Research Article

Comparative Study on the Extraction of Capsaicinoids from *Capsicum chinese* and their Analysis by Phosphomolybdic Acid Reduction and HPLC

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ABSTRACT

The major focus of the present study was to compare and to select a suitable solvent for the extraction and analysis of capsaicinoids from *C. chinense* by qualitative and quantitative methods. In this regard, the solvents acetonitrile and acetone were compared for their high yield of capsaicinoids. TLC was performed for qualitative assay whereas UV spectrophotometer, phosphomolybdic acid reduction and HPLC were used for quantitative analysis. Among the two solvents compared in this study, acetonitrile was found to be the best solvent for obtaining a high yield of capsaicinoid. The TLC plate revealed the presence of capsaicinoids in the two extracts. Preliminary quantification using UV spectrophotometer scanning showed peaks at 220nm and 280nm. The total capsaicin content in the extracts was found to be 4,694,074 SHU and 3,782,120 SHU. The chromatogram of HPLC showed that acetonitrile extract gave an extremely high yield of capsaicinoids with less impurity when compared to acetone. The analytical procedures followed in the quantification of capsaicinoids extracted from *C. chinense* were highly reliable and the results are coherent. The therapeutic analogue capsaicin from *C. chinense* can be eluted and further studied for its pharmaceutical applications.

Keywords: *C. chinense,* capsaicinoid, capsaicin, UV spectrophotometer, Phosphomolybdic acid reduction, HPLC

INTRODUCTION

Capsaicinoids are phenolic compounds normally present in the genus *Capsicum*, of which capsaicin is the most abundant[1]. Several studies have reported on the increase of capsaicinoids in *Capsicum* fruits with regard to the fruit age, size, different stages of development and nutrient stresses. The capsaicinoids begin to accumulate in the early stages of fruit development and they reach a maximum rate as the fruit matures[2]. The level of capsaicinoids varies according to the different pepper cultivars examined [3-5]. *C. chinense* cv. Red Savina was documented as the hottest chilli pepper with a heat level of 577,000 Scoville Units (SHU) [6]. Bosland and Baral[7] reported that ''Bhut Jolokia'', a natural interspecific hybrid between *C. chinense* and *C. frutescens*, is in fact the world's hottest known chilli pepper with a heat level of 879,953 to 1,001,304 SHU. The level of capsaicinoids in the capsicum fruits can be quantified by organoleptic method,[8,9] spectrophotometry,[10,11] thin-layer chromatography [12], gas–liquid chromatography[13] and high-performance liquid chromatography [14]. Of these, high-pressure liquid chromatography (HPLC) is considered the most reliable and rapid method available for the identification and quantification of capsaicinoids [15]. Capsaicin is the active ingredient in several drugs including Capzasin-P (cream) and Icy Hot PM (patch), in topical analgesics used against pain, anti-arthritic and anti-inflammatory ointments [16-19], and also possess powerful antioxidant,[20] anti-mutagenic and anti-tumoral properties.[21-22] In view of its broad biological activities, the preparation of capsaicinoids requires development of efficient extraction protocol. Hence the current work therefore focused on the comparative study on the extraction efficiency of total capsaicinoids of acetone and acetonitrile from *C. chinense* Bhut Jolokia.

MATERIALS AND METHODS

Capsicum chinense fruits were obtained from Manipur, North India. The morphology of the fruit shape, colour, seed colour and size of the *C. chinense* were examined following Moscone[23] and Dias[24]. The fruits were dried by traditional method i.e. sun dried for a day, ground, sieved through 20–30 mesh and kept in air tight containers until further processing. The standard Capsaicin (8-methyl-*N*-vanillyl-trans-6-nonenamide) was purchased from Sigma Chemical Co, St. Louis, MO, USA. All solvents used for capsaicinoids analysis were of HPLC grade from Merck.

Extraction

The extraction and quantification of capsaicinoids in acetone and acetonitrile were performed according to Collins[25] with slight modifications. The chilli powder was mixed with acetone and acetonitrile solvents in the ratio of 1:10 (gram: milliliter). The mixture was placed in 120 ml glass bottles with Teflon lids. The bottles were capped and placed at 65ºC in water bath for an hour and were swirled manually. The samples were removed from the water bath and cooled at room temperature. The supernatant was centrifuged at 10000 rpm and filtered through Whatman No.1 filter paper. The filtrates were evaporated to dryness and the crude obtained was stored at 5ºC in refrigerator until further analysis.

