KKU Res.j. 2015; 20(1) : 54-65
http://resjournal.kku.ac.th

# Analysis of Capsaicin and Dihydrocapsaicin from Sweet-to-Very Hot Chilli Peppers Using an Ultrasound-Assisted Extraction Followed by RP-HPLC-PDA

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## Abstract

In the present study, the optimal conditions of an ultrasound assisted extraction for both capsaicin and dihydrocapsaicin in hot chilli samples were developed. The parameters affecting the yield of the capsaicinoids such as extraction solvents (methanol, ethanol, acetonitrile and water), solvent to sample ratio, extraction temperature and extraction time were investigated. The capsaicinoid extracts were analyzed by reversed phase-HPLC with photodiode array detector (RP-HPLC-PDA). The optimum extraction conditions consist of 20 mL of 80% (v/v) methanol and water as the extraction solvents and 20 min extraction time at 50ºC. The method was applied to determine seven varieties of sweet-to-very hot chilli peppers cultivated. The concentration of the capsaicinoids ranging from 573. 44-15220 µg/g was widely found in these samples.

**Keywords :** *capsaicinoids, chilli pepper, ultrasonic extraction, HPLC*

## 1. Introduction

Hot or spicy peppers are savory food additives that are widely utilized in many parts of the world and they are highly valued for their attributes of colour, pungency and flavour. Capsaicinoids are responsible for the spiciness of peppers and are widely used in food in most parts of the world due to their pungent properties. Among these compounds there are two major capsaicinoids, capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) and dihydrocapsaicin (8-methyl-N-vanillylnonanamide), which represent about 77–98% of capsaicinoids present in peppers (1). Besides these two major capsaicinoids, other minor capsaicinoids have been found in chilli peppers and these capsaicinoids include nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin, nonivamide among more than twenty reported compounds (2-3). The structural

characteristic of capsaicinoids which indicates its pungency depends on the length of an acyl chain, the absence or presence of double bond and its location along the numbered carbon atom of the acyl group. Among many pharmacological applications of capsaicinoids, their uses account for the treatment of pain and inflammation in different diseases, such as rheumatoid arthritis, migraine, and diabetic neuropathic (4).

Many different techniques have been employed for the extraction of capsaicinoids from pepper (5-11), such as maceration (12), magnetic stirring (13), enzymatic extraction (14), accelerated solvent extraction(15), microwave-assisted extraction (16), ultrasound-assisted extraction(17), Soxhlet (18), The conventional extraction methods, like Soxhlet extraction, which have been employed for decades, need long extraction times and require relatively large quantities of solvent (19). Inrecent years, the demand for extraction techniques that shorten extraction times and reduce the consumption of organic solvents has been increasing. Among these techniques, more efficient extraction techniques are ultrasound-assisted extraction (UAE), microwave-assisted extraction, supercritical fluid extraction and accelerated solvent extraction. The UAE technique is based on the use of the energy derived from ultrasounds (sound waves with frequencies higher than 20 kHz) to facilitate the extraction of analytes from the solid sample by the organic solvent, which is selected in conjunction with the nature of solutes to be extracted (20). This technique has been successfully employed to extract a variety of compounds from fruits, vegetables, and other biological tissues. The application of ultrasound-assisted extraction offers many advantages including the reduction of solvents, temperature and the time for extraction, which is very useful for the extraction of thermolabile and in unstable compounds (21). Ultrasonic extraction is considered as one of the simplest extraction techniques because it is easy to perform in common laboratory equipment (i.e. ultrasonic bath). The aims of this study were to optimize the sample preparation of capsaicinoids using ultrasound assisted extraction by ultrasonic bath and determination of the two major capsaicinoids in hot chilli peppers (Figure 1).

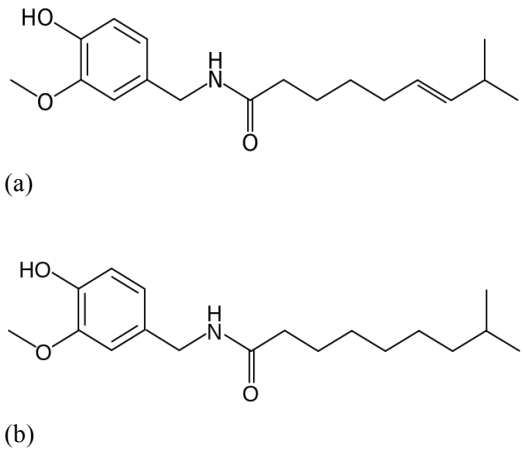


**Figure 1.** Chemical structure of two main components of capsaicinoids (a) capsaicin and (b) dihydrocapsaicin

## 2. Materials and methods

### 2.1 Equipments

An ultrasonic cleaning bath model RF103H (Bandelin Sonorex, Germany) with 140 W of potency, 35 KHz of frequency. The interior dimensions of the water bath were of 24 cm×14 cm×15 cm was used. The bath was kept at constant frequency during all the extraction processes.

