ISOLATION, DEVELOPMENT AND VALIDATION OF QUERCETIN FROM THE ALLIUM ASCALONICUM'S TUNIC BY HPLC METHOD

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Abstract:

Allium ascalonicum, the tunic of which holds various bioactive compounds including including quercetin - a potent flavonoid, is renowned for its medicinal properties. This study presents the development and validation of a High-Performance Liquid Chromatography (HPLC) method for quantifying quercetin in Allium ascalonicum tunic. Validation of the method was conducted following International Conference on Harmonization (ICH) guidelines, evaluating parameters such as linearity, precision, and accuracy. This validated HPLC method offers an efficient and reliable means of quantifying quercetin in Allium ascalonicum tunic. Its application holds promise for routine analysis in pharmaceutical and nutraceutical industries, offering insights into the medicinal potential of Allium ascalonicum peels as a natural source of quercetin.

Keywords: *Allium ascalonicum; HPLC; Quercetin; Validation.*

1. Introduction

Allium ascalonicum, commonly known as the shallot, is a distinctive member of the *Allium* genus, revered for its culinary excellence and diverse medicinal properties. Originating from the ancient region of Mesopotamia, the shallot has been cultivated and esteemed for its unique flavor and therapeutic benefits for millennia (Moldovan et al., 2022). As a close relative of *Allium cepa* (common onion), *Allium ascalonicum* shares certain botanical similarities while possessing its own set of distinctive attributes, making it a fascinating subject for academic inquiry. The shallot is used as a traditional remedy for the treatment of lipoma, poultice, and onchoceriasis, a neglected tropical disease listed by WHO (Elufioye & Ogunlude, 2018; Srisawat et al., 2016). *A. ascalonicum* possess various biological activities including antifungal, antiviral, antibacterial, anti-inflammatory, and antioxidant activities, which could be ascribed on shallot's high flavonoid content, especially quercetin, making it a subject of growing interest in both pharmaceutical and

dietary contexts (Chaaban et al., 2017). The inherent urgency in this extraction lies in the pursuit of harnessing and maximizing the therapeutic potential of quercetin for human well-being. Thus, this study focused on the isolation, development and validation of quercetin from the skins of shallot through the utilization of an HPLC technique.

2. Research overview

Validation of analytical methods is crucial for establishing reference methods and ensuring the reliability of analytical data generated by a laboratory. This validation process is specifically applied within the context of generating chemical data. Analytical method validation involves assessing various performance indicators, such as accuracy, precision, linearity, limit of detection (LOD), and limit of quantification (LOQ) to ensure the quality and accuracy of analytical methods (Boqué et al., 2002). The choice to employ a high-performance chromatography method is driven by the method's superior analytical capabilities. High-performance chromatography allows for precise separation, identification, and quantification of compounds within complex mixtures, which is particularly relevant for quercetin extraction due to its presence amidst various other phytochemicals in shallot skin. This method's high sensitivity and accuracy ensure reliable and robust results, essential for advancing the understanding of quercetin's concentration and distribution within the shallot skin matrix. To date, there are some researches that has reported the quercetin content within the shallot skins (Vu et al., 2013; Wiczkowski et al., 2008). Nonetheless, the validation of the analytical methodologies employed in these studies has remained a challenge, consequently rendering them unsuitable for routine analytical applications. The application of analytical methods holds considerable potential for routine analysis in the pharmaceutical and nutraceutical industries. It provides valuable insights into the medicinal potential of shallot skins as a natural and abundant source of quercetin.

3. Experimental

3.1.Materials and Methods

Apparatus: NMR data were acquired with the Bruker 500 MHz spectrometers. ESIMS data were obtained using an Agilent 1200 system connected with a 6120 quadrupole MSD with a Phenomenex Luna C18(2) column (5 µm, 150 × 4.6 mm). The quantitative analyses were carried out on an HPLC chromatography (Agilent, Palo Alto, CA, USA) and YMC-Triart C_{18} column. Column chromatography was carried out using Merck silica gel. TLC was performed using a Merck pre-coated silica gel plate.

