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*Original Research Article*

# Development and Validation of HPLC Method for Determination of Quercetin in Hydrogel Transdermal Patches Loaded with Red Onion Peel Extract

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ABSTRACT

Quercetin is the major flavonoid present in red onion (*Allium cepa* L.). The determination of quercetin in red onion extract has been investigated previously. However, the HPLC method for analyzing quercetin in hydrogel transdermal patches with red onion peel extract has not been reported. This study aimed to develop and validate the novel HPLC methods for the determination of quercetin in hydrogel transdermal patches loaded with red onion peel extract. The HPLC condition and system configuration were optimized to obtain acceptable validation parameters. Method validations were performed for the quantitative analyses of quercetin in hydrogel transdermal patches with red onion peel extract. The reversed phase HPLC analytical method was developed. The $C_{18}$ column and 100% acetonitrile and 0.1% aqueous phosphoric acid solution as a mobile phase were applied at a flow rate of 1.0 mL/min with gradient elution. The injection volume was 10 µL. The separation was performed at ambient temperature with UV detection at 364 nm. The retention time of quercetin was 23.9 min, with a total run time of 27 min. The linearity range of calibration curves was 5-150 µg/mL ($R^2$>0.999). The limit of detection (LOD) and limit of quantification (LOQ) were 0.18 and 0.53 µg/mL, respectively. The mean recoveries were reported as 99.35–108.29%, indicating accuracy of the method. The %RSD study was less than 2.0%, suggesting the precision of the method. This method successfully determined quercetin in hydrogel transdermal patches with red onion peel extract.

***Keywords***: Quercetin, HPLC, Hydrogel, Transdermal, Red onion peel.

## Introduction

Flavonoids have been found in the onion (*Allium cepa* L.) peel, including quercetin, kaempferol, isorhamnetin, apigenin-7-O-β-D-glucopyranoside, quercetin-3-O-β-D-glucopyranoside, kaempferol-7-O-β-D-glucopyranoside and rutin.[1] Quercetin is a flavonoid produced in various fruits and vegetables such as onions, grapes, berries, broccoli, and citrus. Several studies have shown that the main flavonoid component in *Allium cepa* L. was quercetin and glycosides.[2-5] Quercetin has an antioxidant property, providing many benefits on health, including protection against various diseases by acting as an anti-inflammatory, antihypertensive, vasodilator, anti-obesity, anti-hypercholesterolemic, and anti-atherosclerotic agent.[6] In addition, antibacterial activity, antioxidant activity, protein kinase inhibitory activity and antineoplastic activities were also reported in quercetin. Quercetin plays an important role in wound healing by reversing the proliferative and inflammatory responses in hypertrophic scars.[7] Quercetin is the predominant compound in red onion peel.[8]

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Pagano *et al*. reported that hydrogel film containing red onion peel extract demonstrated the wound healing potential due to its antioxidant, radical scavenging, antibacterial and anti-inflammatory activities.[9] The chemical structure of quercetin is pentahydroxy flavone having five hydroxy groups at the 3-, 3'-, 4'-, 5- and 7-positions. Quercetin is a yellow powder solubilizing in ethanol, methanol, acetone, pyridine, glacial acetic acid, and alkaline solution. Quercetin can be dissolved in absolute ethanol (1 g in 290 mL), and the solubility of quercetin in absolute ethanol was increased up to 1 g in 25 mL of boiling ethanol. The ethanolic solution of quercetin has a maximum UV absorption at 258 and 375 nm. According to the chemical structure of quercetin, the pKa of quercetin are as follow; pKa1 = 7.17, pKa2 = 8.26, pKa3 = 10.13, pKa4 = 12.30, and pKa5 = 13.11.[10] Hydrogel transdermal patches are widely used for the transdermal delivery of various drugs. Hydrogels are suitable for drug delivery as they are biocompatible and can be degraded when made from biodegradable polymers.[11] The *in vitro* drug release studies and drug content are required to control the hydrogel transdermal patch formulation quality. Spectrophotometry, spectrofluorometry and HPLC have been used to determine the amount of quercetin in cosmetic formulations.[12-17] However, no studies on the determination of quercetin in hydrogel transdermal patches with red onion peel extract by HPLC have been reported.

