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Determination of capsaicin and dihydrocapsaicin in some hot chilli varieties by RP-HPLC-PDA after magnetic stirring extraction and clean up with C_{18} cartridge

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Abstract: Reversed phase-HPLC separation of capsaicin and dihydrocapsaicin in some hot chilli varieties was achieved using photodiode array (PDA) detector. Under optimum conditions, their analytical figures of merit for the HPLC method were validated. The linearity range of calibration curve was 1.0-25 µg/mL with multiple determination coefficients higher than 0.995. Limits of detection and quantitation were 0.5 and 1.0 µg/mL, respectively. The repeatability and reproducibility of both retention time and peak area for these compounds were in good precision with their relative standard deviations (RSDs) lower than 1% and 4%, respectively. Solvent extraction using methanol for both compounds were conducted by magnetic stirring extraction (MSE) method and then cleaned up with C_{18} solid-phase extraction (SPE) prior to analysis by HPLC. Recoveries of SPE of all sample extracts spiked with 16 µg of each capsaicin and dihydrocapsaicin were ranged from 90.0-95.0% and 87.8-95.7%, respectively. The optimized conditions were applied for the determination of both capsaicinoids in ten varieties of the chilli samples. Total contents of the capsaicinoids were found in the range of 1,758.2-7068.9 µg/g DW with corresponding to their Scoville heat unit (SHU) obtained in the range of 26,400-106,000. Additionally, the contents of capsaicinoids determined by external calibration method comparing with those of standard addition were not significantly different, indicating less matrix effect. Mostly, the average amounts of capsaicin (62%) found in these real samples were rather higher than those of dihydrocapsaicin (38%).

Keywords: Capsaicinoids, capsaicin, dihydrocapsaicin, chilli pepper, magnetic stirring extraction, solid-phase extraction, photodiode array (PDA), RP-HPLC

Introduction

Chilli peppers are generally known as ripen fruits of various species of genus capsicum. They play an important role as one of the most commercial crops used both as condiment or culinary supplement and as vegetable. Commonly, their hot sensory taste is due to capsaicinoids as the major group of organic compounds which is closely related to the family of alkaloids, and are known to be biosynthesized and accumulated in the placenta of Capsicum fruits (Pruthi, 1976; Tapia *et al*., 1993; Prasad *et al*., 2006). Capsaicinoids are soluble in moderate polar organic solvents e.g. chloroform, acetone, ethyl acetate, methylene chloride, methanol, ethanol, acetonitrile, and among others (Duarte *et al*., 2004; Santamaria *et al.*, 2000). The major capsaicinoids present in most varieties of the chilli pepper are capsaicin (tran-8-methyl-N-vanillyl-6-nonenamide)and dihydrocapsaicin (8-methyl-N-vanillylnonanamide). In addition, other minor ones are also found such as nordihydrocapsaicin, norcapsaicin, homocapsaicin, nornorcapsaicin, nornornorcapsaicin and nonivamide (Barbero *et al*., 2006). Capsaicin has been used in neurological research to stimulate sensory nerves and also to treat bladder inflammation. It is also found in topical ointments used for arthritis and neuralgia (Kaale *et al.*, 2002), and exerts its effect on the sensory nerves by interacting with the vanilloid receptor, promoting the release of substance P as well as other cytokines (Surh *et al.*, 2005).

The determination of capsaicinoids in chilli peppers, topical cream (Kaale *et al*., 2002), self-defense weapons (Reilly *et al*., 2001a) and aerosol defense sprays (Spicer and Almirall, 2005) has been of increasing interest for many reasons. Extraction methods of the capsaicinoids from chilli pepper sample have been conducted using various extraction techniques including liquid-liquid extraction (LLE) (Tapia *et al*., 1993; Kaale *et al*., 2002; Spicer and

