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Original Article

Rapid determination of capsaicinoids by colorimetric method

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ABSTRACT

Capsaicinoids, the pungent component of chili peppers, are generally analyzed by precise analytical techniques, such as gas chromatography and high-performance liquid chromatography (HPLC), but these are not practical for the mass analyses of samples. To analyze mass samples rapidly, a colorimetric method was suggested. In this work, pigments and capsaicinoids were efficiently separated from chili pepper extract by sequential solid–liquid extraction and liquid–liquid extraction in test tubes followed by a colorimetric analysis on the capsaicinoids by a selective chromogenic reaction with Gibbs reagent (2,6-dichloroquinone-4-chloroimide). In the comparison of the capsaicinoid content by the colorimetric method and HPLC using acetone extracts of fresh pepper and dry red pepper as samples, R^2 was 0.9973 and 0.9816, respectively, which shows a high linear correlation. In addition, a minimum of 1 μg/mL capsaicinoids can be detected and it was therefore determined that the method can efficiently analyze a great quantity of samples in a short time.

1. Introduction

The secondary metabolites retaining the pungency of chili peppers, called capsaicinoids, are alkaloids composed of vanillylamide and an acyl chain, which are classified by the acyl chain structure into a capsaicin group, dihydrocapsaicin group, and *N*-vanillyl-*n*-acrylamide group [1]. The biocomponents of capsaicinoid derivatives from chili peppers are affected by various factors such as varieties of chili pepper, cultivation conditions, level of aging, and processing methods [2–5]. Among capsaicinoid derivatives, capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide) account for 80–90% of the capsaicinoids in chili peppers and are therefore the main determinants of pungency [6–10].

The annual global output of chili peppers was approximately 34.5 million tons in 2012 [11] and this is cultivated as a spice crop. Chili peppers are used in natural pigments and drug substances, and have a high economic value. Thus, rapid and simple quantification of the capsaicinoid content is very

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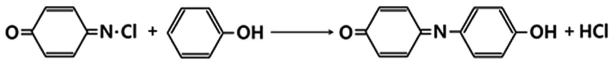
important for breeding a variety of chili peppers and quality control of processed products.

Various methods to measure the capsaicinoid content have been used. The pungency in chili pepper fruit or food is traditionally measured by the Scoville heat test [12], which gradually dilutes a sample to measure pungency. It quantifies the heat level and is a useful scale to indicate pungency. However, this method is dependent on human senses and requires trained personnel. It can be difficult to measure the pungency of food, such as kimchi groups, that utilizes a lot of spices. Instrumental analytical methods to quantify pungency include high-performance liquid chromatography (HPLC) [13–16], gas chromatography [17–19], and UV spectrophotometry [20]. The aforementioned chromatographic analysis can measure the amount of capsaicinoid derivatives and total capsaicinoids. However, it requires a lot of time, money, effort, and equipment so it is inefficient to apply to the breeding of chili peppers or to the quality control of processed products where a large quantity of samples needs to be analyzed quickly. Meanwhile, the Scoville heat unit (SHU) uses a universal unit indicating pungency, and therefore the amount of capsaicinoids analyzed by instrumental analysis is sometimes converted to SHU [21].

2,6-Dichloroquinone-4-chloroimide (DCQ) forms color by reaction with phenols and can be used to detect capsaicinoids. However, pigments contained in chili pepper include capsanthin, carotenoids, chlorophyll, etc., [22,23] and pepper extracts



cannot be directly quantified by the colorimetric method. To overcome these disadvantages, colorimetry has been suggested for the evaluation of extracted capsaicinoids and pigments after separating them from a chili pepper extract by thin-layer chromatography (TLC) or paper chromatography [24,25].

Capsaicinoids share a phenolic hydroxyl group in the molecular structure. A phenolic hydroxyl group forms phenolate ions in basic conditions, and forms salt with metal ions. In this process, changes in basicity of a solution affect relative solubilities in a solvent and so capsaicinoids can be selectively transferred from an organic solvent to an aqueous solution or from an aqueous solution to an organic solvent.

Liquid–liquid extraction is used to separate compounds based on the difference in relative solubilities between two solvents, such as a water-immiscible solvent and a water-miscible solvent, which are not mixed together. In this study, pigments and capsaicinoids were selectively separated from chili pepper extract in test tubes based on the principles of solid–liquid extraction and liquid–liquid extraction. The vanillyl group of capsaicinoids was reacted with DCQ for color formation to suggest a colorimetric method able to quantitatively measure total capsaicinoid content.