The present work aimed to develop a simple and reliable HPLC-UV methodology for determining quercetin in hydrogel transdermal patches loaded with red onion peel extracts and validate the method according to ICH guidelines.

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## Materials and Methods

*Chemicals and reagents*

Quercetin standard (>95% purity) was purchased from Sigma-Aldrich (St.Louise, MO, USA). HPLC grade-acetonitrile and methanol were purchased from LiChrosolve, Darmstadt, Germany. Red onion peel extract and hydrogel patches loaded with red onion peel extract were gifted by Dr. Sirivan Atikomkulchai and Dr. Pimpon Uttayarat.

*Preparation of quercetin standard solution*

The stock standard quercetin solution was prepared in methanol at 0.5 mg/mL.[18] The standard solution was prepared by diluting quercetin with methanol to make final concentrations of 5-150 µg/mL.

*HPLC method development and validation*

*High-performance liquid chromatography; HPLC*

HPLC analytical separation was performed on Agilent InfinityLab LC Series DAD WR, equipped with 1260 Infinity II Diode Array Detector (Agilent, Germany). The separation was carried out on an ACE® C18-AR column, 5 µm, 4.6 x 250 mm at ambient temperature. The mobile phase consisted of acetonitrile and 0.1% aqueous solution of phosphoric acid.[19] The gradient elution was applied by running 0-15 min linear gradient from 5% to 25% acetonitrile, 15-25 min linear gradient from 25% to 50% acetonitrile, 25-26 min of 50% acetonitrile, 26-26.5 min linear gradient from 50% to 25% acetonitrile and 26.5-27 min linear gradient from 25% to 5% acetonitrile. The injection volume was 10 µL. The total run time was 27 min with a flow rate of 1.0 mL/min. The detection wavelength was 364 nm. The software used for operating HPLC separation was Agilent OpenLab CDS 2.X software (Agilent, Germany).

*Method validation*

Method validation followed the ICH Guidelines.[20.] Specificity, range, linearity, accuracy, precision, limit of quantification (LOQ) and limit of detection (LOD) were the validation parameters investigated in this study. The specificity of the method was evaluated by injecting standard solutions of quercetin, methanol, red onion peel extract, and hydrogel transdermal patches containing red onion peel extract separately. The linearity test was investigated using five concentrations of quercetin in the range of 5-150 µg/mL. For each concentration, three injections were analyzed under the same condition. Linear regression was determined as the coefficient of variations ($R^2$). The precision of the method was ascertained by intra-day precision and inter-day precision at three concentrations of samples (5, 50 and 100 µg/mL for low, medium, and high concentrations, respectively). The repeatability was evaluated at these concentrations during the same day. The intermediate precision was evaluated by repeating the studies on three different days and comparing the obtained results. The accuracy of the method was determined by adding known quantities of the quercetin to the transdermal patches with red onion peel extract. The amounts of the quercetin were added at the same concentration levels of the precision for recovery studies. The accuracy and precision of the developed method were stated by percent recovery (% mean recovery) and percent relative standard deviation (%RSD), respectively. The mean recovery (%) was calculated using the following equation.[21]

$$Mean\ recovery\ (\%) = \frac{[(C_t - C_u)}{C_a} \times 100\%$$

Where $C_t$ is the total concentration of quercetin found; $C_u$ is the concentration of quercetin present in the hydrogel transdermal patch; and $C_a$ is the concentration of quercetin standard added to the hydrogel.

*Sample Preparation*

Red onion was bought from Chiang Mai, Thailand in October 2021. The voucher specimen was SIRA004 and deposited at the Faculty of Pharmacy, Srinakharinwirot University. Dried red onion peel (1 g) was extracted by 70% ethanol (40 mL) at 60°C for 90 min. The concentrated extract was suspended by ultrapure water and then centrifuged at 4000 rpm for 20 min. The supernatant was freeze-dried and kept at -20°C until use.[9] The hydrogel patch prepared in this study was composed of hydrophilic compounds. After adding deionized water, the hydrogel patch swelled and released red onion peel extract containing quercetin. Methanol was used to dissolve quercetin released from the patches. Hydrogel transdermal patch containing 10 mg/cm$^2$ of red onion peel extract was cut into small pieces and weighed approximately 1 g before adding 5 mL of water. Then the samples were mixed vigorously using a vortex mixer for 30 seconds and sonicated by an ultrasonic bath for 10 minutes. Methanol (20 mL) was added and mixed for 30 seconds. The samples were sonicated for another 2 hours with temperature-controlled below 35 °C. After leaving the mixture at room temperature for 1 hour, the supernatants were collected and adjusted to 25 ml with methanol. The extraction solution was filtered through nylon filter membrane, pore size of 0.45 µm (CNW Technologies, China) prior to the HPLC analysis.