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Almirall, 2005), enzymatic extraction (Santamaria *et al.*, 2000), supercritical fluid extraction (SFE) (Sato *et al*., 1999; Gnayfeed *et al*., 2001; Kaale *et al.*, 2002; Duarte *et al.,* 2004; Uzunalic *et al*., 2004), pressurized liquid extraction (PLE) (Barbero *et al.,* 2006a), magnetic stirring extraction (MSE) (Kaale *et al*., 2002), solid-phase microextraction (SPME) (Spicer and Almirall, 2005), reflux heating (Peusch *et al.*, 1997), microwave assisted extraction (MAE) (Barbero *et al*., 2006b), maceration (Titze et al., 2002), Soxhlet extraction (Korel *et al.*, 2002) and ultrasonic assisted extraction (UAE) (Karnka et al., 2002). The extraction of capsaicinoids in Capsicum plants is, in fact, influenced by their chemical nature, extraction method, sample particle size, storage time as well as the presence of interfering substances. Thus, the extracts of the plants are always a mixture of different classes of organic compounds that are soluble in the solvents used. Methanol, ethanol, propanol, acetone, ethyl acetate, n-butyl chloride, acetonitrile, dichloromethane, hexane and their combinations are frequently used for the extraction of capsaicinoids. Peusch *et al*. (1997) reported that SFE of capsaicinoids in some pepper samples led to a six-fold higher extraction yield compared to organic solvent extraction using various solvents. However, SFE is cost effective equipment and is not common in routine laboratories. Besides, an ordinary extraction method is still adopted with relative ease to carry out by using magnetic stirring solvent system (Kaale *et al.*, 2002). But it takes time, and then the extract containing the analyte is subjected for centrifugation and or filtration.

Although many extraction methods of plant samples can be performed by sophisticated instruments, the extracts obtained are mostly followed by a further sample cleanup step using various techniques including solid-phase extraction (SPE) (Attuquayefio and Buckle, 1987; Betts, 1999; Monnerville, 1999; Karnka *et al*., 2002; Kim *et al*., 2002; Korel et al., 2002). After extraction of the capsaicinoids from chilli sample using organic solvents, an analysis was performed by gas chromatography (GC) (Spicer and Almirall, 2005; Thomas *et al*., 1998) and or high-performance liquid chromatography (HPLC) (Barbero *et al.*, 2006b; Higashiguchi *et al*., 2006; Schweiggert *et al*., 2006). Previous chromatographic methods have been reported for analytical separation, quantitation and identification of naturally occurring capsaicinoids by HPLC (Heresch and Jurenitsch, 1979; Hoffman *et al*., 1983; Attuquayefio and Buckle, 1987; Cooper *et al*., 1991; Tapia *et al*., 1993; Constant *et al*., 1995; Peusch *et al.*, 1997; Padilla and Yahia, 1998; Betts, 1999; Estrada *et al*., 1999; Santamaria *et al*., 2000; Gnayfeed *et al*., 2001; Reilly *et al*., 2001; Estrada *et al.*, 2002; Kaale *et al.*, 2002; Karnka *et al.,* 2002; Kim *et al*., 2002; Korel *et al.,* 2002; Kurian and Starks, 2002; Titze *et al.*, 2002; Duarte *et al*., 2004; Martin *et al*., 2004; Uzunalic *et al.*, 2004; Kozukue *et al*., 2005; Materska and Perucka, 2005; Thomson, *et al*., 2005a; Barbero *et al*., 2006b; Higashiguchi *et al.*, 2006; Schweiggert *et al.*, 2006), supercritical fluid chromatography (SFC) (Sato et al.,1999), thin layer chromatography (TLC) (Materska and Perucka, 2005), GC (Thomas *et al.*, 1998; Spicer and Almirall, 2005), capillary electrophoresis (CE) (Monnerville, 1999) and spectrophotometry (Bajaj and Kaur, 1979; Perucka and Oleszek, 2000).

Previously, determination of total capsaicinoids have relied on direct measurement of ultraviolet absorption or colorimetric method using Folin-Ciocalteu reagent by UV-Visible spectrometry (Bajaj and Kaur, 1979; Perucka and Oleszek, 2000). The reagent is not specific for capsaicinoids and hence other compounds may interfere. So, disadvantages of direct spectrophotometric measurements can be attributed to lack of selectivity and are not specific because they give only estimate the capsaicinoid contents. Separation and identification of an individual capsaicinoids has been carried out by gas chromatography-mass spectrometry (GC-MS). Recently, an excellent resolving power and detection capabilities of GC and particularly GC-MS have been exploited for the analysis of capsaicinoids (Thomas *et al*., 1998; Spicer and Almirall, 2005). Also, CE has recently been used for the analysis of capsaicinoids (Monnerville, 1999). It is characterized by high separation efficiency, clean, small sample and electrolyte consumption and rapid analysis as the separation required only several minutes. But its equipment is still not so cheap, tedious maintenance and is not versatile in routine laboratories. The direct connection of SFE and SFC has received much attention as well (Sato *et al*., 1999). This method has advantages of not requiring a concentrating procedure or a cleanup procedure before analysis. However, the SFC equipment is also cost effective one, difficult use and is uncommon in routine analysis.

