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Qualitative and Quantitative Analysis of Capsaicin from Capsicum annum Grown in Jordan

Ali Al-Samydai*[1], Talal Aburjai[1], Walhan Alshaer[2], Hanan Azzam[3], Farah Al-Mamoori[1]

[1]Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Jordan, Amman, Jordan
[2]Cell Therapy Center, The University of Jordan, Amman
[3]Hamdi Mango Center for Scientific Research, University of Jordan, Amman, Jordan

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ABSTRACT

As there is an increasing demand for Capsaicin from pharmaceutical firms worldwide, the aim of the present study is to find a simple, fast, and reproducible method for its extraction from the fruit of *capsicum annuum*. Soxhlet extraction method using n-hexan as a solvent was used. Capsaicin was isolated and purified using a combination of column chromatography (CC), and thin-layer chromatography (TLC). High-Performance Liquid Chromatography (HPLC) was used for qualitative and quantitative analysis. Capsaicin was further identified by a spectroscopic method, including nuclear magnetic resonance (NMR) and Mass spectroscopy). The extraction yield was 11.5%, and Capsaicin concentrations in the extract was 8332.3 mg/kg with purity up to 73.95%, then purification of the extract was done by column chromatography, through using two solvent system until the purified Capsaicin detected and collected. Our results present a simple, fast, and reproducible method for the extraction of Capsaicin from the fruit of *C. annuum* with an almost high yield. The main advantage of the present method, in addition to its simplicity, it cut many steps that usually found in the literature.

*Corresponding Author

Name: Ali Al-Samydai
Phone: 0788106069
Email: phalimahmoud2012@yahoo.com

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INTRODUCTION

From ancient civilizations, plants have been used by humans as medicines. From that point, traditional medical has been developed as a systems where plants incorporated in therapy as one of the important sources of drug discovery (Al-Mamoori *et al.*, 2019; Alsamydai *et al.*, 2018; Al-Samydai, 2018).

Capsicum annuum (C.*annuum*) is one of the popular peppers worldwide, usually used to provide a hot and spicy taste. Capsaicinoids are flavor test in red chili peppers come from Capsaicinoids compounds, mainly composed of Capsaicin (8-methyl-N-vanillyl-6-nonenamide) a secondary metabolite of chilli peppers (Figure 1). From ancient times capsicum plants are cultivated throughout the world (Puvača, 2018). Nowadays, Capsaicin demand in pharmaceutical firms is growing (Hayman and Kam, 2008).

Capsaicin is an irritant compound and produces a feeling of burning when it taught in any tissue (Simonovska *et al.*, 2016). Pure Capsaicin is a hydrophobic, colorless, odorless, crystalline to waxy compound (Simonovska *et al.*, 2016). The main Capsaicinoid in *C. annuum* is Capsaicin and then followed by dihydrocapsaicin (Mongkolporn, 2018).

Numerous research have demonstrated that Capsaicin has anti-inflammatory, anti-cancer, anti-diabetic, anti-coagulant and hypolipidemic

activity (Reyes-Escogido *et al.*, 2011; Liu *et al.*, 2012; Zeyrek and Oguz, 2005; Tsuchiya, 2001; Kar *et al.*, 2016).

In the present study, we focus on analytical methodologies, which include the extraction, isolation, purification by new approaches, and characterization of Capsaicin in Jordanian *C. annuum.*

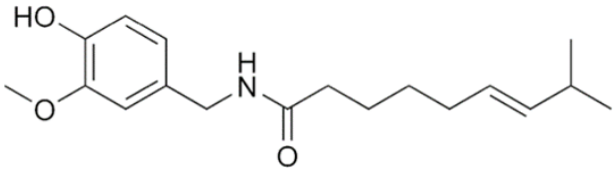

Figure 1: Structures of capsaicin

Table 1: Parameters of system suitability

No.	Parameters	Capsaicin
1	Retention Time	3.22
2	Theoretical plate	1449
3	Area (AUC)	750.355
4	Asymmetry	1.52