*Statistical analysis*

The results were expressed by mean, standard deviation (SD) and relative standard deviation (RSD). The regression analysis of standard curves was performed by using Microsoft Office 365 Excel. Statistical evaluation of data was performed using an analysis of variance (one-way ANOVA). A value of $p < 0.05$ was accepted.

## Results and Discussion

*High-performance liquid chromatography; HPLC*

*System suitability*

The chromatographic separation of quercetin was performed in the gradient mode. The mobile phase system consisted of acetonitrile and 0.1% aqueous solution of phosphoric acid. The retention time of quercetin is 23.9 minutes (Figure 1). The system suitability test was assessed by six replicate injections of hydrogel transdermal patches containing red onion peel extract solution. The precision of injections for quercetin peak was acceptable. The percent relative standard deviation (RSD) for the retention time and peak area responses were 0.1% and 1.0%, respectively. The capacity factor (K), tailing factor (T), and theoretical plate number (N) were calculated. The results for the system suitability parameters compared to the recommended limits are shown in Table 1. The proposed method met these requirements within the accepted limits.[22]

*HPLC method validation*

The specificity of the method was assessed by using methanol, red onion peel extract and hydrogel transdermal patches containing red onion peel extract. Other substances in hydrogel transdermal patches containing red onion peel extract did not interfere with the analyses of quercetin, as shown in Figure 1D. The calibration curve was linear over the concentration range of 5-150 µg/mL.

**Table 1:** The system suitability parameters for quercetin in hydrogel transdermal patches with red onion peel extract (n=6).

| Parameters | Recommended limits | Results |
|---|---|---|
| Retention time (min) | - | 23.9 |
| %RSD of peak area* | %RSD < 1% | 1.0 |
| Resolution (Rs) | Rs > 1.5 | 18.9 |
| Capacity factor (K') | K' > 1.5 | 7.3 |
| Tailing factor (T) | T < 2 | 1.2 |
| Theoretical plate number (N) | N > 2000 | 321,266 |