Qualitative analysis - Thin layer Chromatography

The presence of capsaicinoid in the solvents was indentified using thin layer chromatography. It was performed on TLC silica gel 60 F254 aluminum sheets

(Merck). The standard capsaicin at concentration of 1mg/ml was spotted as a reference on the TLC plate. Aliquots of 10µl of the two extracts were spotted onto the plates. The plates were dried in a hood for 10 min. Petroleum ether: Chloroform: Acetonitrile in the ratio of 40: 45: 15 as used as the mobile phase. The chromatogram was developed in iodine chamber and viewed under the UV light at 302 nm. The standard capsaicin Rf value was calculated and compared with the extracts.

Quantification by UV spectrophotometer

A simple linear regression curve was plotted using standard capsaicin, purchased from Sigma Chemical. A stock solution of 1mg/ml capsaicin in ethanol prepared and different concentrations from 10 µg to 100 µg were prepared from the stock solution. The optical density was recorded at 280 nm. The linear regression equation was generated using the online Statistics and forecasting software (www. wessa.net). The capsaicinoid extracted from solvents was estimated by UV visible spectrophotometer (Hitachi- U1800). The crude extract was diluted to 300X using the respective solvent. The optical density was recorded at 280 nm. The capsaicinoid concentrations in the samples were calculated using capsaicin linear regression equation and it was expressed as µg/ml of capsaicin and finally converted to Scoville Heat Unit.

Quantification of Total capsaicin

Capsaicin is a protoalkaloid which is responsible for the pungency and the quality of the chilli fruit. The phenolic group in capsaicin reduces the Phosphomolybdic acid to lower acids of molybdenum. The resulting compound appeared blue incolour which was directly proportional to the concentration of capsaicin and was read at 650 nm (Fig1). Standard capsaicin solution was diluted to the range of 100 µg, 80 µg, 60 µg, 40 µg and 20 µg and linear regression curve was generated using the online Statistics and forecasting software (www. wessa.net). Five hundred miligram of dry chilli powder was weighed into a glass stopper test tube and 10 ml of dry acetone (25 g anhydrous sodium sulphate mixed into 500ml acetone of analytical grade at least 1 day before use) was added and kept in shaker for an hour. The content was centrifuged at 10000 rpm for 10 min. One mililitre of the clear supernatant was pipetted out and was dried in a hot water bath. The residue was dissolved in 5 ml of 0.4% sodium hydroxide solution and 3 ml of Phosphomolybdic acid was added and kept for an hour. The solution was centrifuged to remove the floating debris. The coloured solution was directly read at 650 nm. The amount of capsaicin was expressed in µg/ml and finally converted to Scoville Heat Unit.

Quantification by Liquid chromatography

The samples were filtered through 0.45 µm (Millipore filter) using a 5 ml disposable syringe (Millipore, Bedford, MA) into a sample vial. A HPLC system Shimadzu (LC-10, Shimadzu, Japan) equipped with LC- 10AS multisolvent delivery system, a SPD-10A UV-Vis detector at wavelength fixed at 280 nm and controlled parameters with system controller unit (SCL-10A) was used. The analysis was carried out under the following conditions: column temperature 30^{o}C, flow rate of mobile phase 1 ml/min and data acquisition was made using Class LC-10 software. All analyses were performed isocratically using degassed HPLC grade 50% acetonitrile (Merck, Germany) and 50% milli 'Q' water as a mobile phase.

The reverse-phase chromatographic column (Discovery C18 (250 x 4.6 mm, 5 mm), Supelco, Bellefonte, PA, USA) was used for the detection of capsaicin and dihydrocapsaicin (Margaret *et al*., 1995).

Simple linear regression curve for standard capsaicin by HPLC

The simple linear regression curve was plotted using standard capsaicin purchased from Sigma Chemicals. A stock solution of 1mg/ml capsaicin per milliliter of ethanol was prepared and different concentrations of 1000 µg, 600 µg, 200 µg, 50 µg and 1 µg were prepared. Standard curves for capsaicin and dihydrocapsaicin were plotted using concentration versus peak area. Total capsaicinoids in the test samples were quantified using linear equation.