Thus, regarding with HPLC as mentioned above it is currently the most popular and reliable technique for the analysis of capsaicinoids. The technique has been mainly associated with UV absorption detection (Hoffman *et al*., 1983; Betts, 1999; Perucka and Oleszek, 2000; Santamaria *et al.*, 2000; Kaale *et al.*, 2002; Karnka *et al*., 2002; Kim *et al.*, 2002; Korel *et al*., 2002; Kurian and Starks, 2002; Duarte *et al.,* 2004; Materska and Perucka, 2005; Lui *et al.*, 2007), fluorescence detection (Gnayfeed *et al.*, 2001; Titze

et al., 2002; Barbero *et al*., 2006a), photodiode array (PDA) (Cooper *et al*., 1991; Constant *et al*.,1995; Padilla and Yahia, 1998; Estrada *et al*., 1999 & 2002) and or coupled with fluorescence detection (Cooper *et al*., 1991; Peusch *et al*., 1997; Uzumalic *et al*., 2004; Barbero *et al.,* 2006b). The recent development of LC-MS provides a useful tool for the determination of these compounds (Surh and Lee, 1995; Reilly *et al.*, 2001; Martin *et al*., 2004; Kozukue *et al.*, 2005; Thomson *et al.*, 2005b; Barbero *et al.*, 2006a; Higashiguchi *et al.,* 2006; Schweiggert *et al*., 2006). However, LC-MS is quite complicated. Since, detection of capsaicinoids can be measured by UV absorption with common wavelength at 280 nm. Thus, HPLC-PDA constitutes a crucial, utterly reliable technique, which is routinely employed in the analysis of capsaicinoids. The PDA is an indispensable tool for the provisional identification of the main capsaicinoid structures. Thus, PDA detection mostly allows the identification of the compounds using absorption spectrum and peak purity. Therefore, the present study is aimed to develop a suitable MSE method for capsaicinoids. The extraction yields obtained under the optimized MSE and their contents determined by HPLC-PDA method were discussed

Materials and Methods

Chemicals

All reagents used were at least analytical reagent (AR) grade such as ethanol (EtOH) (Carlo Erba, Italy). Methanol (MeOH) and acetonitrile (ACN) were of HPLC grade obtained from Lab Scan (Thailand). The highest purity of standard capsaicinoids was used. Capsaicin and dihydrocapsaicin were obtained from Sigma (USA). Aqueous solutions were prepared with de-ionized water obtained from RiOsTM type 1 simplicity 185 (Millipore Waters, USA) throughout the experiments. All standards and samples were kept cool at 4°C before use.

Instruments

The experiment was carried out on a Waters liquid chromatograph (Waters, USA). It consists of a Waters 600E multisolvent delivery system, a Waters in-line degasser AF, a Rheodyne injector with sample loop of 20 µL, a Waters 2996 photodiode array detector, and a Waters temperature control system. Empower software was used for data acquisition. A Hypersil ODS C18 column (4.6 mm i.d. × 100 mm, 3 µm particle diameter) was used. C18 cartridge (500 mg/4 cm^3) for solid-phase extraction was obtained from Alltech (USA).

Plant materials

Ten varieties of hot chilli peppers were collected from cultivating sites and seed products Co Ltd. Most of the samples were *Capsicum annuum L.* (S01, Munpama; S02, Super hot; S05, Yodson, Nonethai-Nakhon Ratchasima; S07, Jinda, Chumsang- Nakhon Sawan; S08, Jinda, Muang-Phetchaboon; S09, Yodson, Theparak-Nakhon Ratchasima; S10, small Jinda), Capsicum chinense L. (S03, Pag-puan; S06, South Africa), and Capsicum frutescens L. (S04, Doi-ded-gan) obtained from Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University. The dried samples were ground using a kitchen grinder, kept in plastic bag and stored in desiccator before use.

Magnetic stirring extraction (MSE) and Solid-phase extraction (SPE)

Two grams of ground chilli sample were macerated using magnetic stirring with three kinds of moderate polar solvents (methanol, 80%v/v ethanol in water and acetonitrile) at four heating temperatures (60, 70, 80 and 90°C) for three extraction time intervals (1, 2 and 3 h). The optimum conditions for MSE method were investigated in detail by varying these experimental parameters. The extracts were centrifuged at 5000 rpm for 10 min and then filtered through a Whatman No. 42 filter paper. The solvent in the extracts was evaporated to dryness using a rotary evaporator and the residue was dissolved with suitable solvent to make a final volume of 5 mL. This extract was then cleaned up using C_{18} SPE cartridge.