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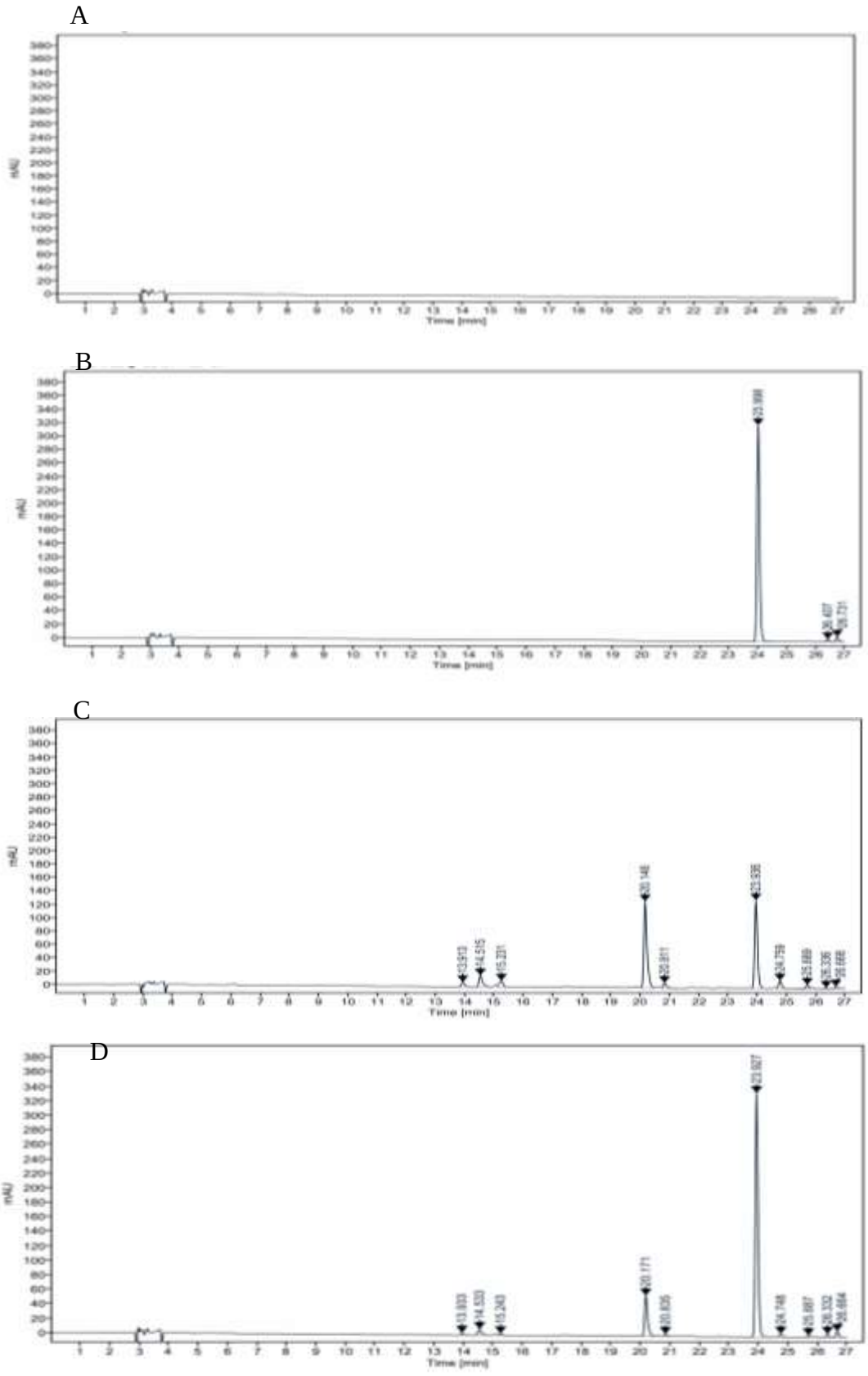


**Figure 1:** HPLC chromatograms of (A) methanol (extraction solvent), (B) quercetin standard solution 50 µg/mL, (C) red onion peel extract in methanol 1 mg/mL, and (D) hydrogel transdermal patches with red onion peel extract.

**Table 2:** The %recovery and precision of HPLC analysis

| Concentration (µg/mL) | Mean recovery (%) | Intra-day precision (%RSD) | Inter-day precision (%RSD) |
|---|---|---|---|
| 5 (low) | 108.29 | 1.02 | 1.02 |
| 50 (medium) | 103.08 | 0.78 | 0.88 |
| 100 (high) | 99.35 | 1.19 | 2.10 |

The results revealed a good correlation between the peak area of analyses and concentration with $R^2 > 0.997$ (Table 2).[23] %Recovery ranged between 99-108%, with %RSD of 0.78-2.10%, indicating that the method was reliable and reproducible (Table 2). In Figure 1C and 1D, the compound showing a retention of 20.1 min might be quercetin glycoside.[2, 11, 24] The peak area at a retention time of 20.1 min in hydrogel transdermal patches was smaller than that of methanolic extract. This might be because red onion extract was dissolved in methanol, whereas hydrogel patches were extracted by using methanol and water mixture. Therefore, the amount of quercetin found in these two samples was different, resulting in different peak areas at a retention time of 20.1 min. The peak area of quercetin in hydrogel patch shown in Figure 1D was higher than that of quercetin in the extract because the concentration of quercetin in hydrogel transdermal patches was higher than that of the red onion extract in methanolic solution (Figure 1C).

LOD and LOQ were obtained from the equations of 3.3 σ/slope and 10 σ/slope, respectively. σ represented a standard deviation of the curve response and slope was obtained from the calibration curve. The results of LOD and LOQ are present in Table 3.

