Original Article

Application of Zwitterionic Ion Chromatography-Hydrophilic Interaction Liquid Chromatography for the Quantification and separation of Naringenin in Royal Jelly and Pollen extracts

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Abstract

Natural products provide a large source of biological activities with a wide range of possible applications. In developed nations, natural drugs not only fulfill primary health criteria, they also raise health-care costs as well. For this, they resort to naturally occurring compounds such as flavonoids. Flavonoids are nutrients from plants that can be non-toxic as well as beneficial to the human body when eaten in the form of fruits vegetables, beverage like tea and wine. Numerous health advantages have been related to the intake of food containing flavonoids. One of the most important flavonoids is naringenin. Naringenin is a compound of the flavonoids family found in all citrus fruit like oranges, lemons, grapes and bee products. Naringenin was separated in the ZIC®-HILIC column by acetonitrile (ACN) and sodium acetate (NaOAc) buffer as mobile phase constituents. The findings revealed that the HILIC mode was clear and efficient and could be used to identify royal jelly and pollen samples in naringenin. The calibration curve was developed for a wonderful type of commercial ZIC®-HILIC column and linear range (0.05-5 μgmL-1), RSD% (0.22 ± 0.045), LOD (0.008 μgmL-1), LOQ (0.024 μgmL-1).

Keywords: Flavonoids, Naringenin, Bee products, Royal jelly, Pollen extracts.

1. Introduction

Natural products have many structurally diverse substances with a wide range of biological activities that have significant benefits in the treatment and prevention of many diseases, the most important of which are flavonoids [1,2]. Flavonoids are naturally present in fruits, vegetables, nuts, and drink coffee, red wine and bee products as well as herbs. Flavonoids are characterized by having important pharmacological activities, such as antioxidants, anti-allergies, antibacterial, anti-inflammatory, antibacterial and anti-cancer activities [3, 4]. There are many essential flavonoids are available and naringenin is one of the most common.

Naringenin is one of the most important natural flavonoids found in certain food fruit and some bee products. Naringenin (Figure 1) has strong effects on human health as it improves antioxidant defences, reduces reactive oxygen species, controls immune system activity, and also plays an anti-atherosclerotic and anti-inflammatory function [5-7].

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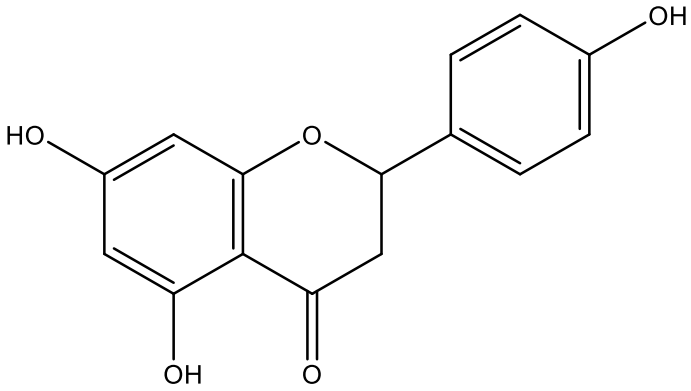

Figure 1: Structure of naringenin

Studies showed that naringenin has been analyzed using HPLC and the columns used by RPLC C18. However, few studies have been performed on naringenin separation using a technique of hydrophilic interaction liquid chromatography (HILIC). In response to the lack of retention of polar compounds in the conventional reversed-phase (RPLC), HILIC was relied on as a relatively new chromatographic mode. Although HILIC most common theory is that the mechanism of HILIC retention is partitioning (between two layers, one of which is an aqueous layer near the solid surface and a highly organic mobile phase) [8, 9]. However, several research shows a multimodal separation mechanism that involves hydrogen bonding and also dipole-dipole interactions are either between the analyte and the water bounded on it [10- 13]. HILIC separation has been widely used in different applications in recent years, as several studies have been published. Among these studies were presented by Rasheed and co-workers [14-28]. Based on the above-mentioned importance of HILIC columns, the first objective of this research is to investigate the mechanism of naringenin separation using a ZIC®-HILIC column with a UV detector and this will be an important incentive because this study has not been verified before. The second objective is to develop a new method for the determination of naringenin in some essential bee products such as royal jelly and pollen. Bees make royal jelly and bee pollen because of their biologically beneficial to human beings [29]. Royal jelly has been used in ancient medicine it is currently used in cosmetics as well as pharmaceuticals and it's healthy food. Where several studies have demonstrated various applications of royal jelly in the field of antimicrobials and against bacteria, fungi and viruses, antihypertensive, anti-tumour, anti-hyper cholesterol and anti-inflammatory medications [30]. Pollen is another traditionally used bee product that includes a variety of antioxidant compounds, including phenolic acids and flavonoids which have a great impact on cardiac disease prevention, liver safety, anti-inflammatory, anti-bacterial disease protection, anti-cancer protection and also the immune booster [31, 32].

