Validated HPLC Method and Quantitative Analysis of Capsaicin in Capsicum Cream

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Abstract

The study was aimed to validate analytical method for analysis of capsaicin in capsicum cream using high performance liquid chromatography (HPLC). The analytical method is a requirement for quality control in the drug manufacturing. In this present study the analysis condition was optimized from AOAC official method. The HPLC analysis was performed on a reversed phase C_{18} phenyl column at 35 ^{o}C and the mobile phase was isocratic ratio of 1% acetic acid in water and acetonitrile (45:55). The flow rate was 1 mL/min and detected at the wavelength of 280 nm. The validated parameters were linearity, accuracy, precision of three concentration levels (50-150% of test concentration, n=2, 5 replicates), limit of detection, limit of quantitation, specificity, reproducibility and robustness. The results indicated good linearity (R^2 > 0.999), accuracy (%recovery = 97-100%), precision and reproducibility (%RSD < 2.0) of this HPLC method. The method was specific and selective for capsaicin and also robust to changing of column temperature by 1 ^{O}C. The quantitative analysis of capsaicin in capsicum cream was within 90-110%w/w according to USP standard criteria. In conclusion, this optimized method can be practically used in routine analysis of capsicum cream efficiently.

Keywords**:** *Capsaicin, Capsicum cream, HPLC, Method validation*

บทคัดย่อ

การศึกษานี้มีจุดมุ่งหมายเพื่อตรวจสอบความน่าเชื่อถือของวิธีวิเคราะห์แคปไซซินในครีมเตรียมจากสารสกัดพริกด้วยเครื่องโครมาโต-กราฟีชนิดของเหลวสมรรถนะสูง วิธีวิเคราะห์เป็นข้อกำหนดสำหรับการควบคุมคุณภาพในอุตสาหกรรมการผลิตยา สภาวะการวิเคราะห์ที่ใช้ในการศึกษาครั้งนี้ปรับจากวิธีมาตรฐานที่กำหนดโดย AOAC การวิเคราะห์ใช้คอลัมน์ชนิดรีเวอร์เฟส คาร์บอน18 เฟนิล ที่อุณหภูมิ 35 องศาเซลเซียส เฟสเคลื่อนที่ คือ น้ำที่มีกรดอะซิติกความเข้มข้นร้อยละ 1 และอะซิโตไนไตรล์อัตราส่วน 45 ต่อ 55 โดยปริมาตร อัตราการไหลของเฟสเคลื่อนที่เป็น 1 มิลลิลิตรต่อนาที และตรวจวัดสัญญาณที่ความยาวคลื่น 280 นาโนเมตร ค่าพารามิเตอร์ที่ตรวจสอบคือ ความเป็นเส้นตรง ความถูกต้อง ความแม่นยำ (ที่ 3 ระดับความเข้มข้น ในช่วงร้อยละ 50-150 ของความเข้มข้นที่ทดสอบ จำนวน 2 ชุดและฉีด 5 ซ้ำ) ขีดจำกัดของการตรวจสอบ ขีดจำกัดของการวิเคราะห์ปริมาณ ความเฉพาะเจาะจง และความทนทานของวิธีวิเคราะห์ ผลการศึกษาแสดงให้เห็นความสัมพันธ์เชิงเส้นตรง (ค่าสัมประสิทธ์ความเป็นเส้นตรงมากกว่า 0.999) ความถูกต้อง (ร้อยละการคืนกลับอยู่ในช่วง 97-100) ความแม่นยำ (ค่าร้อยละของส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์น้อยกว่า 2.0) ของวิธีวิเคราะห์ วิธีวิเคราะห์นี้ยังมีความเฉพาะเจาะจงต่อแคปไซซินและมีความทนทานต่อการเปลี่ยนแปลงอุณหภูมิของคอลัมน์ไป 1 องศาเซลเซียส ผลการวิเคราะห์แคปไซซินในตำรับครีมพริกมีค่าอยู่ในช่วงร้อยละ 90-110 ตามที่กำหนดโดยมาตรฐานตำรายาประเทศสหรัฐอเมริกา สรุปว่าวิธีวิเคราะห์ที่พัฒนานี้สามารถนำมาใช้ในงานประจำของการวิเคราะห์ครีมพริกในทางปฏิบัติได้อย่างมีประสิทธิภาพ