The SPE cartridge was conditioned with 2 mL of methanol and followed by 2 mL of de-ionized water. The sample extract obtained (400 µL) was diluted with 600 µL de-ionized water and subjected into the conditioned C_{18} cartridge. The capsaicinoids were eluted with 2 mL of methanol twice and adjusted the final volume to 10 mL and then an aliquot of this sample was filtered through 0.45 µm nylon membrane prior to HPLC analysis.

Analysis of capsaicin and dihydrocapsaicin by RP-HPLC-PDA

Preparation of capsaicin and dihydrocapsaicin standard solutions

For a stock solution of capsaicin (2,640 µg/mL), standard capsaicin was prepared by dissolving of 0.0132 g in 5.0 mL of methanol, transferred to vial and kept cool at 4°C before use. Also for dihydrocapsaicin solution (2,140 µg/mL), standard dihydrocapsaicin was prepared by dissolving of 0.0107 g in 5.0 mL of methanol in the same manner as the capsaicin solution. Working solutions of both

capsaicinoids were prepared daily by an appropriate dilution in methanol.

Optimization of RP-HPLC-PDA separation

The optimization for HPLC analysis of capsaicinoids was investigated by varying the composition of mobile phase whereas the other conditions used throughout the experiment were as follows: flow rate of 0.9 mL/min, ambient temperature, and photodiode array detector at 280 nm. Binary solvent mixtures (50-70% v/v) of methanol and de-ionized water were used as a mobile phase. The mobile phase was filtered through a 0.45 µm nylon membrane and degassed before use. The column was equilibrated with the mobile phase for 30 min or until a steady detector baseline was achieved.

Method validation of RP-HPLC-PDA

To determine whether the proposed method provided suitable conditions for both quantitative and qualitative analyses of the capsaicinoids, the following validated data were deduced. To determine the linearity of the HPLC method, a series of standard solution were prepared covering a concentration range of 1.0-25 µg/mL by serial dilution of the stock standard solutions of both capsaicin and dihydrocapsaicin for external calibration curve and 0.0-10 µg/mL for standard addition method. The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as the analyte concentration giving a signal to noise ratios (S/N) of 3 and 10, respectively. Mixed standard solutions of the capsaicinoids were diluted from their stock standard solutions for both LOQ and LOD determination. The precision of the method was presented as the repeatability and reproducibility of both retention time and peak area. The repeatability (intra-day precision) was deduced from ten replicates within a day (n = 10) and reproducibility (inter-day precision) was calculated from the data of the experiment carried out in three consecutive days (n = 3×5). Mixed standard solutions of both capsaicinoids were used at the concentration of 10.0 µg/mL.

Results and Discussion

Optimization of RP-HPLC separation conditions

The chromatographic conditions used were optimized with the aim of obtaining the separation with a good resolution of adjacent peaks within a short analysis time. Vaious mobile phases have been described for the analysis of capsaicinoids using the reversed phase column (Kaale *et al.*, 2002; Karnka *et al.*, 2002; Kim *et al.*, 2002; Korel *et al.*, 2002; Kurian and Starks, 2002; Titze *et al.*, 2002; Duarte *et al.*, 2004; Martin *et al.*, 2004; Uzunalic *et al.*, 2004; Kozukue *et al.*, 2005; Materska and Perucka, 2005; Thomson *et al.*, 2005b; Barbero *et al.,* 2006b; Higashiguchi *et al.,* 2006; Schweiggert *et al.*, 2006). In this study, the organic solvent selected for the preliminary experiments was methanol (MeOH) due to the solubility of capsaicinoids. The mobile phases containing various percents of methanol in de-ionized (DI) water were investigated. The mobile phase containing MeOH and DI (66:44, v/v) was chosen which the solvent peaks were completely separated from the analytes. Thus, under the optimal isocratic conditions, both capsaicin (tR 4.04 min) and dihydrocapsaicin (t_R 5.76 min) were separated within 7 min with resolution of 1.2 as shown in Figure 1(a). Since molecular structures of both capsaicin

Table 1. Repeatability and reproducibility of retention time and peak area of RP-HPLC-PDA

Capsaicinoids	Repeatability (n = 10)				Reproducibility (n = 3×5)			
	t_R (min)		Area		t_R (min)		Area	
	Mean	%RSD	Mean	%RSD	Mean	%RSD	Mean	%RSD
Capsaicin	4.04	0.42	128495.4	3.21	4.04	0.87	129021.2	3.87
Dihydrocapsaicin	5.76	0.27	106061.4	1.53	5.79	0.64	107254.9	2.01

Each concentration of standard capsaicinoids used was 10 µg/mL.

