T.; Iwai, K. Capsaicinoid formation in the protoplast from the placenta of *Capsicum* fruits. *Agric. Biol. Chem.* **1982**, *46*, 2591−2592.

Iwai, K.; Suzuki, T.; Fujiwake, H. Formation and accumulation of pungent principle of hot pepper fruits, capsaicin and its analogues, in *Capsicum annuum* var. *annuum* cv. Karayatsubusa at different growth stages after flowering. *Agric. Biol. Chem.* **1979**, *43*, 2493−2498.

Knowles, D. E.; Richter B. E.; Wygant, M. B.; Nixon, L.; Andersen, M. R. Supercritical fluid chromatography: A new technique for AOAC. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 451−457.

Nam, K.-S.; King, J. W. Coupled SFE/SFC/GC for the trace analysis of pesticide residues in fatty food samples. *J. High Resolut. Chromatogr.* **1994**, *17*, 577−582.

Ong, C. P.; Lee, H. K.; Li, S. F. Y. Direct coupling of supercritical fluid extraction-supercritical fluid chromatography for the determination of selected polycyclic aromatic hydrocarbons in aqueous environmental samples. *Environ. Monit. Assess.* **1991**, *19*, 63−71.

Peusch, M.; Müller-Seitz, E.; Petz, M.; Müller, A.; Anklam, E. Extraction of capsaicinoids from chillies (*Capsicum frutescens* L.) and paprika (*Capsicum annuum* L.) using supercritical fluids and organic solvents. *Z. Lebensm. Unters. Forsch. A* **1997**, *204*, 351−355.

Ryan, T. W.; Yocklovich, S. G.; Watkins, J. C.; Levy, E. J. Quantitative analysis of additives in polymers using coupled supercritical fluid extraction-supercritical fluid chromatography. *J. Chromatogr.* **1990**, *505*, 273−282.

Saito, M.; Yamauchi, Y.; Inomata, K.; Kottkamp, W. Enrichment of tocopherols in wheat germ by directly coupled supercritical fluid extraction with semipreparative supercritical fluid chromatography. *J. Chromatogr. Sci.* **1989**, *27*, 79−85.

Sugiyama, K.; Saito, M.; Hondo, T.; Senda, M. New double-stage separation analysis method. Directly coupled laboratory-scale supercritical fluid extraction-supercritical fluid chromatography, monitored with a multiwavelength ultraviolet detector. *J. Chromatogr.* **1985**, *332*, 107−116.

Thomas, B. V.; Schreiber, A. A.; Weisskopf, C. P. Simple method for quantitation of capsaicinoids in peppers using capillary gas chromatography. *J. Agric. Food Chem.* **1998**, *46*, 2655−2663.

Todd, P. H. Determination of pungency due to capsicum by gas−liquid chromatography. *J. Food Sci.* **1977**, *42*, 660−665.

Yao, J.; Nair, M. G.; Chandra, A. Supercritical carbon dioxide extraction of Scotch Bonnet (*Capsicum annuum*) and quantification of capsaicin and dihydrocapsaicin. *J. Agric. Food Chem.* **1994**, *42*, 1303−1305.

Yasumoto, M.; Inakuma, T.; Okazaki, T.; Kobayashi, T. Extraction of capsaicin from red pepper with liquid carbon dioxide. *Nippon Shokuhin Kogyo Gakkaishi* **1994**, *41*, 565−567.

Received for review May 10, 1999. Revised manuscript received August 16, 1999. Accepted September 2, 1999. This study was supported in part by a grant from the Japan Health Sciences Foundation.

JF990488R