2. Materials and Methods

2.1. Materials

Capsaicin, dihydrocapsaicin, ethanol, DCQ, HPLC-grade acetonitrile, and HPLC-grade water were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Chili peppers (*Capsicum annuum*) were cultivated on a farm near Chuncheon, Korea. We obtained fresh pepper from the farm and dry red pepper was purchased on sale.

2.2. Extraction of capsaicinoids

Five-gram whole fresh peppers and dry red pepper were ground with a home blender for 3 minutes and then a fivefold volume of acetone was added, respectively, to the extract at 50°C for 1 hour in triplicate. Centrifuged supernatant was taken for HPLC and colorimetric analysis.

2.3. HPLC and TLC analysis

For HPLC analysis, the acetone extract was filtered with a 0.22-μm membrane filter and then directly injected into the HPLC system (GTS 30, Young Lin, Anyang-si, Republic of Korea) using YMC hydrosphere C_{18} S-5 (4.6 × 150 nm) as a column. The isocratic mobile phase was acetonitrile/1% acetic acid in water (40:60, v/v) with a flow rate of 1.0 mL/min. The absorbance was measured at 280 nm (see Supplementary Material online). For TLC, silica gel 60 F_{254} precoated plates (Merck, Darmstadt, Germany) were used with toluene/chloroform /acetone (55/26/19, v/v/v) in the solvent system. Capsaicinoids were detected by spraying 0.1% DCQ solution and placing the plate in a chamber saturated with ammonia vapor.

2.4. Experimental design

For direct colorimetric quantification of capsaicinoids from pepper extracts, these pigments act as interfering substances so it is important to separate the pigments and capsaicinoids. When pepper extracts are developed by TLC, there are several components affecting chromogenic reactions, besides pigments. To investigate the partition efficiency of capsaicinoids from an organic solvent layer to an alkali solution, 250 μg capsaicin was dissolved in 5 mL of dichloromethane (polarity 3.1), tetrachloromethane (polarity 1.6), and *n*-hexane (polarity 0.0), which are hydrophobic organic solvents with different polarities. Then, 10 mL of 0.05N NaOH was added to each capsaicin solution followed by intense vortexing. Finally, capsaicin transferred into a water layer was identified by TLC.

Because of liquid–liquid extraction between an *n*-hexane solution and NaOH solution, capsaicin is solubilized to be an NaOH solution for complete partitioning. Therefore, the *n*-hexane solution obtained through solid–liquid extraction was used for liquid–liquid extraction with an NaOH solution. A 4-mL *n*-hexane solution containing capsaicinoids and pigments was taken and then partitioned by 0.05N NaOH solution (10 mL).

2.5. Colorimetric quantification of capsaicinoids

Chromogenic substances by origin are water-soluble components with high polarity extracted with acetone and they affect the chromogenic reactions of capsaicinoids. To remove them, extracts in the test tube were completely dried and then *n*-hexane was added to selectively extract capsaicinoids and pigments from the remaining solids; 1 mL acetone extract of

chili pepper was transferred into a glass test tube and completely dried by nitrogen gas. Five milliliters *n*-hexane was added and the mixture was allowed to remain at room temperature for 10 minutes to dissolve the extract. Then, the 4-mL *n*-hexane layer was carefully taken to a new tube without any solid materials tagging along. To 4 mL of *n*-hexane solution, 10 mL of 0.05N NaOH was added and the mixture was intensely vortexed. The supernatant was removed. One milliliter from the NaOH layer left was taken and then mixed with 50 μL of 1N HCl, 50 μL of 0.1% DCQ, and 50 μL of 2.5% ammonia solution in order. The mixture was reacted at room temperature for 10 minutes and the absorbance was measured at 600 nm.

For the standard, 5 mg capsaicin was dissolved in 10 mL of methanol and 50 μL, 100 μL, 200 μL, and 400 μL of the solution were transferred into glass tubes with the methanol removed by nitrogen gas. Each standard was dissolved in 4 mL of hexane and then extracted with the NaOH solution for color development as mentioned earlier.