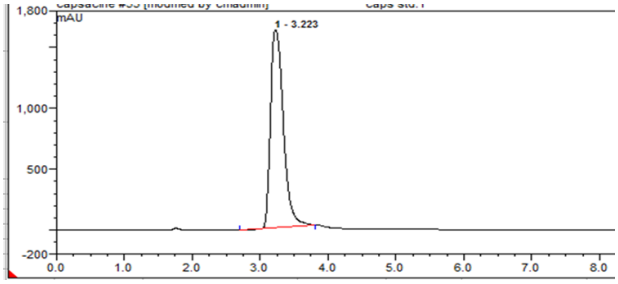

Figure 2: Chromatogram of standard Capsaicin

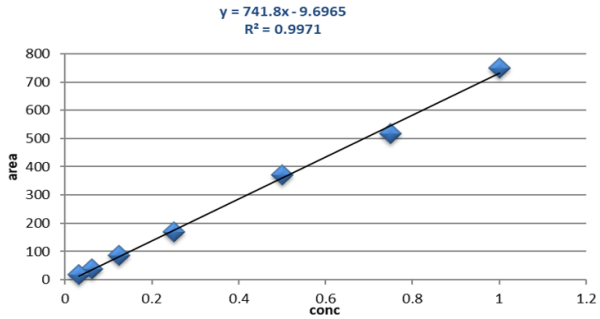

Figure 3: Calibration curve of peak area versus concentration for capsaicin

MATERIALS AND METHODS

Plant material

C. annuum was collected from the Al-Gor region (30km South-West Amman) in Spring 2019. The plant fruits were taxonomically identified by direct comparison with an authenticated sample at the herbaium of Faculty of Science, The University of Jordan, and with the help of Prof. Dr. Dawud AL-Eisawi, Department of Biological Science, Faculty of Science. The University of Jordan. A voucher specimen No. CAP. 2019 was deposited at the Department of Pharmaceutical Sciences, School of Pharmacy, the University of Jordan (Phytochemistry lab.). Three kgs of plant fruits were oven-dried (Temp. 30^{o} C) and then powdered finely.

Table 2: Precision values of Capsaicin

Sample name	Capsaicin AUC
Injection 1	356.194
Injection 2	355.242
Injection 3	355.187
Average	355.541
Standard Deviation (SD)	0.566183
Relative Standard Deviation (% RSD)	0.159245

Chemical and reagents

Chloroform, methanol, n-hexane, p-ether, and acetonitrile. All the above chemicals were of analytical and HPLC grade, were purchased from Fisher Scientific. Capsaicin standard were got from Sigma (USA). de-ionized water which used to prepare the aqueous solutions was 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.

Preparation of plant extracts

The plants extract from *C. annuum* was obtained by Soxhlet extraction; n-hexane was used as an extraction solvent. A rotary evaporator was used to concentrate the extract under pressure and heating. The Dried crude extract was stored in amber tubes and placed under 4°C for farther studies.

Separation of Capsaicin by silica gel column chromatography

Two mobile phase systems were used for Capsaicin separation. In the first column n-hexan: p-ether (1:1 v/v) was used for washing out (de-fatting), then methanol was added gradually (1% increment up to 8%).

Fractions collected were inspected in comparison with standard capsaicin. Fractions containing Capsaicin were collected and dried under reduced pressure using a rotary evaporator. The dried materials were subjected for a new column (n-silica gel) using chloroform: p-ether (1:1v/v) with increasing polarity up to 8:2.