Analytical methods: The quantitative analyses were conducted on an Agilent (Palo Alto, CA, USA) series 1200 liquid chromatograph and a YMC-Triart C_{18} column packed with 3 µm particles (4.6 × 250 mm) maintained in a 25 °C column oven. Formic acid (0.1%) in both acetonitrile (A) and water (B) was used as a mobile phase to achieve a better peak shape (Table 1). The injection volume was 10 µL, the flow rate was 0.7 mL/min, and UV detection was performed at 260 nm.

Table 1. Mobile phase for chromatographic method

Time (min)	Flow (mL/min)	%A	%B
0	0.7	5	95
15	0.7	20	80
37	0.7	20	80
45	0.7	25	75
55	0.7	50	50
60	0.7	50	50
63	0.7	100	0

Preparation of standard solution: Preparation of quercetin standard was done by dissolving 6 mg of quercetin in 10 mL of methanol. The appropriate concentrations for content determination were obtained by diluting with MeOH. The solutions were transferred to 10 mL glass brown vials, sealed using plastic film and stored in refrigerator for analysis.

Method validation: Validation was executed through an assessment of various method parameters, including the range of linearity, limits of detection and quantification, precision, and accuracy, which was determined by evaluating the recovery of the HPLC assay. Identification of the peak was accomplished by comparing its retention time with that obtained

from the analysis of a standard compound and by fortifying samples with the standard compound to validate the identification. For the quantification of quercetin, an external calibration curve was established using calibrators at concentration levels of 9.375, 18.75, 37.5, 75, 150, and 300 mg/L.

The determination of linearity range involved plotting the peak area attributed to quercetin against varying analyte concentrations. Subsequently, the least squares method was employed to compute the corresponding regression coefficients and the determination coefficient (r^2). In accordance with the validation protocol, the determination coefficient was required to satisfy the criterion of $r^2 > 0.999$.

Assay precision was assured by repeatedly analyzing the extract under optimized conditions (see Table 1). Intra-day (relative standard deviation ≤ 1.95 %) and inter-day (relative standard deviation ≤ 1.69 %) variance was shown to be in the acceptable range (Table 2)

Table 2. Intra-day and inter-day precision and accuracy of quercetin in shallot skin extract.

	Fortified conc. (µg/mL)	Sample conc. (µg/mL)	Observed (µg/mL)	SD	Accuracy (%)	Precision (%)
Intra-day	9.38	34.47	41.24	0.81	106.34	0.26
	18.75	34.47	52.12	0.30	102.10	0.57
	75.0	34.47	116.47	0.30	93.99	1.95
Inter-day	9.38	34.47	41.32	0.31	106.11	0.75
	18.75	34.47	52.95	0.72	100.51	1.38
	75.0	34.47	118.73	1.99	92.22	1.69

Accuracy was determined by spiking a defined amount of grounded, dried material with three different concentrations of quercetin (low, medium, and high) prior to sample preparation. The observed recovery rates showed the maximum recovery rate of 92.22 % (low spike), confirming the validity of this parameter.

The limit of detection (LOD) and limit of quantification (LOQ) for each organic acid were calculated by taking into account the residual standard deviation (SD) of the analytical signal and slope of the calibration curve (s), according to the formulas: LOD = 3.3 SD/s, LOQ = 10 SD/s, respectively.

Data analysis was carried out by employing the Microsoft Excel software.

3.2. Method of extraction

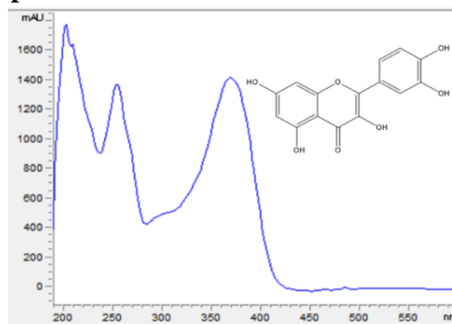
Isolation and characterization of the isolated compound: The dried skins of shallot were ground and extract with ethanol three times at 70°C for 3 h. The crude ethanol extract was suspended in water and partitioned with *n*-hexan and *n*-BuOH. The *n*-BuOH fraction was chromatographed using a silica gel column, eluting with a CH_2Cl_2/MeOH gradient system (0:1 to 1:0, v/v) to yield seven sub-fractions (A1 – A7). Sub-fraction A5 was applied to a Sephadex LH-20 open column and eluted with MeOH to obtain compound 1.