The chromatograph for separation was a LC-20A HPLC instrument (Shimadzu, Japan) equipped with the following modules: SIL-20A HT autosampler; LC-20AD HPLC pump; CTO-20A column; SPD-M20A photodiode array detector. The Class VP software was used. The chromatographic separation was carried out using an ODS-C18 RP (4.6 mm × 250 mm) column.

**2.2 Chemicals**

The solvents (ethanol, methanol and acetonitrile) purchase from Merck (Germany) were used as analytical quality. The reference standards of capsaicin (96%) and dihydrocapsaicin (90%) were obtained from Sigma-Aldrich (U.S.A.).

**2.3 Plant materials**

Seven kinds of chilli pepper samples**,** which their codes and species type were given Table 1. The chilli peppers were obtained from Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kean University, Khon Kean, Thailand. All samples were dried in an oven at 60 ºC for 48 h. and ground using a kitchen grinder to pass a 100-mesh sieve. The ground samples were kept storing in desiccator before use.

**Table 1.** List of chilli pepper samples used in this study

| Sample code | Plant variety | Species |
|---|---|---|
| S01 | KKU-p31065 | *Capsicum annuum.* L. |
| S02 | MS4 | *Capsicum annuum.* L. |
| S03 | PC2-1 | *Capsicum annuum.* L. |
| S04 | YTP16-98-46-37-1-1 | *Capsicum annuum.* L. |
| S05 | Yodson Khem 80 | *Capsicum annuum.* L. |
| S06 | Akkanee Piroth | *Capsicum chinense.* L. |
| S07 | PB8-1-1 | *Capsicum chinense.* L. |

Sample code: S01 and S02 are sweet chilli pepper
S03, S04 and S5 are hot chilli pepper
S06 and S07 are very hot chilli pepper

**2.4 Extraction procedure**

The optimum conditions for the extraction of capsaicinoids in hot chilli pepper were studied. The parameters affecting the yield of the capsaicinoids were investigated. There were extraction solvent, ratio of solvent to water, ratio of solvent to sample, extraction temperature and extraction time. Sample code S06 was used for the following experiment test based on the single parameter experiments.

**(1) Effect of extraction solvent**

For the extraction solvent, three kinds of organic solvents including methanol, ethanol and acetonitrile were used. The conditions were tested as following: 0.5 g sample, 20 mL of the solvent as extraction solvent, extraction temperatures at 50 ºC and extraction time 20 min.

**(2) Effect of different ratios of methanol and water**

The different ratios of methanol and water, the percentage of methanol including 50-100% were investigated. The rest of conditions were tested as following: 0.5 g sample, 20 mL of the methanol as extraction solvent was varied percentage of methanol, extraction temperatures at 50 ºC and extraction time 20 min.

**(3) Effect of solvent volume to sample mass ratio**

For the ratio of solvent volume (mL) to sample mass (g), a series of that ratio was carried out with different solvent volumes (5, 10, 15, 20 and 25 mL/ 0.5 g). The rest of conditions were tested as following: 0.5 g sample, 80% methanol solvent was varied volume, extraction temperatures at 50 ºC and extraction time 20 min.

**(4) Effect of extraction temperature**

The extraction temperature was also evaluated ranging from 30-60 ºC. The rest of conditions were tested as following: 0.5 g sample, 20 mL of 80% methanol solvent, extraction temperatures was varied temperature for 39, 40, 50 and 60 ºC and extraction time 20 min.

**(5) Extraction time**

The extraction time was also evaluated ranging from 10-60 min. The rest of conditions were tested as following: 0.5 g sample, 20 mL of 80% methanol solvent, extraction temperatures at 50 ºC temperatures and extraction time was varied time for 10, 20, 40 and 60 min.