Characterization of Capsaicin

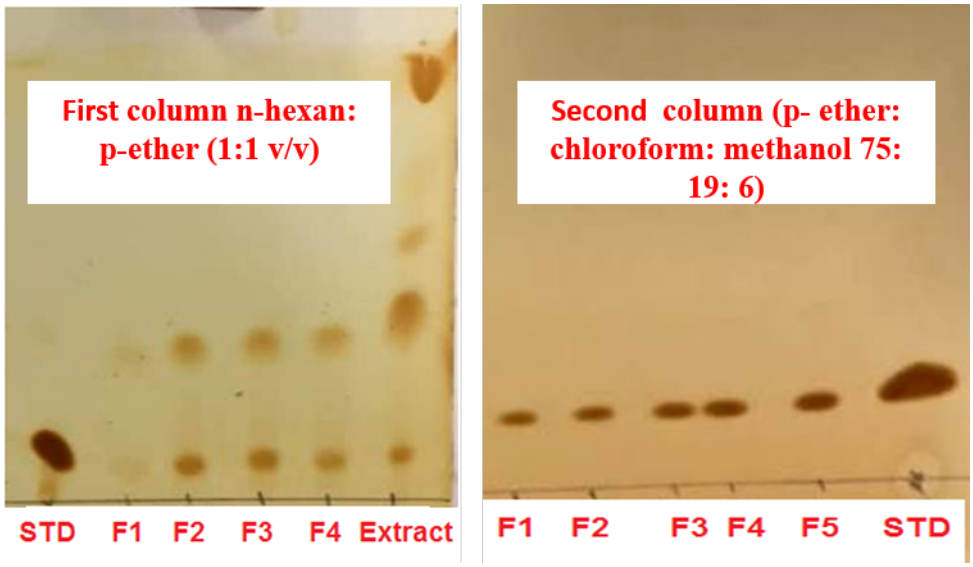

Figure 4: TLC of stander Capsaicin, extract *C. annuum* and purified Capsaicin from Jordanian *C. annuum;* Mobile phase (p- ether: chloroform: methanol 75: 19: 6)

TLC analysis

The fractions were exposed to TLC (20×20 cm aluminum sheet coated with silica gel 60 F254) to detect the presence of capsaicin.

TLC plates were immersed in the mobile phase (p-ether: chloroform: methanol 75: 19: 6) and sprayed with iodine, each Rf spot compared with standard Capsaicin spot.

HPLC analysis

Instrumentation and chromatographic conditions

An HPLC (DIONEX UltiMate^{TM}3000). (Thermo Fisher Scientific, Waltham, MA, USA). The detector (UV-VIS-PDA Detector). the pump (solvent delivery systems pump) (UltiMate^{TM} 3000) and the auto sampler (UltiMate^{TM} 3000).

The computer software used was Chromeleon®. HPLC system was set at a wavelength of 220 nm (Othman *et al.*, 2011), and coupled with aKromasil®C-18 Column (KNAUER, Germany); (150 mm x 4.6 mm, 5μm) with a flow rate of 0.5 ml/min and column temperature 40^{o} C using a 20 μl injection volume. The mobile phase was (80% methanol, 20% acetonitrile).

Preparation of standard Capsaicin solution

Ethanol was used to dissolve Standard capsaicin, 6 mg of Capsaicin in 6ml ethanol to obtain a final stock solution with a concentration of (1.00mg/ml). More dilutions were made to obtain (0.75, 0.50, 0.25, 0.125, 0.0625, 0.03125) mg/ml then standard curve was drawing.

Method development

During the development of the procedure, various conditions were optimized to find the most appropriate methods for the determination of capsaicin. Several wavelengths have been proceeding, but to achieve high sensitivity, the detection was performed at 220nm (Othman *et al.*, 2011). And different mobile phase compositions and ratios were applied. The mobile phase composed of (80% methanol, 20% acetonitrile) at a flow rate of 0.5 ml/min generated a sharp peak (Aburjai *et al.*, 2019)

Validation

System Suitability Parameters

The stock solution was injected into the chromatographic system, and system suitability parameters were determined (Table 1).

Specificity

The method was found to be specific since there was no interference of the mobile phase in the retention time of the analytical peak (Figure 2).

Linearity

The linearity range was found to 0.0 mg /ml to 1 mg /ml for Capsaicin calibration curves were plotted between the peak area and the concentrations. The linear regression coefficients for Capsaicin found to be 0.997 (Figure 3).

Precision

In the method precision study %, RSD was found to be less than 2 %. This indicates that the method has good repeatability (Table 2).