คำสำคัญ: *แคปไซซิน ครีมพริก เครื่องโครมาโตกราฟีชนิดของเหลวสมรรถนะสูง การตรวจสอบความน่าเชื่อถือของวิธีวิเคราะห์*

1. Introduction

It is well known that capsicum fruits (chili fruits) have been used in culinary. There are several varieties of *Capsicum* spp. around the world, most of them are *C. annuum*, *C. frutescens*, *C. Chinese*, *C. baccatum*, and *C. pubescens*. The main component of capsicum extract is capsaicinoids which is recognized for their spicy flavor and different biological activities. It has been reported earlier that capsaicin has analgesic and anti-inflammatory effects (Altman et al., 1994; Dray, 1992; Winter et al., 1995) as well as it can cause pungent taste, neurogenic inflammation, lacrimation and erythema (Hyder, 1996). Therefore capsicum extracts have been used in topical formulations for musculoskeletal treatment and neuropathic pain. It has been used in self-defense weapon also such as pepper spray (Reilly et al., 2001).

Capsicum extracts are mixtures of capsaicin and its analogues, named capsaicinoids (Barceloux, 2009). The major capsaicinoids are capsaicin (E-8-methyl-N-vanilly-6-nonenamide, $C_{18}H_{27}NO_3$, M.W. = 305.42) and dihydrocapsaicin (8-methyl-N-vanillynonenamide/N-(4-Hydroxy-3-methoxybenzyl-8-

methylnonanamide, $C_{18}H_{29}NO_3$, M.W. = 307.43). Capsaicinoids is an alkaloid which contains an amide bond connecting a vanillyl ring and acyl chain. The only difference between capsaicin and dihydrocapsaicin is the presence of carbon-carbon double bond of capsaicin. Capsicum extracts are used to manufacture several pharmaceutical formulations (tincture, solution, ointment, gel and cream) for external uses. The United State Pharmacopoeia (USP) Official monograph displays capsicum which is obtained from dried ripe fruit of *Capsicum* spp. and capsaicin which is defined as percent of capsaicin in total capsaicinoids (USP, 2014).

A number of analytical methods have been developed and optimized for analysis of capsicum extracts from many species and capsaicin and its analogues in pharmaceutical formulations. The volatile extracts of *C. annuum* were identified by gas chromatography (GC) (Jang et al., 2008) and gas chromatography mass spectrometry (GC MS; Pena-Alvarez et al., 2009). The major compounds were sulphides, alcohol and heterocyclic compounds. Some research studies report high performance liquid chromatography (HPLC) with ultraviolet (Al Othman et al., 2011; Butnariu et al., 2012; Musfiroh et al., 2013), fluorescence (Lu and Cwik, 1997; Latimer, 2012) and mass spectrometry detection (Reilly et al., 2001; Wang et al., 2014) for analysis of capsaicin and dihydrocapsaicin. Most of them report using of reversed phase HPLC with C_{18} column and aqueous methanol or acetonitrile as mobile phase while USP suggests to use phenyl column (L11) and the official method of analysis describes using of C_{18} column in the method (AOAC 995.03) for analysis of capsaicinoids in capsicums and their extractives (Latimer, 2012). Some studies reported uses of gradient elution (Waite & Aubin, 2008; Hitachi 2009) and few studies used isocratic with more than 2 solvent systems (Kaale et al., 2002; Modi, 2014). Although much works has been done recently, most of them optimized the analytical methods for capsaicin and other capsaicinoids in chilli, varieties of peppers and chilli products (Al Othman et al., 2011; Juangsamoot et al., 2012; Kuzma et al., 2014; Waite & Aubin, 2008; Musfiroh et al., 2013). Few studies developed sample preparation and analysis of capsaicin in the topical formulations (Kaale et al., 2002; Modi, 2014) and one study evaluated capsaicin in liniment using microemulsion liquid chromatography (Pashkova, et al., 2011). Most of these studies used C18 reversed phase columns in the length of 100-250 mm with 3.0 or 4.6 mm id., column temperature from 30 to 60 ^{o}C and flow rate from 0.8 to 1.5 mL/min. This study was carried out to optimize these parameters for analysis the amount of capsaicin in capsicum cream.