**2.5 Determination of two major capsaicinoids**

An extraction of capsaicinoids in the chilli sample (0.5 g) was sonicated by ultrasonic assisted extraction with 20 mL of solvent. The extract was centrifuged and then filtered through Whatman No. 42 filter paper. The extract solvent was evaporated to dryness using a rotary evaporator. The residue was dissolved with the solvent to make up final volume of 5.0 mL. The capsaicinoids extract was then filtered through 0.45 μm filter membrane prior to analysis by RP-HPLC-PDA. The stepwise of extraction of capsaicinoids in hot chilli pepper were shown in Figure 2

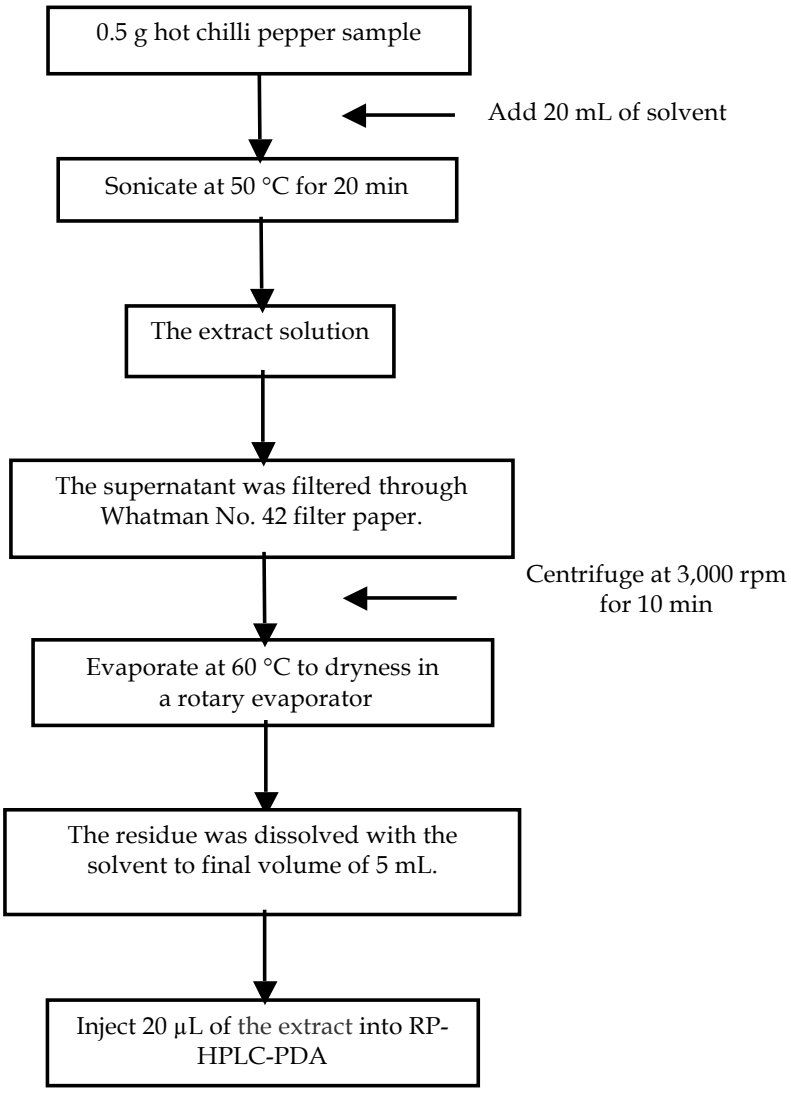


**Figure 2.** The stepwise of extraction of capsaicinoids in chilli pepper sample

The separation and determination of capsaiciniods were performed by RP-HPLC-PDA. For the chromatographic separation, an isocratic method was utilized with 70% acetonitrile as a solvent A; 30% DI water as a solvent B. The total run time was 10 min. The injection volume was 20 µL. Both major capsaicinoids (capsaicin and dihydrocapsaicin) were monitored at 280 nm at a flow rate of 1 mL/min. The chromatogram of two capsaicinoids were shown in Figure 3 and 4.