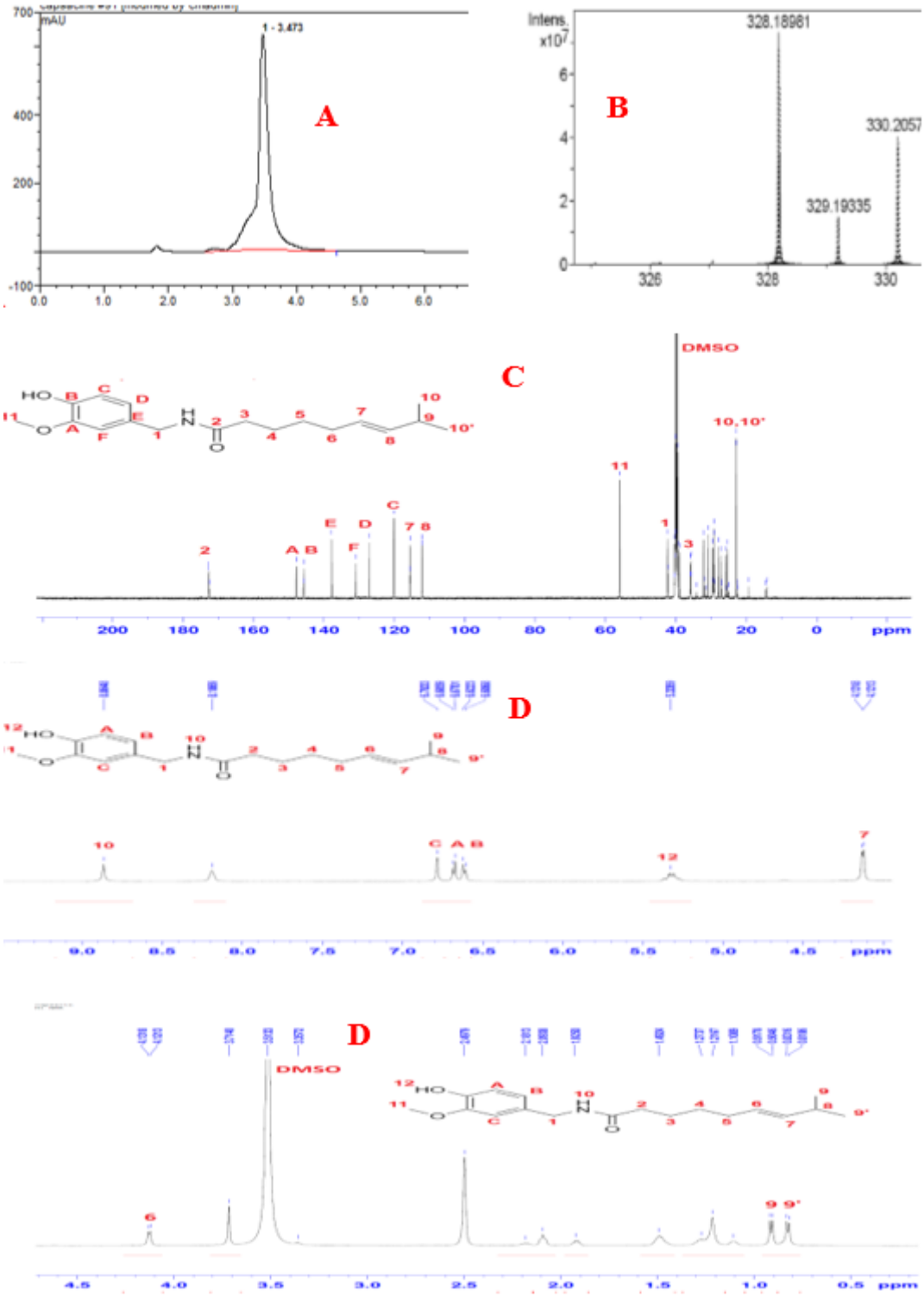

Figure 5: Purified Capsaicin obtained from silica gelcolumn; ^{A}-HPLC methanol: acetonitrile (8:2, vol:vol) extract AUC(507.433); ^{B}- Mass spectrum of capsaicin; ^{C}-^{13}CNMR spectrum of Purified capsaicin, ^{D}- ^{1}H NMR spectrum of Purified Capsaicin

Mass and NMR spectroscopy

Mass spectra were measured in negative ion mode using the electrospray ion trap (ESI) technique, a Bruker Apex-4 (Tesla) instrument (Bremen, Germany). The analytical results for the elements were within±0.4% of the theoretical values.