*Determination of quercetin in hydrogel transdermal patches containing red onion peel extract*

The HPLC method developed in this study was to quantify the amount of quercetin in hydrogel transdermal patches loaded with red onion peel extract. Peak identification of quercetin was based on comparing the retention time of standard quercetin to the sample. Quercetin quantification was determined by using calibration curves fitted by linear regression analysis. The amounts of quercetin in red onion peel red extract and hydrogel transdermal patches are shown in Table 4. This study developed and validated the novel HPLC method for quercetin determination in hydrogel transdermal patches containing red onion peel extract. The examination of various detection wavelengths indicated the wavelength of 364 nm exhibited the best detection. The color of red onion peel did not interfere with the HPLC analysis since the red color of onion absorbed light at wavelengths ranging from 380-700 nm. Quercetin is a phenolic compound in red onion peel extract that requires an acidic mobile phase for satisfactory separation and peak shapes. The previous studies used 100% methanol or trifluoroacetic acid as mobile phase solvents.[15, 16] However, this mobile phase system could not separate quercetin from other components, and trifluoroacetic acid is a corrosive chemical. The isocratic elution, 0.1% phosphoric acid: acetonitrile (60:40 v/v), was effective for quantifying quercetin standard solution. However, isocratic elution could not separate the other matrixes from quercetin in red onion peel extract, while gradient elution provides good separation between compounds and enhances peak resolution. Kim et al has reported HPLC analysis for identifying bioactive compounds, including quercetin in red onion peel using methanol-water with 1%formic acid and 100%methanol as a mobile phase.[25] This study used acetonitrile and 0.1% orthophosphoric acid as a mobile phase with gradient elution for better resolution and peak symmetry. The following gradient elution was applied; 0-15 min linear gradient from 25% to 40% acetonitrile, 15.1- 25 min linear gradient from 40% to 25% acetonitrile. However, this system could not separate the other matrixes from quercetin in hydrogel transdermal patches containing the red onion peel extract. The percentage of acetonitrile was therefore increased up to 50%, as follows: 0-15 min linear gradient from 5% to 25% acetonitrile, 15-25 min linear gradient from 25% to 50% acetonitrile, 25-26 min of 50% acetonitrile, 26-26.5 min linear gradient from 50% to 25% acetonitrile and 26.5-27 min linear gradient from 25% to 5% acetonitrile. This gradient elution of chromatographic analysis was simple. The system of the mobile phase in this method showed that a peak resolution was more than 1.5. The quercetin demonstrated a retention time of 23.9 min. This method completes the separation of quercetin from other compositions. The methanol and quercetin chromatograms showed a good resolution without interference from the hydrogel base peak. Thus, this developed HPLC method was selective and specific to quercetin. This study revealed that the range of quantitative analysis for quercetin covered the quantity of quercetin in hydrogel transdermal patch samples. The peak areas showed linear regression with an $R^2$ of 0.9993, a y-intercept value of 0.4676, and a slope of 39.181. Linearity solutions were prepared by quantitative dilution of the stock solution of working standard, and solutions were obtained in the range of 5-150 µg/mL. The standard curves of quercetin showed good linearity ($R^2 > 0.999$). The %recovery was in the range of 99.35-108.29%, and the %RSD was 1.19 and 2.10 for intra-day and inter-day precision, respectively. It could be concluded that this developed method was precise and reproducible for the prescribed conditions during different runs. It provided acceptable sensitivity with LOD and LOQ of 0.18 and 0.53 µg/mL, respectively. The developed and validated method was successfully applied to determine quercetin in the hydrogel transdermal patches containing red onion peel extract.

**Table 3:** Summary of validation parameters of quercetin

| Parameter | Result |
|---|---|
| Concentration (µg/mL) | 5-150 |
| Linearity ($R^2$) | 0.9993 |
| Equation | $y = 39.181x + 0.4676$ |
| LOD (µg/mL) | 0.18 |
| LOQ (µg/mL) | 0.53 |

## Conclusion

The HPLC method was successfully developed for quercetin determination in hydrogel transdermal patches with red onion peel extract. It was validated and achieved good linearity, accuracy, precision, and system suitability parameters. The advantages of this method were simplicity and a short run time. Quercetin was successfully isolated from the hydrogel transdermal patch matrix, and good analyte recoveries were obtained.

## Conflict of Interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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