Quantification of capsaicinoids in acetone and acetonitrile extracts by HPLC

Five µl aliquot of the sample was used for each HPLC injection. The capsaicinoids were identified with reference to retention time of standards and by spiking the samples with standards. The major capsaicinoids in peppers, capsaicin and dihydrocapsaicin, were determined by comparison to external reference standards injected under the same conditions. Their identification was based on the retention times measured under identical HPLC conditions while the quantitative determination in the different peppers samples was carried out using the peak areas. The ratio between these capsaicinoids was calculated by dividing capsaicin and dihydrocapsaicin contents by the total capsaicinoids[26]. The capsaicinoids concentrations in samples were expressed as µg/g pepper.

Scoville Heat Unit Conversion

According to the commonly accepted Scoville organoleptic test, the spicy strength of the investigated samples was calculated by converting the capsaicin content expressed in grams of capsaicin per gram of pepper. This conversion to Scoville heat units was done by multiplying the capsaicin content in pepper dry weight by the coefficient corresponding to the heat value for pure capsaicin.[27]

RESULTS

The simple linear regression curve was plotted for standard capsaicin purchased from Sigma Chemical. The linear regression equation was generated using the online

statistics and forecasting software (www. wessa.net). The amount of capsaicin in acetone and acetonitrile extract was calculated using the following equation (Y = 0.00919X – 0.0084) for UV spectrophotometer estimation (Fig 1a) and (Y= 0.0367 + 0.006275) for the total capsaicin estimation (Fig 1b). The HPLC analysis of capsaicin and dihydrocapsaicin standard linear regression curve was generated using (Y=13044.1603X + 3607.917) and (Y = 100545.430X + 3521.201) respectively (Fig 1 c&d). The calibration curve was used to determine the reference concentrations for the acetone and acetonitrile extract samples. The capsaicinoid contents obtained in µg/g were converted to Scoville heat units in order to classify them according to their various pungency levels. The standard capsaicin Rf value 0.078 coincided with the spot observed in extracts. The TLC profile of acetonitrile has capsaicin spot with less impurities when compared to acetone (Fig.3a). In UV estimation acetone and acetonitrile extracts showed high pungency level with 1,347,439 SHU and 1,266,250 SHU respectively (Fig 3 b & c). The total capsaicin content in the extracts was estimated and found that acetonitrile extracts have high pungency of 4,694,074 SHU with better yield of capsaicin. The chromatograms obtained for acetone and acetonitrile crude extracts of Bhut Jolokia showed two major peaks, identified as capsaicin and dihydrocapsaicin, which registered a difference of 0.38 min between the retention periods of the capsaicin (5.18 min) and the dihydrocapsaicin (5.56 min). The HPLC analysis of acetonitrile and acetone extracts showed 11,161,030 SHU and 5,948,120 SHU for capsaicin and 4,830,189 SHU and 2,826,335 SHU for dihydrocapsaicin respectively (Fig 5). The chromatogram of HPLC showed that acetonitrile has extremely high pungent capsaicinoids with less impurity (Fig.4).