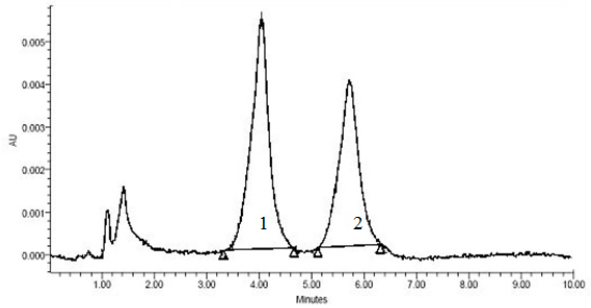

Figure 1(a). Chromatogram of two standard capsaicinoids (1, capsaicin and 2, dihydrocapsaicin) using UV detection at 280 nm

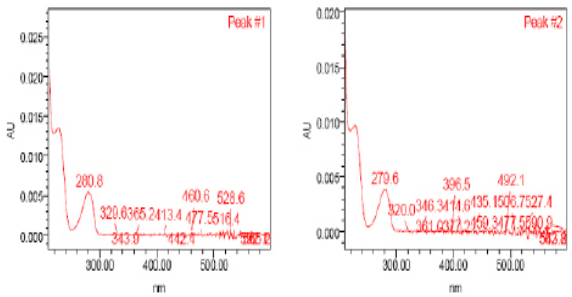

Figure 1(b). The UV-Visible spectra of two standard capsaicinoids (1, capsaicin and 2, dihydrocapsaicin) using PDA detection

Table 2. The contents of capsaicin and dihydrocapsaicin obtained from various extraction conditions using methanol as a solvent

Series No.	Extraction condition	Contents (µg/g)			
		Capsaicin	%RSD	Dihydrocapsaicin	%RSD
1	MeOH/60°C/1h	1229.2	3.25	631.5	1.46
2	MeOH/60°C/2h	1592.0	5.09	843.9	5.14
3	MeOH/60°C/3h	1307.9	1.87	688.0	4.16
4	MeOH/70°C/1h	1065.2	1.66	513.3	3.03
5	MeOH/70°C/2h	1007.5	1.26	764.2	2.87
6	MeOH/70°C/3h	776.1	1.83	526.7	3.30
7	MeOH/80°C/1h	667.1	1.41	491.9	2.36
8	MeOH/80°C/2h	938.7	8.48	552.6	2.72
9	MeOH/80°C/3h	1199.0	2.31	568.8	5.22
10	MeOH/90°C/1h	1038.2	2.15	699.8	4.88
11	MeOH/90°C/2h	722.7	2.85	545.4	4.83
12	MeOH/90°C/3h	538.2	9.01	416.7	9.32

and dihydrocapsain are very similar, the maximum absorption wavelengths determined by PDA are also nearly the same and found to be 280.8 and 279.6 nm, respectively (Figure 1(b)). The PDA using Empower software detects the absorbance at the chosen wavelengths of the analytes and simultaneously provides their absorption spectra. Identification of compounds was achieved by retention time and absorption spectrum of standard and sample. However, both compounds were detected with PDA at 280 nm.

Method validation

The quantitative features of the HPLC method were studied under the optimum separation conditions. The linearity was found in the range of 1.0-25.0 µg/mL for both compounds. The external calibration curves were found as Y=12572x-5093.7 (r^2 = 0.9943) for capsaicin, and Y=11199x-8675.4 (r^2 = 0.9959) for dihydrocapsaicin (Table 1). Both LOD (0.5 µg/mL) and LOQ (1.0 µg/mL) of the two compounds were the same. The precision of the method using 10 µg/mL of each capsaicinoid was expressed in term of repeatability and reproducibility of both retention time and peak area. The repeatability and reproducibility of the retention time and peak area for the two capsaicinoids were in good precision with their relative standard deviations (RSDs) were lower than 1% and 4%, respectively.