2. Objectives

The objectives of the study were to develop and validate HPLC analytical method for capsaicin in capsicum cream.

3. Materials and methods

Chemicals and reagents

USP Capsaicin RS (Catalog No. 1091108, CAS No. 404-86-4) was purchased from USP Pharmacopeial Convention (Rockville, MD). Glacial acetic acid was obtained from Carlo Erba. Capsaicin extract was prepared by Kaewmungkornphaesaj Ratchaburi. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Burdick&Jackson (Korea). Three strengths (0.025, 0.075 and 0.100 %w/w) of capsicum cream were obtained from Paradigm Pharma (Thailand). Ultrapure water was prepared using Puris water purification system.

Preparation of standard solution

USP Capsaicin RS was accurately weighed (10 mg) and dissolved in 10 mL of methanol to obtain a stock solution (1,000 µg/mL). Then it was diluted to six different concentrations: 15.625, 31.25, 62.50, 125, 250 and 500 µg/mL (n=3), respectively. The solutions were filtered through 0.45 µm PTFE syringe filter and injected into HPLC (five replications). The standard curve was constructed by plotting peak areas against the concentration of the standard capsaicin solutions.

Preparation of sample solution

Capsicum cream was accurately weighed (2 g of each strength, n=2) and dissolved in 25 ml of methanol. Then it was sonicated for 20 minutes and centrifuged at 5,000 rpm and 25 ^{o}C for 10 minutes. The supernatant solution was filtered through 0.45 µm PTFE syringe filter and injected into HPLC (five replications).

Method validation

The HPLC analytical method was validated for linearity, accuracy, repeatability, intermediate precision, specificity, limit of detection and limit of quantitation in accordance with the current ICH (ICH, 2005) and USP guidelines (USP, 2014).

For linearity the standard capsaicin stock solution was diluted to 500 µg/mL and further diluted to 3 different concentration levels: 25, 50 and 75 µg/mL, respectively. The concentrations of standard capsaicin were plot against their peak areas. Accuracy was evaluated by standard addition method carried out by preparing the standard capsaicin solution at three concentration levels: 25, 50 and 75 µg/mL (n=2). The standard capsaicin solution was added at the same concentrations in the 0.075% capsicum cream (n=2). The supernatant was filtered with 0.45 µm syringe filter and injected into HPLC for 10 µL (five replications). The %recovery of capsaicin was calculated. Intra-day and inter-day precisions were obtained from recorded peak areas and retention times of three strengths of capsicum creams prepared as mentioned above. The average values and %relative standard deviation (RSD) were calculated on the same analysis day (intra-day) and on different days for three days (inter-day). Limit of detection (LOD) and limit of quantitation (LOQ) were determined by diluting the 0.025% capsicum cream sample solution and considering the signal-to-noise ratio (S/N = 3:1 and 10:1, respectively). Specificity was evaluated by observing the absorbance in the range of 200-400 nm of the standard capsaicin compared with that of capsicum cream. The absorption spectrum should be the same for three points (the beginning, middle and the end). Reproducibility was evaluated from the analysis of capsaicin contents in the formulation by three different analysts. Robustness was compared by changing the column temperature (34, 35 and 36 ^{o}C) and flow rate of the mobile phase (0.9, 1.0 and 1.1 mL/min) of standard capsaicin at the concentration of 100 µg/mL.