3. Results

3.1. *Selective solubilization of capsaicinoids*

In the results, the higher polarity solvent shows a lower transfer efficiency of capsaicin into a water layer. However, in case of hexane, most of the capsaicin was transferred into a water layer by a primary partition. The color formation resulting after partitioning with a 0.05N NaOH solution in capsaicin standards at each concentration dissolved in *n*-hexane is shown in Figure 1. R^2 was 0.9996. It was confirmed that the partition was proportional to the capsaicin concentration.

3.2. *Chromogenic reaction of capsaicinoids from chili pepper extract*

Acetone extracts of fresh pepper and dry red pepper have a unique color. Unique pigments of chili pepper are not limited to acetone extracts and those are also extracted in methanol and ethanol extracts. In Lanes 1 and 5 of Figure 2, it is indicated that there are many components in each extract of fresh pepper and dry red pepper with chromogenic reactions by DCQ in the origin [26,27]. These pigments in extracts act as interfering substances so it is important to separate the pigments and capsaicinoids. Water-soluble substances in the origin were removed by solid–liquid extraction using *n*-hexane because they remained solids (Figure 2, Lanes 2 and 6).

Capsaicinoids were solubilized and completely transferred to an NaOH layer, whereas pigments were left in an *n*-hexane layer (Figure 2, Lanes 3 and 7). Therefore, capsaicinoids and pigments were efficiently separated by liquid–liquid extraction.

The NaOH solution partitioned in this process was used for a chromogenic reaction according to the experimental method and to measure the absorbance. After a 10-minute color development, the blue color was stabilized and maintained for more than 60 minutes. Lastly, as shown in Figure 1, samples for chromogenic reactions of capsaicinoids in Lanes 4 and 8 showed some color-forming components in the origin but the blue color formation of capsaicinoids was not affected. The capsaicinoids content in the acetone extract of chili pepper was analyzed by HPLC and the total capsaicinoid content was calculated by adding up areas of nordihydrocapsaicin, capsaicin, and dihydrocapsaicin, which were the three main peaks. Tables 1 and 2 compared the results of the quantification of fresh pepper and dry red pepper, respectively. The regression coefficients (R^2) of the total

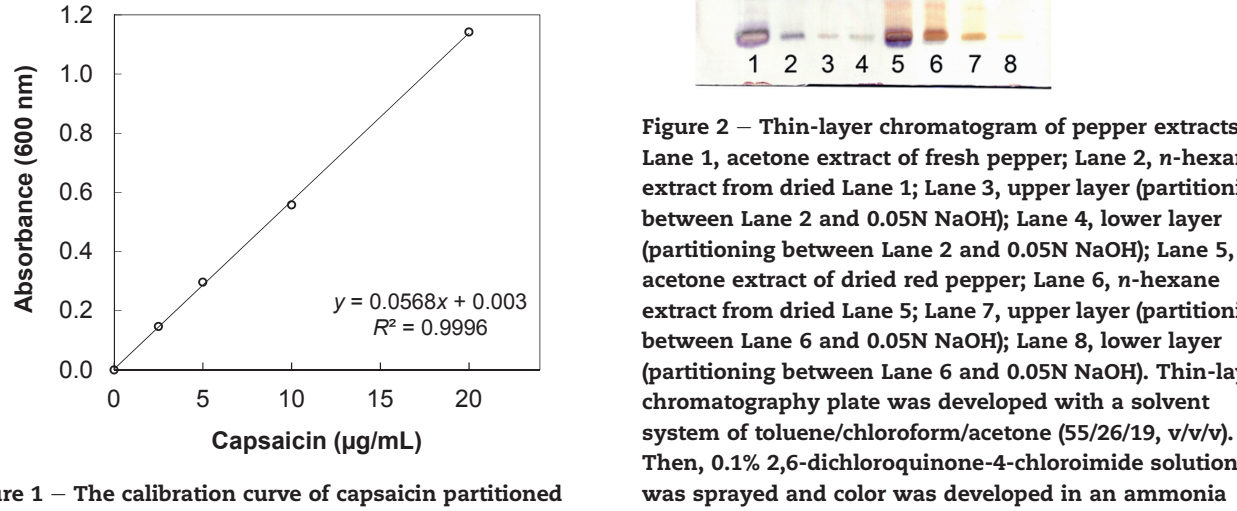


Figure 1 – The calibration curve of capsaicin partitioned from *n*-hexane to 0.05N NaOH solution.