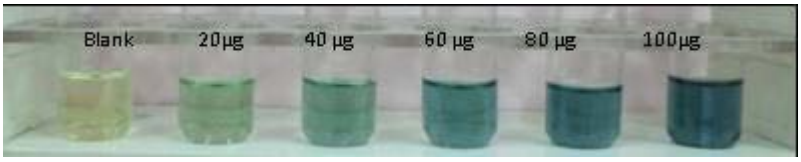


Figure 1: Phosphomolybdic acid reduction of capsaicin

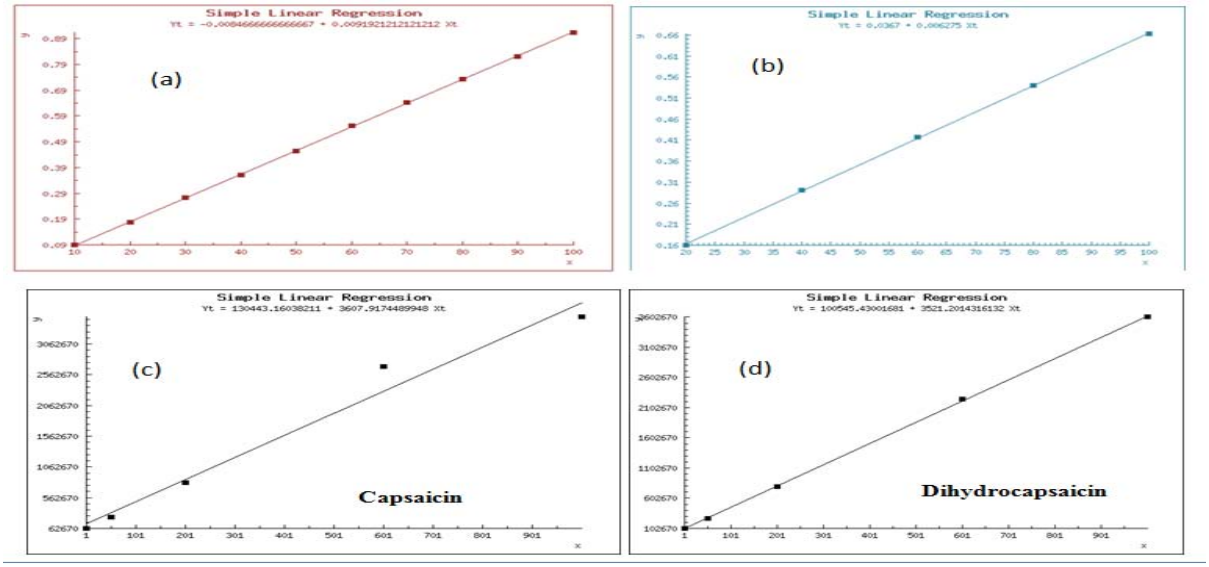


Figure 2: Standard linear regression curve for capsaicin by (a) UV spectrophotometer, (b) Phosphomolybdic acid reduction and (c) capsaicin, (d) dihydrocapsaicin, quantified by high pressure liquid chromatography (HPLC)

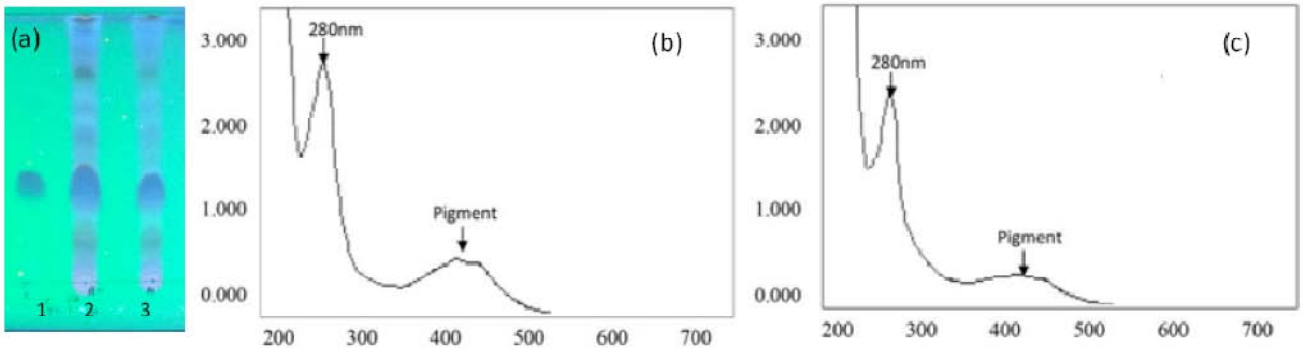
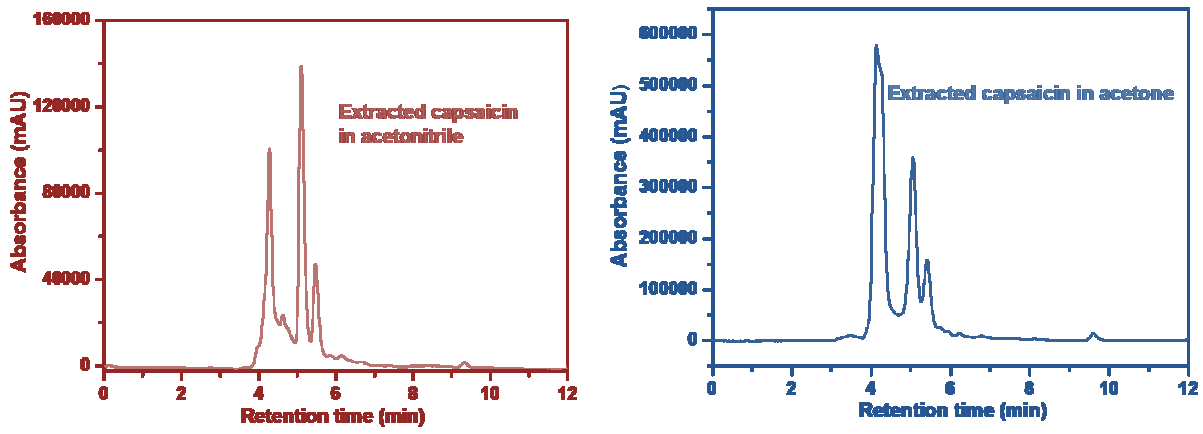


Figure 3: (a)TLC plate under UV light (spot 1- standard capsaicin, spot 2- acetonitrile and spot 3- acetone), UV spectrum of (b) Acetone and (c) Acetonitrile extract of *Capsicum chinense* fruit



(a) (b)

Figure 4: High-performance liquid chromatography (HPLC) chromatogram: (a) acetonitrile extract (b) acetone extract with the peaks of capsaicin and dihydrocapsaicin were identified by comparing retention times.