2. Experimental

2.1. Chemicals

The standard of naringenin has been obtained from Sigma-Aldrich. HPLC-grade acetonitrile (ACN), methanol (MeOH) and ethanol were purchased from Merck. All reagents were prepared with Ultrapure water, with a conductivity of lower than 0.1 μS/cm, obtained with the Milli-Q system (Millipore, USA). The solution was purified using a filter (0.45 μm).

2.2 Preparation of standards

Stock solutions of (100 μg ml-1), exactly dissolving the amount of naringenin (0.01 g) in 100 ml of ACN. The result was then dissolved and filtered through a 0.45 μm filter. The solution was stored in a refrigerator at 6 oC before analysis.

2.3 Preparation of samples and extraction techniques

Most samples were collected from the local market in Iraq in 2020. Samples were stored in the refrigerator for a maximum of three weeks prior to the analysis and preparation of the extract. 1.5 g of accurately royal jelly and pollen samples are dissolved in 100 mL of 80 % ethanol and 20 % Millipore water as a solvent solution. The mixtures were separated by ultrasound-assisted for 40 minutes. After completion of the sonication process, the mixtures were centrifuged at 4000 rpm at 30 minutes. This extraction process was performed three times to ensure complete extraction. The solution was shaken and filtered using a 0.45 μm filter. The solution was stored at 4 °C in a dark position before it was used.

2.4 Instrumentation

HPLC equipment (Merck Hitachi) consisting of analytical commercial column ZIC®-HILIC from Merck SeQuant. The HPLC system coupled with a UV detector (type L-4200) was used for the quantitative determination of naringenin as well as it was used L-6200 Pump. Data were analyzed with the software of the N2000 workstation. In an ultrasonic bath, the centrifuge was used as a pH 740 (WTW).

2.5 Chromatographic conditions

The Mobile Phase consist of acetonitrile (ACN) and sodium acetate (NaOAc) buffer at a flow rate of 0.5 mL/min at 30 oC (column oven temperature) and the injection volume was 20 μL. The UV detector was set at 260 nm.

3. Results and discussion

The goal of this study was to establish a simple and rapid (HPLC-ZIC-HILIC) method for naringenin determination. Where the mechanisms of the separation of naringenin have been studied using ZIC®-HILIC column, the effect of such as the ACN content, the effect of the buffer concentration used, and the pH influence of eluent. Naringenin chromatogram shows in Figure 2. Under condition 30 mM (pH 5.5) of acetate buffer and 90% of ACN.

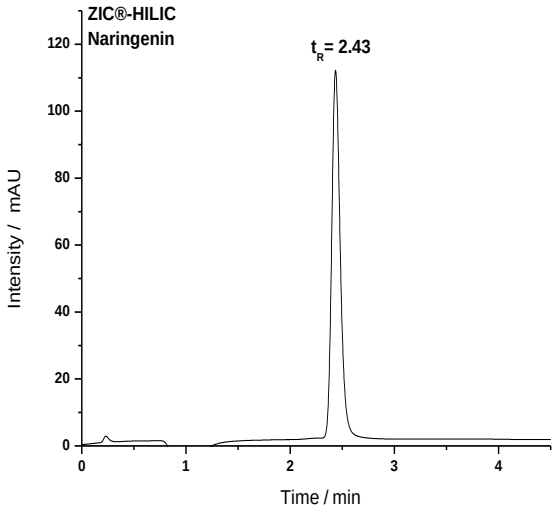

Figure 2: A chromatogram of naringenin.