HPLC instrumentation and chromatographic conditions

The capsaicin content was determined using HPLC system (Agilent 1260 with OpenLab EZChrom software). It was equipped with a quaternary pump (G1311C), a degasser, an autosampler (G1329B), an injector with a 100 µL loop, a column oven (G1316A) and a diode array detector (DAD, G1315D). The analytical method was performed on a VertiSep^{TM} pH endure Phenyl-Hexyl column (250 x 4.6 mm i.d., 5 µm) at the temperature of 35 ^{o}C. The mobile phase was a mixture of 1% acetic acid in water and acetonitrile (45 : 55) at a flow rate of 1 mL/min. The injection volume was 10 µL and detected at a wavelength of 280 nm. The total run time was 10 minutes.

4. Results and Discussion

The HPLC analytical method was developed and validated for analysis of capsaicin in capsicum cream. Figure 1 (A and B) illustrated the linearity of standard capsaicin in both concentration ranges 15.625-500 and 25-75 µg/mL with the correlation coefficient higher than 0.999.

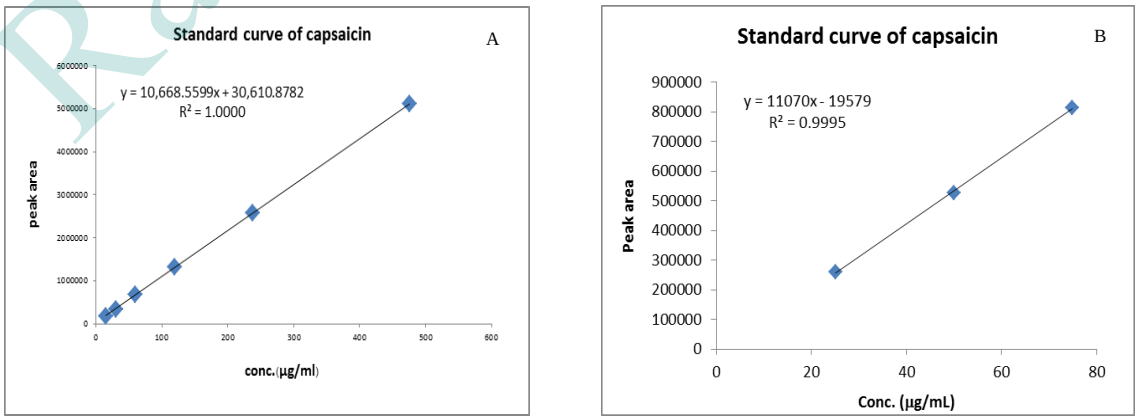

Figure 1 (A) The standard curve of capsaicin (concentration range 15.625-500 µg/mL)
(B) Linearity of standard capsaicin solution (concentration 25, 50, 75 µg/mL)

This method was accurate and precise for analysis all strengths of capsicum cream. Percentage recovery was 96.59 – 100.16% (Tables 1) and %RSD was less than 2% for both intra-day and inter-day precision as shown in Table 2. The reproducibility of this analytical method showed %RSD less than 2% for peak area and retention time. LOD and LOQ were the concentrations of 0.12±0.04 and 0.34±0.04 µg/mL which gave the S/N equal to 3:1 and 10:1, respectively. Their HPLC chromatograms are shown in Figures 2 (A and B). The acceptance criteria for active pharmaceutical ingredient (API) in finish product was 97-103% recovery, %RSD less than 2.0% for precision and linearity $\geq$ 0.9900 for linear response plot (ICH, 2005; USP, 2014).