Optimization conditions for MSE and Sample clean up

For conventional solvent extraction (Kaale *et al.*, 2002), the sample preparation with an appropriate solvent was investigated using magnetic stirring extraction (MSE) method under different conditions

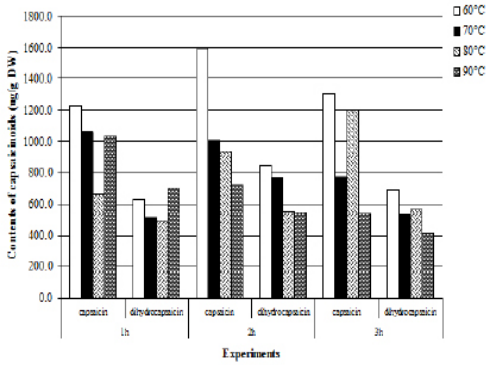

Figure 2. The contents of capsaicin and dihydrocapsaicin obtained from various extraction conditions using methanol as a solvent

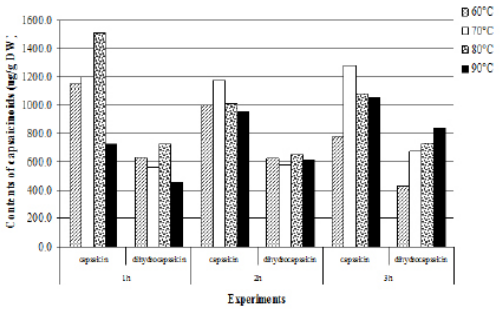

Figure 3. The contents of capsaicin and dihydrocapsaicin obtained from various extraction conditions using acetonitrile as a solvent

according to 4 levels of extraction temperature and 3 ranges of extraction time. The sample extracts were then cleaned up with C_{18} cartridge. The extraction yields of both capsaicinoids when using methanol as the extraction solvent are shown in Table 2 and Figure 2. The yields were in the range of 538.2-1592.0 µg/g DW for capsaicin and 416.7-843.9 µg/g DW for dihydrocapsaicin. Their RSDs were lower than 10.0%. It was found that the suitable conditions of this system was MeOH/60°C/2h giving the highest contents of capsaicin (1592.0 µg/g DW) and dihydrocapsaicin (843.9 µg/g DW). However, the extraction yields were also varied not so much big differences in their amounts among the extraction temperature and its extraction time used. For example, at 60°C the contents of both capsaicinoids obtained were relatively comparable including at 70°C for 1-2 h, and at 80°C for 3 h or at 90°C for 1 h. Therefore, the extraction condition with short analysis time is choice of the appropriate procedure.

When using 80% ethanol in water as the extraction solvent in association with the same levels of both extraction temperature and extraction time, the results are shown in Figure 3. The contents of capsaicin were found in the range of 606.7-1189.6 µg/g DW. And those of dihydrocapsaicin were in the range of 364.3-741.9 µg/g DW. Their RSDs were less than 9.0%. However, the contents of capsaicinoids obtained from 80%EtOH/60°C/3h (capsaicin, 1189.6

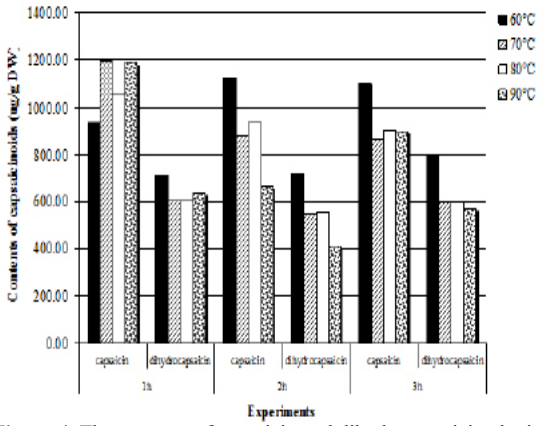

Figure 4. The contents of capsaicin and dihydrocapsaicin obtained from various extraction conditions using 80% ethanol in water as a solvent

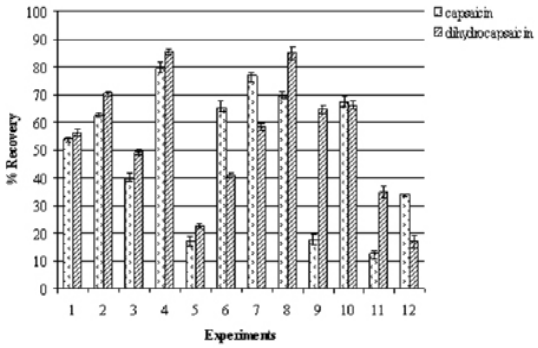

Figure 5. The percentage recoveries of capsaicin and dihydrocapsaicin obtained from 12 experimental trials in Table 2 (mean ± SD, n = 3)