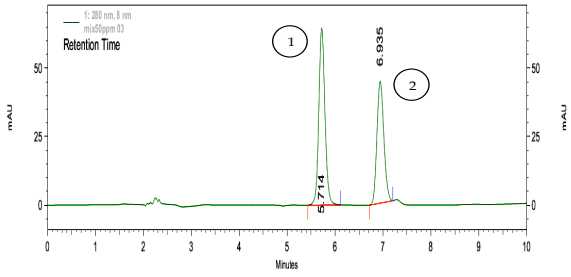


**Figure 3.** The chromatogram of two standard capsaicinoids (peak 1 : capsaicin and peak 2:dihydro-capsaicin)

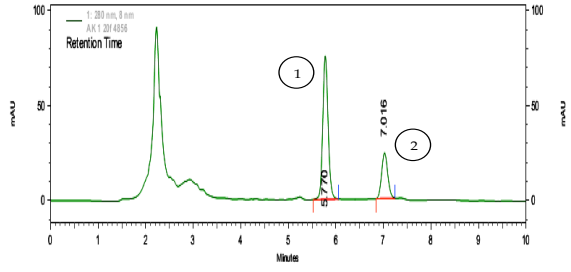


**Figure 4.** The chromatogram of two capsaicinoids extract from chilli pepper sample code S06 (peak 1 : capsaicin and peak 2:dihydro-capsaicin)

## 3. Results and discussion

### 3.1 Effect of extraction solvent

To select the suitable extraction solvent for capsaicinoids, three kinds of organic solvents including methanol, ethanol and acetonitrile were investigated. the capsaicinoids should be soluble in the solvent that is used for the extraction The results were shown in Figure 5.

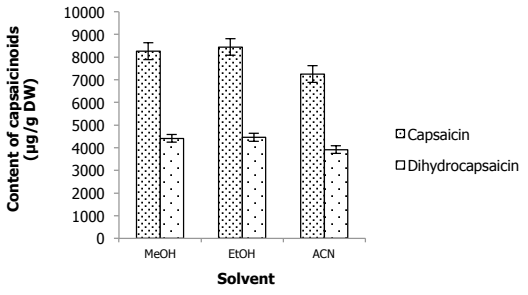


**Figure 5.** Effect of different extraction solvents on the extraction yields of capsaicinoids in chilli pepper sample code S06

As shown in Figure 5, The contents of the capsaicinoids were found rather different when using three kinds of the solvents extracted. It can be noted that both methanol and ethanol extracts gave similar amounts of the capsaicinoids. Acetonitrile was a fairly efficacious solvent. So, in this case, methanol was used as the extraction solvent because baseline of chromatogram was slightly better than ethanol extract.

### 3.2 Effect of different ratios of methanol and water

The different ratios of methanol in water were an important factor that must be optimized to increase the extraction yield of capsaicinoids. The results were shown in Figure 6.

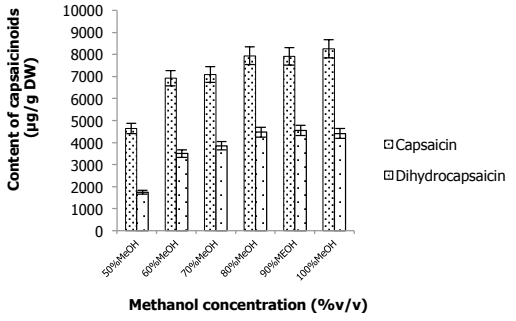


**Figure 6.** Effect of different methanol on the extraction yield of capsaici noids in chilli pepper sample code S06

As shown in Figure 6, the contents of the capsaicinoids were different depending on the mixed ratios of methanol and water. In the study of capsaicinoids, the addition of water to the solvent does not improve the extraction properties of methanol. The percentage of water in the methanol is increased, there is a decrease in the amount of capsaicinoids extracted. This may be because of the good solubility of capsaicinoids in methanol, which diminishes as the polarity of the solvent is increased by the addition of more water (22). It was found that the polarity of the solvent mixture decreased, resulted in an increase in the capsaicinoids contents when using up to 80-100% MeOH/$H_2O$. Thus, 80% methanol can be used as the extraction solvent because using a large amount of solvent was considered as a cost of solvents consumption.

### 3.3 Effect of solvent volume to sample mass ratio

The ratio of solvent volume (mL) to sample mass (g) is a factor that must be studied to increase the extraction yield of capsaicinoids. In this study, by varying the volume of solvent while holding the quantity of sample constant was condueted to evaluate the effect of the volume of the solvent. The results were shown in Figure 7.