Table 1 Percentage recovery of capsaicin

Concentration of standard capsaicin (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Spiked sample (µg/mL)	%Recovery
25	25.00	25.31 ± 0.26	90.09 ± 0.15	96.59 ± 1.21
50	50.00	49.38 ± 0.87	114.60 ± 2.43	99.10 ± 4.00
75	75.00	75.31 ± 0.35	141.07 ± 0.16	100.16 ± 0.79

Table 2 Intra-day and inter-day precision

Strength of capsicum cream (%w/w)	%RSD (Retention time)				%RSD (Peak area)			
	Intra-day			Inter-day	Intra-day			Inter-day
	Day 1	Day 2	Day 3		Day 1	Day 2	Day 3	
0.025	0.00	0.05	0.07	0.04	0.20	1.18	0.23	0.54
0.075	0.07	0.04	0.06	0.06	0.94	0.69	0.09	0.57
0.100	0.04	0.08	0.05	0.06	1.05	0.74	0.19	0.66

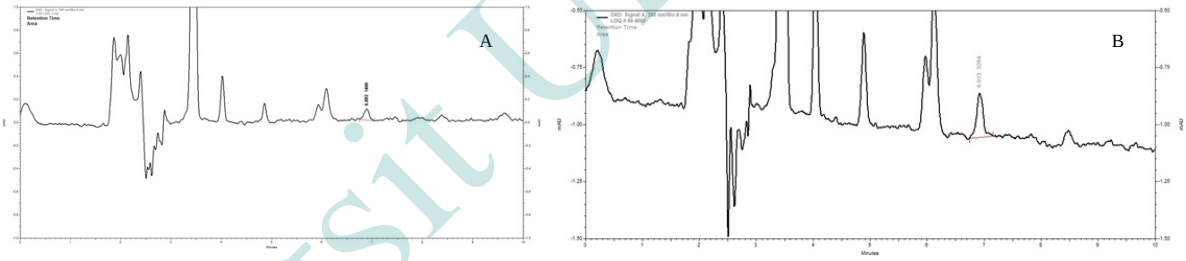

Figure 2 HPLC chromatograms of limit of detection (A) and limit of quantitation (B)

This analytical method was specified and selective for capsaicin. The HPLC condition was specified to differentiate capsaicin and its analogues such as dihydrocapsaicin. The maximum absorption wavelengths of standard capsaicin were 228 and 280 nm which was the same as capsaicin in topical cream at the same HPLC analysis condition (Figure 3 A-D).

The analytical method showed relatively high reproducibility for the analysis of capsaicin in cream among different analysts using the same analysis condition and instrument (Table 3). This method demonstrated its robustness when changing the column temperature by 3% and changing the flow rate by 10%. Percentage differences of retention time and peak area were within 5% of the optimal condition when changing the column temperature. However, changing the flow rate caused approximately 9-10%difference in retention time. Decreasing the flow rate caused higher percentage difference of peak area than increasing it (Table 4).

Earlier studies have been reported the separation and quantitation of capsaicin and its analogues in varieties of *Capsicum* spp. Those methods used either water (Al Othman et al., 2011) or mixtures of aqueous acid and acetonitrile in different ratios. The mobile phase compositions which were 2% acetic acid (Musfiroh et al., 2013) or 0.5% phosphoric acid (Hitachi, 2009; Kuzma et al., 2014) and 0.1% formic acid with acetonitrile (Wang et al., 2014) gave the pH about 2.4 – 2.8 which can protonate amide functional group in capsaicin and other capsaicinoids. The retention time was slightly faster with mixtures of aqueous acid and acetonitrile and less background interferences compared with methanol. The higher the ratio of acetonitrile was, the faster the retention time of capsaicin. This validated method showed fast analysis with acceptable resolution between capsaicin and dihydrocapsaicin (Figure 4 A-D). In this method capsaicin and

dihydrocapsaicin were eluted at the retention times of 6.97 and 8.55 minutes which showed resolution of more than 2.0 and illustrated baseline resolution with other capsaicinoids such as nordihydrocapsaicin were well separated from other components in the formulation.