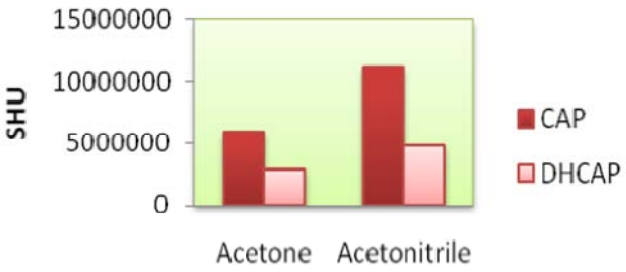


Figure 5: HPLC analysis acetone and acetonitrile extracts of capsaicin and Dihydrocapsaicin of *C. chinense*

DISCUSSION

Capsaicin and dihydrocapsaicin are the two major compounds responsible for the pungency of *Capsicum* fruits. Quality of the pepper was graded according to the pungency level, and therefore reliable methods aimed to extract and analyze these compounds are needed. According to our previous report, acetone and acetonitrile solvents showed higher content of capsaicinoids, among the non polar solvents, polar aprotic solvents and polar protic solvents extractions [28]. Capsaicinoids were determined using a validated HPLC method, which allowed reliable identification and quantification of capsaicin and dihydrocapsaicin. This analytical method is used for compound determination on HPLC retention time. Earlier determination of total capsaicinoids has relied on direct measurement of ultraviolet absorption or colorimetric method using Folin-Ciocalteu reagent by UV-Visible spectrometry[29-30]. The reagent is not specific for capsaicinoids and hence other compounds may interfere. According to Sadasivam and Manikkam [31] total capsaicin content in the samples was estimated by spectrophotometric measurement of the blue coloured component formed as a result of reduction of phosphomolybdic acid to lower acids of molybdenum which is directly proportional to the capsaicin. In our experiment the different concentration of capsaicin solution were reduced to give blue colour and the intensity of the colour was directly proportional to the concentration of the capsaicin (Fig 1). Qualitative analysis of the extracts was done by thin layer chromatography. TLC method is provides a chromatographic plant extract fingerprint[32]. In our study the capsaicin spot was analogous to the reference spot confirming the qualitative test. The HPLC chromatogram was obtained with a commercial capsaicin using absorbance at 280 nm. The sample gave a chromatogram of capsaicin and dihydrocapsaicin along with minor amounts of the other capsaicinoids. Standard solutions of commercially available capsaicin were prepared and subsequently used to prepare an HPLC calibration curve of total peak area and the sum of the peak areas for capsaicin and dihydrocapsaicin gave the total concentration of the two major capsaicinoids. The peaks eluting prior to 4.5 min on this chromatogram are carotenoids and other pigment materials in the sample. In the chromatogram, both capsaicin and dihydrocapsaicin give strong and well-resolved peaks. Among the two test solvents used in this study, acetonitrile showed less pigments with high capsaicin content when compared to the acetone. At the time interval of 4.5 min the pure capsaicin can be eluted for commercial applications. According to Joseph Amruthraj[33] the acetonitrile extracts of *C. chinense* fruits showed wide antibacterial activities against the human pathogens and concluded that capsaicin present in *C. chinense* fruits is very effective in the prevention of many diseases.

CONCLUSION

C. chinense has immense ethnopharmacological potential. The scientific literature on the impact of capsaicinoids in therapy as potential molecules is the current interest. The preparation of capsaicinoids requires efficient extraction

and estimation protocol. We conclude that the analytical procedures followed in the quantification of capsaicinoids extracted from *C. chinense* were highly reliable and the results were coherent. The results also suggested that the therapeutic analogue capsaicin from *C. chinense* can be eluted and quantified by the above mentioned protocols of the current study. Further more extensive ethnomedicinal investigations are to be carried out for understanding its pharmaceutical applications.

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