µg/g DW and dihydrocapsaicin 741.9 µg/g DW) were comparatively higher than using 80%EtOH/60°C/2h (capsaicin, 1080.5 µg/g DW and dihydrocapsaicin 633.5 µg/g DW) and 80%EtOH/90°C/1h (capsaicin, 1088.1 µg/g DW and dihydrocapsaicin 594.4 µg/g DW). But the content obtained under the extraction at 70°C for 1 h was also comparable. Thus the extraction at 90°C for 1 h would be preferable for short extraction time.

In the same manner when using acetonitrile as the extraction solvent under the same extraction conditions as mentioned above, the capsaicin contents were found in the range of 579.9-1371.0 µg/g DW (Figure 4). Among these solvent systems, ACN/60°C/1h gave the highest content of capsaicin (1371.0 µg/g DW). While ACN/80°C/1h (capsaicin 1082.5 µg/g DW) and ACN/90°C/3h (capsaicin 1098.1 µg/g DW) gave comparatively the same contents. In case of dihydrocapsaicin, it was found in the range of 340.5-673.7 µg/g DW. Thus using ACN/90°C/3h showed in the highest content of dihydrocapsaicin (673.7 µg/g DW).

From these results, the optimum conditions of each solvent system were depended on both extraction temperature and extraction time due to using the similar moderate polar solvents. However, it was demonstrated that one can should the suitable conditions at which consist of 60°C extraction temperature, 2 h extraction time and methanol as an extraction solvent with relative ease. The extraction of the capsaicinoids from ten varieties of hot chilli pepper samples was conducted. The extracts were cleaned up by SPE before analysis by HPLC. Since SPE was necessary to remove matrices from the analyte prior to analysis, in this study, the efficiency of the clean up procedure was also conducted with all real samples spiked with 16 µg of each capsaicin and dihydrocapsaicin. The percentage recoveries for this clean up step were found to be 90.0-95.0 and 87.8-95.7, for capsaicin and dihydrocapsaicin, respectively (data not shown). However, the method recoveries were found in ranges of 12.6-79.5% and 22.7-85.4% for capsaicin and dihydrocapsaicin, respectively, when using the experimental conditions as shown Figure 5. The range of the recoveries was relatively wide under those various conditions used. But for such suitable system (MeOH/70°C/1h) the percentage recoveries of the method with spiking of 80 µg of each compound into 2.0 g sample were found to be 79.5 and 85.4 for capsaicin and dihydrocapsaicin, respectively. In this study, the suitable extraction conditions for each solvent were choice of use including MeOH/60°C/2h (Table 2), 80%EtOH/90°C/1h (Figure 2) and ACN/80°C/1h (Figure 3), resulted in comparatively high contents of both capsaicinoids.

Analysis of capsaicinoids in real samples

The proposed method was applied for the determination of capsaicin and dihydrocapsaicin in ten varieties of hot chilli peppers. The amounts of capsaicin and dihydrocapsaicin found in these samples as using calibration curve were ranged of 910.5-4981.4 µg/g DW and 692.6-2087.5 µg/g DW, respectively as shown in Table 3. Since standard addition method was proved to reduce interferences in real sample, all samples of the same lot used for external calibration curve were also analyzed. Both capsaicin and dihydrocapsaicin were found in the ranges of 921.5-5132.3 µg/g DW and 652.2-2004.3 µg/g DW, respectively (Table 3). Figure 6 illustrates the comparison of the capsaicinoid contents in these samples between calibration curve and standard addition methods. Most of all were not significantly different ($p<0.05$), except sample No. S05. For the general taste of chilli pepper, the correlation between Scoville heat unit (SHU) and the two capsaicinoids obtained was calculated as shown in Table 4 by using the relationship between this content (µg/g DW) and its SHU rating of approximately 15 SHU equivalent

Table 3. Comparison of the capsaicinoid contents in hot chilli pepper samples between external calibration curve and standard addition methods