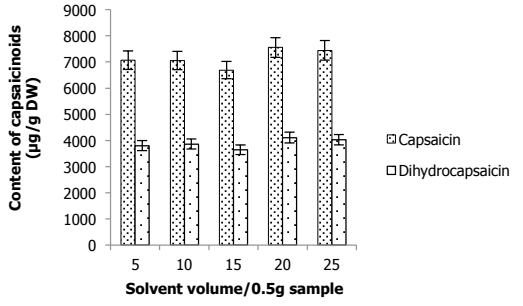


**Figure 7.** Effect of different ratios of solvent to mass on the extraction yield of capsaicinoids in chilli pepper sample code S06

As shown in Figure 7, the contents of the capsaicinoids were not much different when vary differences in the solvent volume/0.5 g sample. The large amount of sample results in longer extraction times and a large particle size demands higher volumes of solvent, the quantities of capsaicinoids extract were increased in higher volume of solvent. It was found that the solvent volume 20 mL give rather higher amount of the capsaicinoids. Therefore, the variable of solvent volume will not be a determining factor when extracting capsaicinoids in these conditions. It was decided to work with a volume of 20 mL of solvent /0.5 g sample.

### 3.4 Effect of extraction temperature

The extraction temperature was also evaluated ranging from 30 to 60ºC to increase the extraction yield of capsaicinoids The results were shown in Figure 8.

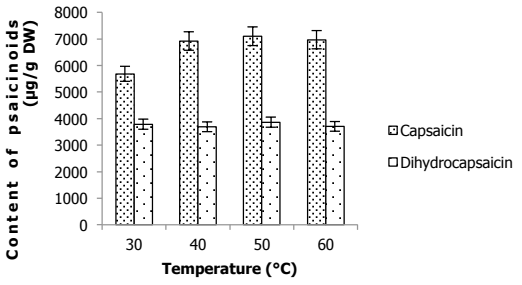


**Figure 8.** Effect of different extraction temperatures on the extraction yield of capsaicinoids in chilli pepper sample code S06

As shown in Figure 8, the contents of the capsaicinoids were different when varying the extraction temperature. It was noted that the extraction temperature around 40-60°C give similar amounts of the capsaicinoids. In generally, the higher the extraction temperature, the higher velocity of solvent molecules could accelerate mass transfer rate; and the larger solubility would enhance yield of target compounds. However, solvent volatilization and some degradation processes may occur at high temperature, which would lead to the lower extraction yield (23). Thus, In this study methanol was selected for the extraction of the capsaicinoids at 50°C due to a low boiling point of methanol (64.7 °C). A higher temperature for UAE means a higher efficiency in the extraction process due to the increase in the number of cavitation bubbles and in the surface contact area, but this effect is less when the temperature is near the boiling point (24).

### 3.5 Effect of extraction time

The effect of extraction time on the extraction yield of capsaicinoids was also evaluated ranging from 10 and 60 min. Generally, by increasing the extraction time, the quantity of analytes extracted is increased, although there is the risk that degradation may occur. To determine the time needed to obtain complete extractions, extractions of samples were performed for different periods of time. The results were shown in Figure 9.

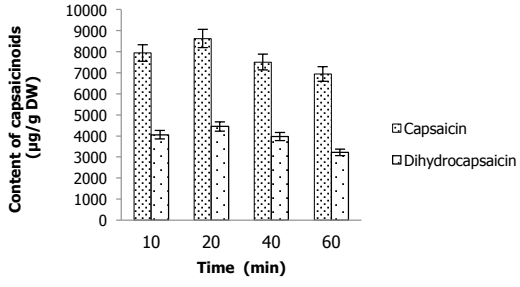


**Figure 9.** Effect of different extraction times on the extraction yield of capsaicinoids in chilli pepper sample code S06

As shown in Figure 9, the contents of the capsaicinoids were also different when the extraction time was varied. It was found that the extraction times longer than 20 min gave differences in the amounts of the capsaicinoids, a decrease in the extraction yield was observed, which may be explained considering the possibility of organic compounds decomposition by the effect of the sound waves. Thus, the extraction time of the capsaicinoids was selected at 20 min.

The summarize of the extraction conditions were shown in Table 2

**Table 2.** The summarize of the extraction conditions

| Parameters | Conditions |
|---|---|
| Extraction solvent | Methanol |
| Methanol concentration (%v/v) | 80 |
| Solvent volume/0.5g sample (mL) | 25 |
| Extraction temperature (°C) | 50 |
| Extraction time (min) | 20 |

**3.6 Determination of two major capsaicinoids**

Under the optimum conditions, the amounts of capsaicinoids were found in chilli pepper samples ranging from 573. 44-15219 µg/g DW, the results were shown in Table 3.