^{1}H-NMR and ^{13}C -NMR spectra were measured on a Bruker UltraShield instrument operating at 500MHz.

RESULTS AND DISCUSSION

The current study presents a fast, cheap, and reproducible method for the extraction of Capsaicin from C. annum. The advantages of the method used in this study for extraction minimize the extraction time up to 60% and increase the yield of Capsaicin 15 folds compared to similar studies.

A large number of extraction methods for Capsaicin from C. annum have been used over the last decades. While designing an extraction process, the selection of a suitable solvent is the first step to produce a higher yield of the desired compound. Besides solvent selection step, several factors must be considered in order to achieve high extraction efficiency, such as time and temperature of extraction, extraction solvent and volume of extraction solvent, sample quantity, the reproducibility and repeatability of the methods. Capsaicin is extracted by a number of methods such (Bajer *et al.*, 2015), magnetic stirring (Santos *et al.*, 2015), enzymatic extraction (Bajaj and Kaur, 1979), microwave - (Thapa *et al.*, 2009) and ultrasound-assisted extraction (UAE) (Peusch *et al.*, 1997; Pruthi, 2003), Soxhlet (Musfiroh *et al.*, 2013), supercritical fluid (Nag *et al.*, 2017), and pressured liquids extraction (PLE) (Jarret *et al.*, 2013).

The Soxhlet extraction is widely applied method to extract the oil from organic matrix, which is used when the chosen compound has limited solubility in a solvent while the impurities are insoluble in this solvent; n-hexanee was used for extracting Capsaicin from C.anuum for 4 hours at 60^{o} C with extraction yield 11.5% close to pervious study (Nag *et al.*, 2017). In Previous studies, Capsaicin was extracted from C.anuum fruit by Soxhlet apparatus using Acetone, Ethanol, Ethyl acetate, Dichloromethane, Ethyl ether, Hexane or acetonitrile alone or in combination as solvents. Most of these studies revealed that solvent nature and condition significantly affected Capsaicin yields (Bajer *et al.*, 2015; Santos *et al.*, 2015). Depended on previous results, which mention that n-hexane is a solvent of choose to extract Capsaicin by using soxhlet (Stoica *et al.*, 2016).

In the present study the separation was done by column chromatography and TLC, with two solvent system first on as washing out (de-fatting), hexan: p-ether (1:1 v/v) then methanol was added gradually (1% increment up to 8%), while the second system is chloroform: p-ether (1:1v/v) with increasing polarity up to 8:2, until the purified spot Capsaicin detected on TLC (p- ether: chloroform: methanol 75: 19: 6), then collect the fraction and HPLC On the other hand, TLC system is (p- ether: chloroform: methanol 75: 19: 6) Use of these systems seems to be interesting because the possibility of determination of capsaicin. The results of Rf values (0.173), as seen in (Figure 4), and collected fractions were tested by HPLC, Mass spectrum, ^{1}H, and ^{13}C NMR, as shown in Figure 5.

After applied Linear regression equation, % yield of Capsaicin was 0.833 with purity up to 73.95% and this results is matching with previous studies (Musfiroh *et al.*, 2013), while mean concentration of Capsaicin in Jordanian C. anuum, was 8332.3 mg/kg which was higher than previous studies using of different methods of extraction and different sources country of C. anuum (Othman *et al.*, 2011; Nag *et al.*, 2017; Sanatombi and Sharma, 1970). Current methods present fast, cheap, reproducible and nearly specific method for the extraction of Capsaicin from C. anuum with almost high yield which could be used to produce the quantities required for in -vivo, in -vitro studies and for clinical trials, in additional to all above HPLC method of Capsaicin detection, which has been developed shown minimizing the retention time up to 65%, increase the sharpness of peak, and used simple mobile phase without interring of acid.

CONCLUSION

We have optimized the isolation and purification of Capsaicin from Jordanian C. anuum fruit. In addition, this method is shown fast, reproducible, and could be commercialized at the industrial level for ensuring the highest quality of C. anuum as raw material for food and pharmaceutical preparation for the extraction with high yield. Also, this procedure is readily accessible to produce the quantities required for in -vivo, in -vitro studies and for clinical trials, or for conversion to derivatives that are added medicinal effect potential.

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