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RESEARCH NOTE

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Comparison of Extraction Procedures for the Determination of Capsaicinoids in Peppers

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Abstract The objective of this study was to compare 3 extraction methods including, solid phase extraction (SPE), acetonitrile extraction, and methanol extraction, for their usefulness as extraction methods to determine capsaicinoids. The determination of capsaicinoids in the extracts was carried out on a reverse-phased high performance liquid chromatography (HPLC) using a fluorescence detector. Three extraction methods, i.e., SPE, acetonitrile extraction, and methanol extraction were compared for the quantification of capsaicinoids using raw peppers and pepper powder. The highest analytical values were observed using methanol extraction and the lowest values using SPE. Also, the analytical method validation parameters such as accuracy, precision, limit of detection, limit of quantitation, and specificity were calculated to ensure the method's validity. This method provides a fast and accurate approach for the determination of capsaicinoids in peppers.

Keywords: capsaicinoid, pepper, extraction method, high performance liquid chromatography (HPLC)

Introduction

Hot pepper is widely cultivated and used all over the world as natural flavoring and coloring agent. The consumption of hot peppers is due, mainly, to their pungent flavor. The pungency of pepper is directly related to the content of capsaicinoids. There are several naturally occurring capsaicinoids such as capsaicin, nordihydrocapsaicin, dihydrocapsaicin, homocapsaicin, and homodihydrocapsaicin (1,2). Capsaicin and dihydrocapsaicin are the main pungent components and constitute more than 80% of the total capsaicinoids (3).

A large number of analytical methods have been published to measure capsaicinoids in peppers and pepper products. They include high performance liquid chromatography (HPLC) (4-6), spectrophotometic method (7,8), gas chromatography (GC) (9), enzyme immunoassay (10), and micellar electrokinetic capillary chromatography (11). At the present time, the method of choice of capsaicinoids quantification is HPLC because it is simpler, more precise, and more efficient than other methods and it has the ability to separate capsaicinoid homologs. Depending on the nature of the sample, various extraction methods, including acetonitrile extraction, methanol extraction, and solid phase extraction (SPE) (12,13), have been used to release capsaicinoids. However, it is still necessary to compare the extraction methods to ensure the complete extraction of capsaicinoids from sample matrices.

Therefore, the effectiveness of 3 extraction methods including SPE, acetonitrile extraction, and methanol extraction was evaluated to determine capsaicinoids from peppers using HPLC. In addition, analytical method validation parameters including accuracy, precision, linearity, and specificity were calculated to ensure the validity of the most effective of the tested procedures.

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Materials and Methods

Samples and chemicals Capsaicin and dihydrocapsaicin were obtained from Fluka (St. Louis, MO, USA). Stock solution of capsaicin and dihydrocapsaicin were prepared in methanol, stored in dark at –20°C, and diluted to the desired concentration with methanol to make a daily working standard. All other reagents and solvents used were of analytical and HPLC grade. Raw peppers and pepper powder used to compare extraction methods were purchased from local retail stores. Peppers of 11 different cultivars were obtained from Chungbuk Eumsung Agricultural Research and Extension Service, Korea.

Sample preparation Raw peppers used in the comparison of extraction methods were washed with tap water after manually removing inedible parts. Peppers were dried on a basket, cut into small pieces, and ground in a grinder. Eleven different pepper cultivars from Chungbuk Eumsung Agricultural Research and Extension Service were treated as described above. These peppers were freeze-dried and then used to determine capsaicinoid contents. The capsaicinoid contents of the raw peppers were expressed as wet weight basis and those of the freeze-dried pepper cultivars expressed as dry weight basis.

SPE extraction Pepper powder (1.0 g) and raw pepper (10 g) were homogenized with 25 mL of acetonitrile using a Ultra Turrax® homogenizer (T25; Janke&Kunkel IKA Labortechnik, Staufen, Germany) for 2 min. The homogenized mixture was filtered through a Whatman filter paper (Whatman No. 2, Whatman International Ltd., Maidstone, UK) and the filtrate was quantitatively transferred to a 100-mL volumetric flask and diluted to volume with acetonitrile. A 1.0 mL aliquot of the acetonitrile extract was taken for an SPE cleanup procedure. A C_{18} Sep-pak was conditioned with about 5 mL of acetonitrile followed by 5 mL of water.

The aliquot was loaded into the conditioned Sep-Pak (Vac 3 cc, 500 mg, Waters, Tauton, MA, USA) column. The capsaicinoids in the column are then eluted with 4 mL of acetonitrile followed by 1.0 mL of acetonitrile containing 1%(v/v) glacial acetic acid. The eluate was filtered through a 0.45-μm nylon membrane filter (MSI Inc., Westboro, MA, USA) and injected onto the HPLC system.

Acetonitrile and methanol extraction Pepper powder (1.0 g) or 10 g of raw pepper is blended with 25 mL of acetonitrile or methanol for 2 min. The homogenized mixture was filtered through a Whatman filter paper (Whatman No. 2, Whatman International Ltd.) and the filterate was transferred to a 100-mL volumetric flask, diluted to volume with the acetonitrile (methanol). The extraction was filtered through a 0.45-μm nylon membrane filter (MSI Inc.) and injected onto the HPLC system. All solvent were HPLC grade.