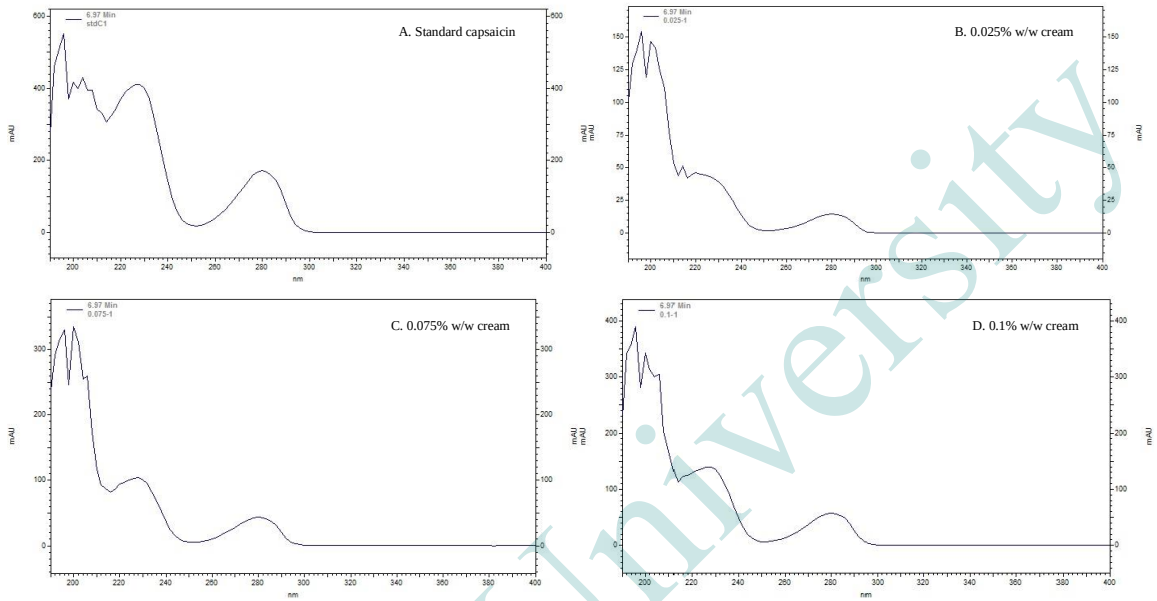

Figure 3 UV-Visible spectra of standard capsaicin (A) and capsaicin cream (B, C, D)

Table 3 Reproducibility of capsaicin peak

Strength of capsicum cream	%RSD (retention time)			%RSD (peak area)		
(%w/w)	Analyst 1	Analyst 2	Analyst 3	Analyst 1	Analyst 2	Analyst 3
0.025	0.08	0.08	0.19	0.34	0.55	1.20
0.075	0.06	0.06	0.18	0.07	0.23	0.50
0.100	0.02	0.06	0.19	0.16	0.16	0.99

Table 4 Robustness of capsaicin peak

Column temperature (^oC)	t_R	%Difference	Peak area	%Difference	Flow rate (mL/min)	t_R	%Difference	Peak area	%Difference
34	6.98±0.01	1.06	1135392±5259	2.03	0.9	7.74±0.03	9.58	1287740±7447	19.84
35	7.06±0.02	0.00	1112755±6064	0.00	1.0	7.06±0.03	0.00	1074584±14471	0.00
36	6.97±0.02	1.22	1140512±6185	2.49	1.1	6.31±0.00	10.59	1047112±7475	2.56

Several methods have been used for capsaicinoid extraction from chilli and the optimum condition was using magnetic stirring extraction in either methanol at 60 ^oC for 2 hours or 80% ethanol at 90 ^oC for 1 hour, then the sample solution was centrifuged and cleaned up with C18 solid phase extraction cartridge (Juangsamoot et al., 2012). One article reported HPLC analysis of capsaicin topical cream (Thermocream®), hexane solvent extraction was used for sample preparation and the 80% methanol aqueous layer was subjected to analysis (Kaale et al., 2002). Another article studies the released of capsaicin in gel formulation, the results showed that 70% ethanol was receptor medium at 32 ^oC and high release through 0.2 μm of synthetic teflon membrane (Modi, 2014). The other study proposed the microemulsion of capsaicin liniment and analyzed with RP-HPLC. The chemical substances were used to form oil in water emulsion and trap the hydrophobic compounds inside micelles and used that microemulsion as the mobile phase (Pashkova, et al., 2011). In this study the sample preparation was modified from earlier studies and solubility of capsaicin. Therefore, capsicum cream was dissolved in methanol, centrifuged and filtered before injecting to HPLC. The results showed acceptable %recovery and within the criteria (97-103%).