**Table 3.** The contents of the major capsaicinoids in chilli pepper samples (mean ± SD, $n = 3$)

| Sample | Capsaicin (µg/g DW) | Dihydrocapsaicin (µg/g DW) | Major Capsaicinoids (µg/g DW) |
|---|---|---|---|
| S01 | 319.10 ± 1.7 | 254.34± 2.3 | 573.44 |
| S02 | 345.47± 1.9 | 263.08± 1.9 | 608.55 |
| S03 | 1540.1±1.2 | 1227.3± 2.0 | 2767.4 |
| S04 | 1809.8± 1.3 | 1597.8± 1.6 | 3407.6 |
| S05 | 3009.5± 1.5 | 1411.7± 1.8 | 4421.2 |
| S06 | 9340.9± 1.0 | 4591.6± 1.6 | 13932.5 |
| S07 | 8209.7± 0.7 | 7009.8± 1.3 | 15219.5 |

In Table 3, Seven kinds of chilli pepper samples were used with their codes and pungent levels including; sweet chilli pepper (S1 & S2), hot chilli pepper (S3, S4 & S5) and very hot chilli pepper (S6 & S7). The amount of capsaicinoids depends on its pungency (25). S07 give high amount of capsaicinoids.

**4. Conclusion**

In this study, the detailed effects of ultrasound assisted extraction for extraction of capsaicinoids from hot chilli peppers were investigated. The determination of two major capsaicinoids in hot chilli samples was conducted. The optimal extraction conditions were obtained consisting of 80%

methanol and water as extraction solvent with 20 mL/0.5 g solvent to mass ratio, extraction temperature at 50°C and 20 min of extraction time. The developed method has been employed to perform the determination of the capsaicinoids present in seven varieties of hot peppers cultivated in Thailand. In a comparison between the others method of extraction, qualitative composition of ultrasonic extracts was the same as found in the extracts obtained using others extraction techniques.

## 5. Acknowledgements

This research was supported by the Higher Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Food and Functional Food Research Cluster of Khon Kaen University, and the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Education are gratefully acknowledged for the financial support and chemicals.

## 6. References

(1) Govindarajan, V. S., Rajalakshmi, D., & Chand, N. Capsicum production technology chemistry and quality. Critical Reviews in Food Science and Nutrition. 1987; 25: 185–282.

(2) Giuffrida, D., Dugo, P., Torre, G., Bignardi, C., Cavazza, A., Corradini, C. Characterization of 12 Capsicum varieties by evaluation of their carotenoid profile and pungency determination. Food Chem. 2013;140: 794–802.

(3) Huang, X. F., Xue, J. Y., Jiang, A. Q., Zhu, H. L. Capsaicin and its analogues:structure-activity relationship study. Current Med Chem. 2013; 20: 2661–2672.

(4) Garcia-Salas P., Morales-Soto A., Segura-Carretero A., Fernández-Gutiérrez A. Phenolic compound extraction systems for fruit and vegetable samples. Molecules. 2010; 15: 8813-8826.

(5) Barbero G.F., Palma M., Barroso C.G. Pressurized liquid extraction of capsaicinoids from peppers. J. Agric. Food. Chem. 2006; 54: 3231−3236.

(6) Chinn S.M., Sharma-Shivappa R. R., Cotter L. J. Solvent extraction and quantification of capsaicinoids from *Capsicum chinense*. Food Bioprod. Process. 2011; 89: 340–345.

(7) Davis CC. B., Markey C. E., Busch M. A., Busch K. W. Determination of capsaicinoids in Habanero peppers by chemometric analysis of UV spectral data. J. Agric. Food. Chem. 2007;55: 5925−5933

(8) Kozukue N., Han J. S., Kozue E.,Lee S. J., Kim J.A., Lee K. R., Levin C., et. al.. Analysis of eight capsaicinoids in peppers and pepper-containing foods by high-performance liquid chromatography and liquid chromatography−mass spectrometry. J. Agric. Food. Chem. 2005; 53: 9172−9181.

(9) Nwokem C.O., Agbaji E.B., Kagbu J.A., E.J. Ekanem. Determination of capsaicin content and pungency level of five different peppers grown in Nigeria. NY Sci. J. 2010; 3(9): 17-21.