Quercetin (1): A yellow amorphous powder; ^{1}H NMR (500 MHz, CD_3OD) δ_H 6.18 (1H, d, *J* = 2.1 Hz), 6.39 (1H, d, *J* = 2.1 Hz), 6.88 (1H, d, *J* = 8.5 Hz), 7.63 (1H, dd, *J* = 8.5, 2.1 Hz), 7.73 (1H, d, *J* = 2.1 Hz). ESIMS *m/z* 325.0 $[M + Na]^+$, calcd for $C_{15}H_{10}O_7Na$.

4. Results

Chromatographic separation and purification of *n*-BuOH fraction from the skins of shallot led to the isolation of compound 1 which was identified as quercetin (Vu et al., 2020) by comparison of its MS and NMR data with reported data (Fig. 1).

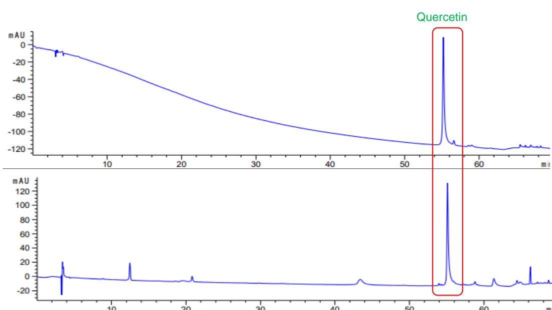
Fig. 1. Structure and UV spectrum of compound 1



4.1. Optimization of sample preparation condition

The dried skins of shallot were ground to make sample homogeneity. In comparison between sonication and reflux method using EtOH as the solvent extraction, the peak area of quercetin when extracted by sonication was superior compared to reflux method. Thus, the sample was extracted two times (30 min each) via sonication at room temperature.

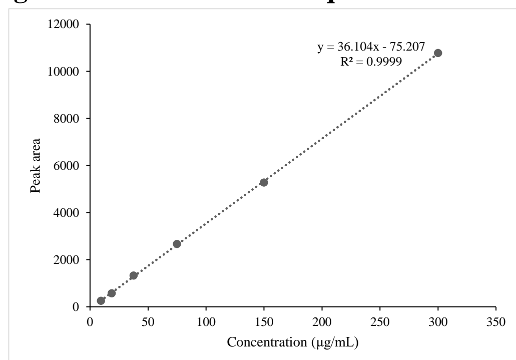
Fig. 2. The HPLC chromatograms at wavelength of 260 nm for the quercetin standard (above) and shallot extract (below).



4.2. Linearity

The linearity of the method was validated by analyzing six concentrations of compound **1** in the range of 9.375 to 300 µg/mL. Triplicate analysis was carried out. The calibration curves of compound **1** displayed good linearity over the tested range (r^2 = 0.9999) (Fig. 3). The LOD of compound **1** was determined to be 0.986 µg/mL and the LOQ was 2.987 µg/mL indicating that the developed method for the skin of shallot is well-established with a good sensitivity.

Fig. 3. Calibration curve of quercetin



4.3. Precision and accuracy

The accuracy evaluation of the developed HPLC method involved the analysis of predetermined quantities of the analyte at three different concentrations intentionally introduced into the ethanol extract solution obtained from the skin of shallot. Subsequently, the recovery studies were conducted to investigate the performance of the solution after the addition of known amounts of the analyte to the initial ethanol extract. The method precision was measured by five successive injections, and the precisions were less than 1.95% in intra-day and 1.69% in inter-day. The accuracies of the method were in the range 93.99 − 106.34% in intra-day and 92.22 − 106.11% in inter-day. The method validation indicated that the regression equations of compound **1** was linear and this method was precise, accurate, and reliable for quantitation of quercetin.