HPLC quantification The reverse-phased HPLC system consisted of a solvent delivery pump (PU-2089; Jasco Corporation, Tokyo, Japan) equipped with a spectro-fluorometric detector (FP-2020; Jasco Corporation) and a Luna 5 μ C18(2) 100 A column (5 μm, 250×4.6 mm, Phenomenex Inc., Torrance, CA, USA). The column temperature was maintained with a column oven (CO-2060; Jasco Corporation). The isocratic mobile phase consisted of acetonitrile-water-glacial acetic acid in ratio of 60:39:1 (v/v/v), and the flow rate was 1.0 mL/min. The wavelengths were set at 280 nm for excitation and 320 nm for emission for the identification and quantification of capsaicin and dihydrocapsaicin. Capsaicin and dihydrocapsaicin peaks were identified by comparing their retention times to those of standards. Concentrations were calculated from peak areas determined by Jasco ChromNav Chromatography Data System (Jasco Corporation).

Method validation and statistical analysis The extraction methods were validated by determining the accuracy (recovery), precision (repeatability and reproducibility), linearity, and specificity (peak purity). One-way analysis of variance (ANOVA) was performed with SAS version 9.1 (SAS Institute, Cary, NC, USA). Means were compared using Duncan tests with a significance level of 0.05.

Results and Discussion

Comparison of extraction methods Three extraction methods including SPE, acetonitrile extraction, and methanol extraction, were compared for the determination of capsaicin and dihydrocapsaicin using pepper powder and raw pepper (Table 1). There were significantly differences between the 3 methods for the determination of capsaicinoids ($p<0.05$). The highest values of capsaicinoids were observed from the methanol extraction and the lowest values from SPE. The capsaicin values in pepper powder obtained by methanol extraction were higher than the values from SPE and acetonitrile extraction by 48.3 and 31.4%, respectively. Although SPE could reduce interference and improve resolution of the capsaicinoid peaks, it would be complicated and time-consuming. Moreover, the capsaicinoid values from SPE were the lowest among the 3 extraction methods. According to our results, the methanol extraction method was proven to be the best method for the determination of capsaicinoids in pepper; therefore, it was chosen for further application in this study.

Method validation Using the methanol extraction method and reverse phased liquid chromatography, analytical method validation parameters such as accuracy, precision, limit of detection, limit of quantitation, and specificity were calculated to prove the validity of the complete procedure for capsaicinoids analysis of pepper powder. A precision study was performed on the pepper powder and the results are given in Table 2. The repeatability and reproducibility (% of coefficient of variation, CV) was usually less than or about 2%. The accuracy was evaluated by analyzing 5 samples of the pepper powder to which known concentrations of each capsaicinoid were added prior to extraction. Recovery data based on 5 trials for capsaicinoids are shown in Table 2. The % mean recovery ±standard deviation (SD) 99.90±0.01 and 100.67±0.03 for capsaicin and dihydrocapsaicin, respectively.

The limit of detection (LOD) and limit of quantitation (LOQ) were determined based on the detector's signal-to-noise (S/N) ratio. The standard deviation of the S/N ratio was calculated and multiplied by factor of 3, then this value was added to the average of S/N ratio to obtain the LOD. For LOQ, 10 was chosen as a factor (14). The LODs in ng/20 μL were 0.32 and 0.23 and LOQs in ng/20 μL were 0.35 and 0.25 for capsaicin and dihydrocapsaicin, respectively. Peak purity was determined using pepper powder by the procedure described by Haroon *et al.* (15) for fluorescence response. Peak heights of capsaicinoids were determined at excitation wavelengths of 270, 280, and 290 nm, while keeping the emission wavelength constant at 320 nm. Then, the ratio of the peak heights from samples was compared with the peak ratio of

Table 1. Concentrations of capsaicinoids in pepper powder and raw peppers measured using 3 different assay methods

Sample	Capsaicinoid	Extraction method[1)]		
		SPE	ACN extraction	MeOH extraction
Peppers, hot chili, powder	Capsaicin	21.83±1.07[a2)]	24.64±0.09[b]	32.37±0.41[c]
	Dihydrocapsaicin	10.41±0.74[a]	14.50±0.02[b]	17.32±0.48[c]
Peppers, hot chili, raw	Capsaicin	20.91±2.08[a]	23.76±0.93[b]	32.07±0.43[c]
	Dihydrocapsaicin	5.31±0.42[a]	6.48±0.23[b]	8.99±0.09[c]

[1)]SPE, solid phase extraction; ACN, acetonitrile; MeOH, methanol. Values that are followed by the different letters are significantly different within rows ($p<0.05$).
[2)]Concentration of capsaicinoids expressed as mg/100 g sample.