(10) Pena-Alvarez A., Ramírez-Maya E., Alvarado-Suárez L. Analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces by solid phase microextraction-gaschromatography–mass spectrometry. J. Chromatogr. A. 2009; 1216: 2843–2847.

(11) Perucka I., and Oleszek W. Extraction and determination of capsaicinoids in fruit of hot pepper *Capsicum annuum* L. by spectrophotometry and high-performance liquid chromatography. Food Chem.2000;71:287-291.

(12) Kirschbaum-Titze P., C. Hiepler, E. Mueller-Seitz, M. Petz. Pungency in Paprika (*Capsicum annuum*) Decrease of Capsaicinoid Content Following Cellular Disruption. J. Agric. Food Chem. 2002; 50: 1260-1263.

(13) Contreras-Padilla M., E.M. Yahia. Changes in Capsaicinoids during Development, Maturation, and Senescence of Chile Peppers and Relation with Peroxidase Activity. J. Agric. Food Chem. 1998; 46 : 2075-2079.

(14) Santamaria R.I., M.D. Reyes-Duarte, E. Barzana, D. Fernando, F.M. Gama, M. Mota, A. Lopez-Munguia. Selective Enzyme-Mediated Extraction of Capsaicinoids and Carotenoids from Chili Guajillo Puya (*Capsicum annuum* L.) Using Ethanol as Solvent. J. Agric. Food Chem. 20004; 8: 3063- 3067.

(15) Chanthai S. Juaugsamoot J., Ruangviriyachai C., Techawongsa-tien S. Determination of capsaicin and dihydrocapsaicin in some chilli varieties using accelerated solvent extraction associated with solid-phase extraction methods and RP-HPLC-Fluorescence. E-J. Chem. 2012; 9(3): 1550-1561.

(16) Barbero G.F., Palma M., Barroso C.G. Determination of capsaicinoids in peppers by microwave-assisted extraction–high performance liquid chromatography with fluorescence detection. Anal. Chim. Acta. 2006; 578: 227–233.

(17) Karnka R., M. Rayanakorn, S. Watanesk, Y. Vaneesorn, Optimization of High-Performance Liquid Chromatographic Parameters for the Determination of Capsaicinoid Compounds Using the Simplex Method. Anal. Sci. 2002; 18: 661-665.

(18) Korel F., N. Bagdatlioglu, M.O. Balaban, Y. Hisil, Ground Red Peppers: Capsaicinoids Content, Scoville Scores, and Discrimination by an Electronic Nos. J. Agric. Food Chem. 2002; 50: 3257-3261.

(19) M.D. Luque de Castro, L.E. Garcia-Ayuso. Soxhlet extraction of solid materials: an outdated technique with a promising innovative future. Anal. Chi. Act. 1998; 369: 1-10.

(20) Barbero G.F., Liazid A., Palma M., Barroso C.G. Ultrasound-assisted extraction of capsaicinoids from peppers. Talanta. 2008; 75: 1332–1337.

(21) S. Balachandran, S.E. Kentish, R. Mawson, M. Ashokkumar, Ultrasonic enhancement of the supercritical extraction from ginger, Ultrason. Sonchem. 2006; 13: 471–479.

(22) Maria Ines Soares Melecchi, Valeria Flores Peres, Claudio Dariva, Claudia Alcaraz Zini, Fernanda Contieri Abad, Migda ´lia Miranda Martinez, Elina Bastos Caramao. Optimization of the sonication extraction method of Hibiscus tiliaceus L. flowers. Ultrason. Sonochem. 2006; 13: 242–250.

(23) Xiang-Yuan Deng, Kun Gao, Xin Huang, John Liu. Optimization of ultrasonic-assisted extraction procedure of capsaicinoids from Chili peppers using orthogonal array experimental design. Afri. J. Biotech. 2012; 11: 13153-13161.

(24) Palma M., C.G. Barroso. Ultrasound-assisted extraction and determination of tartaric andmalic acids from grapes and winemaking by-products. Anal. Chi. Act. 2002; 458: 119–130.

(25) Maria de Lourdes Reyes-Escogido, Edith G.Gonzalez-Mondragon, Erika Vazquez-Tzompantzi. Chemical and Pharmacological Aspects of Capsaicin. Molecules; 2011:16, 1253-1270.