Capsaicinoids	Sample	Content (µg/g) obtained from calibration curve	% RSD	Content (µg/g) obtained from standard addition	% RSD
Capsaicin	S01	1250.2	9.99	1131.3	8.16
	S02	2909.2	6.26	2910.8	9.66
	S03	3214.5	9.02	3187.6	9.71
	S04	4981.4	5.82	5132.3	8.01
	S05	1273.2	9.81	1311.8	9.14
	S06	2410.6	9.95	2351.3	4.92
	S07	2143.3	9.23	2305.2	8.39
	S08	1065.6	9.85	1124.1	8.01
	S09	1295.6	9.26	1358.6	8.05
	S10	910.50	9.88	921.50	5.89
Dihydrocapsaicin	S01	705.30	9.34	652.20	9.66
	S02	1892.5	5.02	2004.3	9.44
	S03	1821.7	9.93	1724.4	9.75
	S04	2087.5	4.31	1898.3	8.90
	S05	749.30	9.41	658.70	7.53
	S06	1142.2	9.98	1201.9	9.34
	S07	1106.0	8.86	1106.6	8.69
	S08	692.60	3.61	712.40	4.98
	S09	815.40	3.07	785.20	8.77
	S10	981.70	8.15	867.20	9.40

t-test for data : < 2.78 no significant ($p < 0.05$)

to 1 µg/g of capsaicinoids (Mathur *et al*., 2000). Therefore, their corresponding SHU were found in the range of 26,400-106,000. From these results, it is indicated that capsaicin and dihydrocapsaicin were primarily responsible for the SHU rating. Thus, the chilli sample S04 gave quite high SHU related with higher contents of the capsaicinoids. Therefore, total yields of capsaicinoids in these chilli peppers were ranged from 1758.2–7068.9 µg/g DW. In addition, capsaicin and dihydrocapsaicin have the same trend in contents of the capsaicinoids, and in particular capsaicin was found in higher contents than dihydrocapsaicin in all samples studied.

Conclusion

In the present study, RP-HPLC-PDA method was used for analysis of major components of capsaicinoids (capsaicin and dihydrocapsaicin) after sample preparation using MSE and SPE methods. Under optimum conditions, the validated method showed high sensitivity and selectivity for the capsaicinoids determination depending on type of solvent, extraction temperature and time. In this case, their methanolic extracts under the optimized conditions of MSE (at 60°C for 2 h) were cleaned up by C_{18} SPE. Recovery of the SPE step for all sample extracts was also higher than 88%. The method recoveries of these samples were also found up to 85.4%. The proposed method was applied for the determination of the two capsaicinoids in ten varieties of hot chilli peppers. Total contents of the capsaicinoids were found in the range of 1,758.2-7068.9 µg/g DW. The contents of capsaicinoids using calibration curve method compared with those of standard addition one almost showed no significantly difference. The contents of capsaicin (62%) found in the real samples were mostly higher than those of dihydrocapsaicin (38%). Their corresponding SHU were also calculated and found in the range of 26,400-106,000.

Table 4. The capsaicinoid contents and their Scoville heat unit (SHU) of ten varieties of hot chilli pepper samples

Sample	Capsaicin (µg/g DW)	Dihydrocapsaicin (µg/g DW)	Total capsaicinoids (µg/g DW)	Scoville heat unit (SHU)
S01	1250.2	856.30	2106.5	31600
S02	2909.2	1892.5	4801.7	72000
S03	3214.5	1821.7	5036.2	75500
S04	4981.4	2087.5	7068.9	106000
S05	1273.2	749.30	2022.5	30300
S06	2410.6	1142.2	3552.8	53300
S07	2143.3	1106.0	3249.3	48700
S08	1065.6	692.60	1758.2	26400
S09	1295.6	815.40	2111.0	31700
S10	910.50	981.70	1892.2	28400

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Abbreviations

RP-HPLC-PDA, reversed phase high-performance liquid chromatography with photodiode array detection; MSE, magnetic stirring extraction; SPE, solid-phase extraction; DW, dry weight; SHU, Scoville heat unit; MeOH, methanol; ACN, acetonitrile; EtOH, ethanol; RSD, relative standard deviation; LLE, liquid-liquid extraction; SFE, supercritical fluid extraction; PLE, pressurized liquid extraction; SPME, solid-phase microextraction; MAE, microwave assisted extraction; UAE, ultrasonic assisted extraction; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; SFC, supercritical fluid chromatography; TLC, thin layer chromatography; CE, capillary electrophoresis; DI, deionized water; LOD, limit of detection; LOQ, limit of quantitation

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