By applying the above developed HPLC method, the concentration of the major component from the skins of shallot, quercetin, was 137.84 ± 0.69 mg/g.

5. Discussion

The present study focused on the development and validation of a HPLC method to quantify quercetin in *Allium ascalonicum* skins, a plant species well-known for its medicinal properties. The importance of this research lies in the fact that shallot skins, a byproduct of shallot processing, have been shown to contain various bioactive compounds, particularly quercetin, which is recognized for its potent antioxidant and health-promoting properties. The validation of the HPLC method was performed following the guidelines set by the International Conference on Harmonization (ICH). This rigorous validation process ensures the reliability, accuracy, and robustness of the analytical method, allowing for consistent and reproducible results. The evaluated parameters, including linearity, precision, and accuracy, are crucial aspects of method validation to ascertain the suitability of the method for routine analysis in different applications. The obtained results from the validation studies demonstrated excellent linearity, indicating a direct correlation between the concentration of quercetin and the

response from the HPLC method. This linear relationship suggests that the developed HPLC method is sensitive enough to accurately measure quercetin levels across a wide range of concentrations in *Allium ascalonicum* skins. Precision, which assesses the method's repeatability and intermediate precision, was found to be satisfactory in this study. The low variability observed in precision studies ensures that the HPLC method can be consistently applied to analyze quercetin content in shallot skins, even when different operators or equipment are used. This attribute is essential for reliable data generation and strengthens the confidence in the method's applicability for routine analysis. Moreover, the accuracy assessment of the HPLC method demonstrated that the measured values closely match the known concentrations of quercetin in the samples. This confirms that the developed method provides reliable and precise quantification of quercetin in *Allium ascalonicum* skins. The accurate determination of quercetin content is of utmost significance for understanding its potential therapeutic effects and ensuring consistent quality in various applications. In contrast to other published methods, which might exhibit limitations in terms of accuracy, sensitivity, or selectivity, HPLC stands out as a robust analytical tool that overcomes these challenges. Its capacity to provide precise, selective, and sensitive analysis while accommodating various sample types underscores its prominence as a preferred approach for quercetin analysis from shallot skins.

6. Conclusion

In this study, the major compound from the skin of shallot was isolated and determined as quercetin by using various chromatography and spectroscopy techniques including UV, NMR and MS. The validation parameters employed for the developed HPLC method of shallot skin encompassed the assessment of linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision was proved to be accurate, precise, and reliable for quantitation of quercetin.

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PHÂN LẬP VÀ NGHIÊN CỨU THẨM ĐỊNH PHƯƠNG PHÁP XÁC ĐỊNH QUERCETIN TỪ VỎ HÀNH TÍM BẰNG HPLC

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Tóm tắt:

Allium ascalonicum (hành tím) nổi tiếng về đặc tính chữa bệnh, với lớp vỏ chứa nhiều hợp chất hoạt tính sinh học khác nhau, bao gồm quercetin, một loại flavonoid mạnh. Nghiên cứu này trình bày sự phát triển và thẩm định phương pháp Sắc ký lỏng hiệu năng cao (HPLC) để định lượng quercetin trong vỏ hành tím. Việc thẩm định phương pháp được tiến hành theo hướng dẫn của Hội nghị quốc tế về hài hòa hóa (ICH), đánh giá các tham số như độ tuyến tính, độ chụm và độ chính xác. Phương pháp HPLC đã được kiểm chứng này cung cấp một phương tiện hiệu quả và đáng tin cậy để định lượng quercetin trong vỏ hành tím. Ứng dụng của nó hứa hẹn cho phân tích thông thường trong ngành công nghiệp dược phẩm và thực phẩm dinh dưỡng, cung cấp cái nhìn sâu sắc về tiềm năng y học của vỏ hành tím như một nguồn quercetin tự nhiên.

Từ khóa: *Allium ascalonicum; HPLC; Quercetin; Thẩm định phương pháp.*