Table 2. Precision and accuracy of the methanol extraction method

	Parameters[1)]	Precision		Accuracy[2)]
		Repeatability[3)]	Reproducibility[4)]	Recovery (%)
Capsaicin	Mean	32.75	32.34	99.90
	SD	0.38	0.68	0.70
	CV%	1.15	2.09	0.70
Dihydrocapsaicin	Mean	17.21	17.18	100.67
	SD	0.20	0.32	0.87
	CV%	1.16	1.86	0.86

[1)]Mean, n=5 (mg/100 g); SD, standard deviation; CV%, coefficient of variation.
[2)]A measure of the closeness of the analytical result to the value evaluated by analyzing a spike sample.
[3)]Refers to the results of independent determinations carried out on a sample by analyzing 5 replicates of the sample on the same day.
[4)]Refers to the results independent determinations carried out a sample by analyzing 5 replicates of the sample at different periods of time.

Table 3. Evaluation of peak purity by fluorescence ratio using pepper powder

Ratio[1)]	Capsaicin		Dihydrocapsaicin	
	Sample	Standard	Sample	Standard
280/290 nm	2.06	2.05	1.98	2.10
270/290 nm	1.55	1.56	1.48	1.59
280/270 nm	1.33	1.32	1.34	1.32

[1)]Fluorescence ratios shown were calculated by dividing the values for the 2 peak heights for each analyte (capsaicin and dihydrocapsaicin) obtained from separate chromatographic runs at 2 different excitation wavelengths, with the emission wavelength constant at 320 nm.

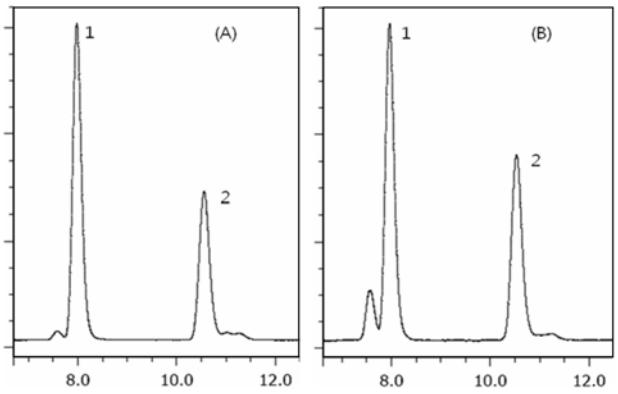

Fig. 1. Reverse-phased LC chromatograms of capsaicinoids using fluorescence detection. Standard (A) and pepper extract sample (B) where 1 is capsaicin and 2 is dihydrocapsaicin ($E_x\lambda$= 290 nm, $E_m\lambda$=320 nm).

standard obtained at the same wavelengths (Table 3). Very similar values were obtained from samples using the methanol extraction method and standards for capsaicin and dihydrocapsaicin, indicating the purity of the peaks.

Determination of capsaicinoids As mentioned earlier, the methanol extraction method was used to quantify the capsaicin and dihydrocapsaicin in samples. The chromatograms of capsaicinoid standard and pepper extract were shown in Fig. 1. The linearity test for quantitation was carried out over the range of 0.0-312.0 and 0.0-168.0 ng/20 μL injected for capsaicin and dihydrocapsaicin, respectively. Regression analysis showed an excellent linear relationship (R^2=0.999). These values suggest that quantitation of the capsaicinoids by HPLC is highly reproducible.

Using the methanol extraction method, the analytical results were presented in Table 4. The amount of capsaicin and dihydrocapsaicin varied in different cultivars ranged from 0.8 to 279.8 and 0.3 to 72.0 mg/100 g sample on dry weight basis, respectively. Among the 11 samples, the highest capsaicinoid level has been detected in *Capsicum annuum* cv. Cheongyang and the lowest in *C. annuum* cv. Sonatabigarim. *C. annuum* cv. Chungyang is very pungent and has been used for soup and preparing pungent foods (16). Our results are consistent with those of Park *et al.* (17) who reported the capsaicinoid contents of *C. annuum* cv. Chungyang within the range of 250-350 mg/100 g.

In this study, three different extraction methods were compared and the methanol extraction method proved to be the best method for the quantifying capsaicinoids in pepper samples. Using the methanol extraction method, the higher analytical values of capsaicinoids were obtained compared to those from other methods. This method provides a fast, accurate approach for the determination of capsaicinoids in peppers.

Table 4. Concentration of capsaicinoids in pepper cultivars

Sample	Capsaicinoids[1)]	
	Capsaicin	Dihydrocapsaicin
Buchon	11.66[2)]	4.74
Hongboseok	63.99	26.03
Joara	56.58	22.85
PR-Manitta	86.44	31.13
Wangdaebak	122.22	43.78
Superbigarim	8.29	5.26
Sonatabigarim	0.80	0.29
Myeongak	21.69	15.61
Manitta	91.52	39.39
Cheongyang	278.81	72.01
Nokkwang	32.03	16.61

[1)]Concentration expressed as mg/100 g sample as dry weight basis.
[2)]All samples were assayed in duplicate.

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