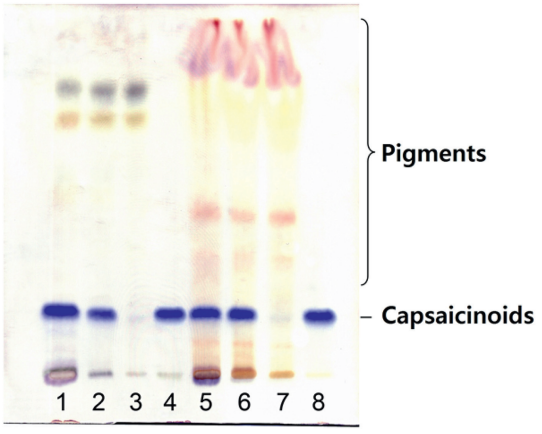


Figure 2 – Thin-layer chromatogram of pepper extracts. Lane 1, acetone extract of fresh pepper; Lane 2, *n*-hexane extract from dried Lane 1; Lane 3, upper layer (partitioning between Lane 2 and 0.05N NaOH); Lane 4, lower layer (partitioning between Lane 2 and 0.05N NaOH); Lane 5, acetone extract of dried red pepper; Lane 6, *n*-hexane extract from dried Lane 5; Lane 7, upper layer (partitioning between Lane 6 and 0.05N NaOH); Lane 8, lower layer (partitioning between Lane 6 and 0.05N NaOH). Thin-layer chromatography plate was developed with a solvent system of toluene/chloroform/acetone (55/26/19, v/v/v). Then, 0.1% 2,6-dichloroquinone-4-chloroimide solution was sprayed and color was developed in an ammonia vapor chamber.

Table 1 – Comparison of the capsaicinoids content according to quantification methods from fresh pepper.

Sample	Capsaicinoids (μg/g)		(Colorimetric/HPLC) × 100 (%)
	HPLC	Colorimetric	
FP 1	144.9	134.7	93.0
FP 2	232.7	221.8	95.3
FP 3	235.3	219.7	93.4
FP 4	426.1	419.0	98.4
FP 5	557.7	551.2	98.8
FP 6	315.8	284.4	90.1
FP 7	278.1	264.6	95.1
FP 8	236.5	228.5	96.6
FP 9	392.8	358.6	91.3
FP 10	94.3	90.5	96.0
FP 11	22.4	22.1	98.7
FP 12	108.1	106.0	98.0
FP 13	145.7	142.5	97.8
FP 14	13.4	12.7	95.0
FP 15	104.0	103.8	99.8
FP 16	336.1	323.9	96.4
FP 17	218.5	215.2	98.5
FP 18	40.6	39.5	97.3
FP 19	266.6	247.8	92.9
FP 20	463.1	432.9	93.5
FP 21	288.2	267.2	92.7
FP 22	69.6	67.5	97.0
FP 23	462.9	450.2	97.3
FP 24	84.0	80.7	96.1
FP 25	183.9	174.5	94.9
FP 26	411.1	382.7	93.1
FP 27	113.9	103.8	91.1
FP 28	45.3	44.1	97.4
FP 29	105.2	104.5	99.4
FP 30	147.4	139.6	94.7
Average ± deviation			95.8 ± 2.5

FP = fresh pepper; HPLC = high-performance liquid chromatography.

Table 2 – Comparison of the capsaicinoids content according to quantification methods from dry red pepper.

Sample	Capsaicinoids (μg/g)		(Colorimetric/HPLC) × 100 (%)
	HPLC	Colorimetric	
DP 1	859.0	901.2	104.9
DP 2	855.2	921.1	107.7
DP 3	988.4	988.0	96.9
DP 4	622.6	676.8	108.7
DP 5	1186.4	1122.0	94.6
DP 6	558.8	580.3	103.8
DP 7	645.6	659.8	102.2
DP 8	174.0	164.2	94.3
DP 9	889.2	882.7	99.3
DP 10	715.2	749.3	104.8
DP 11	798.2	861.4	107.9
Average ± deviation			102.3 ± 5.2

DP = dry red pepper; HPLC = high-performance liquid chromatography.