The results also indicated that the average capsaicin contents in topical cream for three strengths (0.025, 0.075 and 0.100 %w/w) were 103.90 ± 2.23, 105.95 ± 1.52 and 107.32 ± 1.58%, respectively. The capsaicin contents in the formulations were within 90.0-110.0 % label amount according to USP criteria (USP 32, 2009).

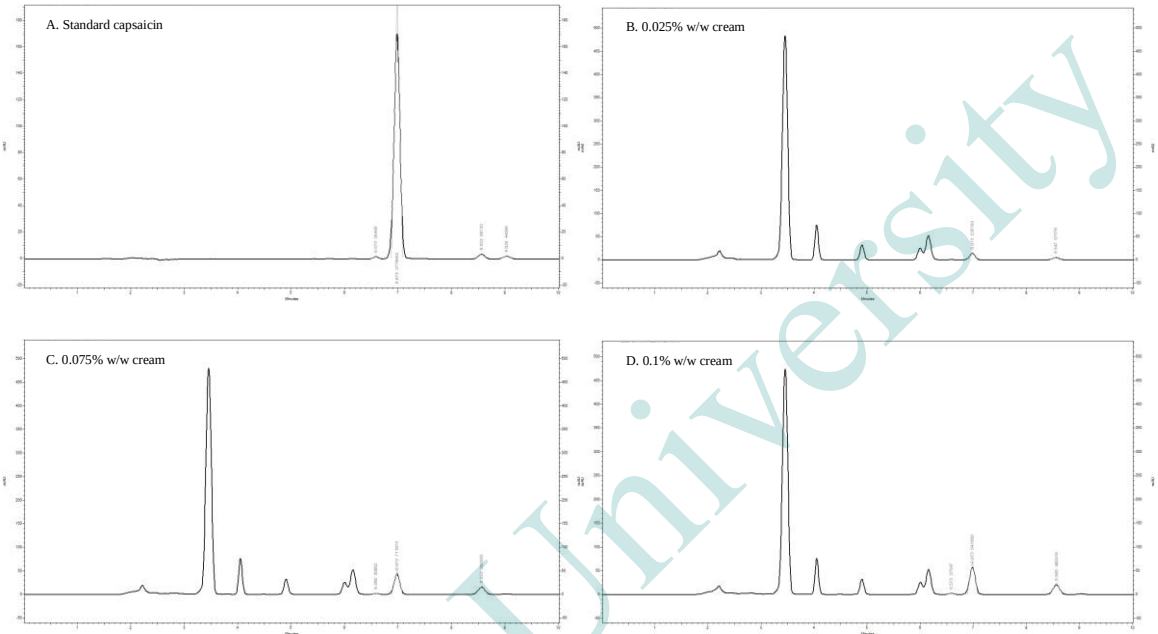

Figure 4 HPLC chromatograms of standard capsaicin (A) and capsaicin cream (B, C, D)

5. Conclusion

The study addresses the validated HPLC method for utilization in the quality control of capsicum cream. The analytical method was optimized from the analysis of capsicum extract. The optimum HPLC condition was performed on C18 phenyl column at 35 ^{o}C, 1% acetic acid in water and acetonitrile (45:55), flow rate of 1 mL/min, detected at 280 nm. It can be used in the routine analysis the amount of capsicum cream in the pharmaceutical industry because the method is accurate, precise, and selective. The analysis is fast and the sample preparation is easy and reproducible.

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