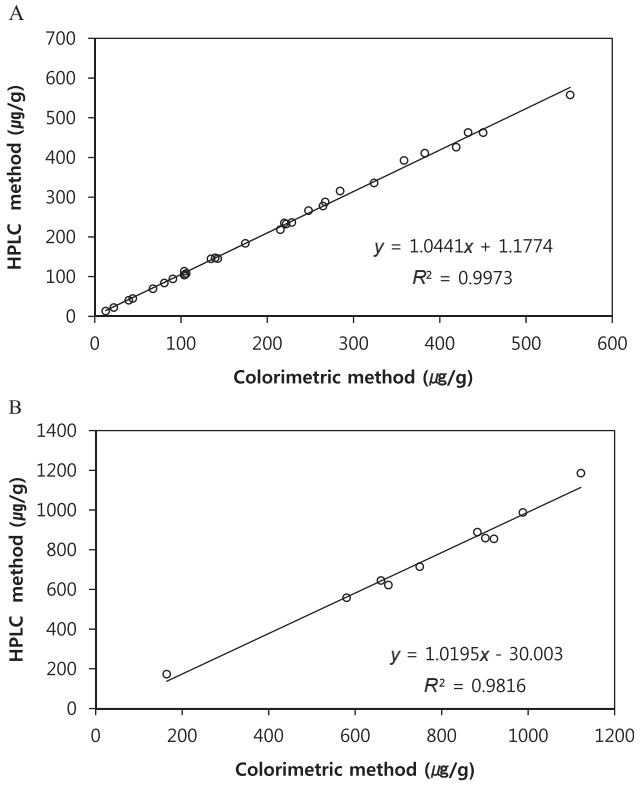


Figure 3 – A correlation between the colorimetric method and high-performance liquid chromatography (HPLC) in quantification of capsaicinoids from (A) fresh pepper and (B) dry red pepper.

capsaicinoid content from the colorimetric and HPLC methods were 0.9973 (Figure 3A) for fresh pepper and 0.9816 (Figure 3B) for dry red pepper, which showed very high correlations.

4. Discussion

Quantification of capsaicinoids retaining the pungency of chili peppers is widely conducted using instrumental analyses such as HPLC [13–16] and gas chromatography [17–19]. These methods universally separate and detect substances by chromatography, and are strong and elaborate methods indicating a high level of analysis to separate even a capsaicinoid isomer. The heat scale is expressed as the SHU [12]. In instrumental analysis, all capsaicinoids were added up to be converted to SHU [21]. When the scale of pungency needs to be large in the case of breeding chili peppers or quality control of chili pepper products, analysis of total capsaicinoids by colorimetry would be a very practical method.

Capsaicinoids have vanillylamide, a branched fatty acid, and a phenolic hydroxyl group in the molecule's structure in common. A phenolic hydroxyl group forms color through a reaction with vanadium chloride [28], phosphotungstic–phosphomolybdic acid [29], and DCQ [30] and is therefore used for the colorimetry of total capsaicinoids. Colorimetric analysis using the aforementioned methods for determination of capsaicinoids was developed and reported [31–34]. However, these methods are an inaccurate quantification of capsaicinoids

because the quantitative methods use pepper extracts without separation of the pigments as interfering substance.

Water-soluble solvents including acetone and alcohol are used as extraction solvents of chili pepper [35,36]. As shown in Figure 2, the extract contains color developing components by DCQ besides capsaicinoids and pigments, so it is necessary to efficiently separate these components, as shown in the results, and successfully remove them (Figure 2).

Upon comparing the results of the capsaicinoid content by the colorimetric method and HPLC using fresh pepper and dry red pepper as samples, the regression coefficient (R^2) value was found to be 0.9973 and 0.9816 (Figures 3A and 3B), respectively. The high value indicates the accuracy and reliability of the method. In particular, a great quantity of sample, which could not be analyzed by instrumental analysis, could be analyzed simultaneously using simple equipment, such as a centrifuge and spectrophotometer, without the need for expensive equipment. Therefore, it is possible to drastically reduce overall analysis time. Usability of the method will be efficient in the breeding process of chili peppers, which need to be analyzed in a short time with hundreds of samples produced simultaneously. In addition, 1 μg/mL capsaicinoids as a minimum can be detected and it is therefore postulated that the method could easily be applied to drug manufacturing and quality control of chili pepper products, such as kimchi and chili pepper sauce.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfda.2016.11.007.

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