3.1 Study the effect of ACN content on the retention of naringenin

This choice of mobile phase (ACN) composition has a significant and powerful influence on retention in the ZIC-HILIC, and when investigated, a substantial number of organic solvents have also been integrated into the column of ZIC®-HILIC to support low water solubility compounds. The retention attitude of the naringenin was observed at 5.5 pH 35 mM NaOAc / HAc. This behaviour of naringenin is reversed-phase (RP), continues to high from 60% to 95%. hydrophobicity naringenin is the reason for this behaviour, naringenin activity is shown in this column in reverse-phase (RP) (Figure 3), that was induced by the naringenin log Pow (2.83).

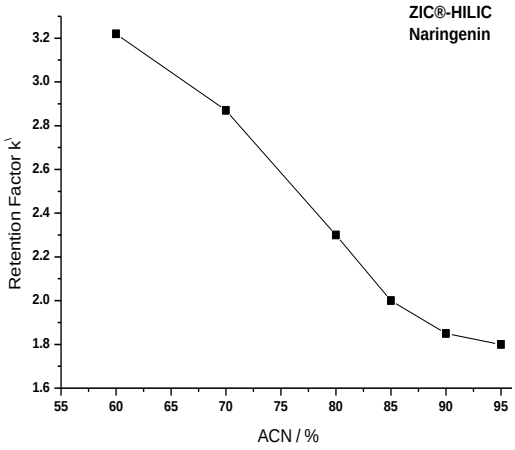

Figure 3: Retention of naringenin as an ACN content variation.

3.2 The effect of salt concentration on naringenin retention

The aim of salts addition to the mobile process is to control the interaction between the charged analytes and the stationary phase. It is obvious that as the concentration of salt increases, it will have an important effect by reducing the electrostatic interactions of the charged analytes in the ZIC-HILIC columns. On this basis, in this study salt sodium acetate (NaOAc) was used because of its strong solubility in the mobile phase. Figure 4, describes the impact of the NaOAc/HAc buffer concentration on the retention of naringenin while retaining a stable ACN percentage at 90 % and pH at 5.5 while changing the NaOAc/HAc buffer concentration (10-80 mM) in the mobile phase.

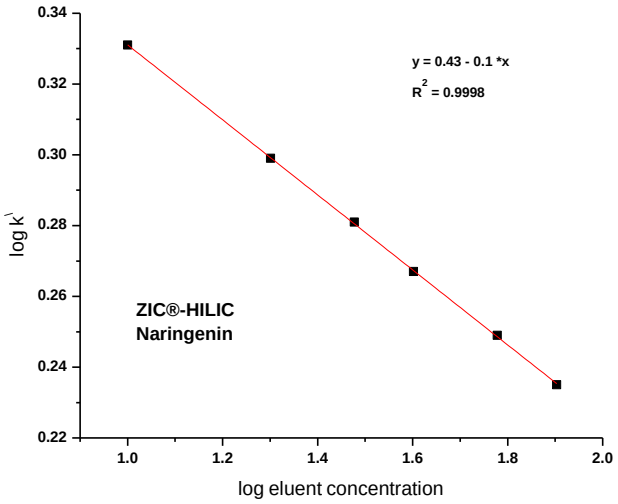

Figure 4: Naringenin retention behaviour as a function of salt concentration.

3.3 Eluent pH influence in naringenin retention

To complete the naringenin separation in HILIC mode, the eluent pH must be changed to decrease the strong electrostatic attraction between charged analyses and stationary HILIC materials. The pH raised from (3 to 5.5) at a constant buffer concentration of 35 mM and 90 % ACN. The retention factor for naringenin increases as shown in Figure 5, this is because naringenin contains OH groups. This is based on the physical and chemical data that naringenin expects. The pka value from just below (7.86) and the analyzes are detonated if the pH in the mobile phase increases to 5.5.

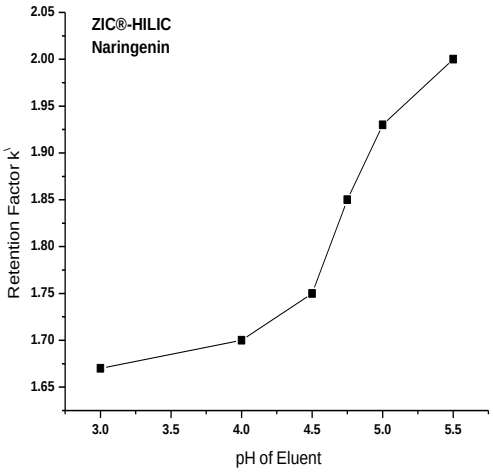

Figure 5: The naringenin retention indifference of eluent pH.

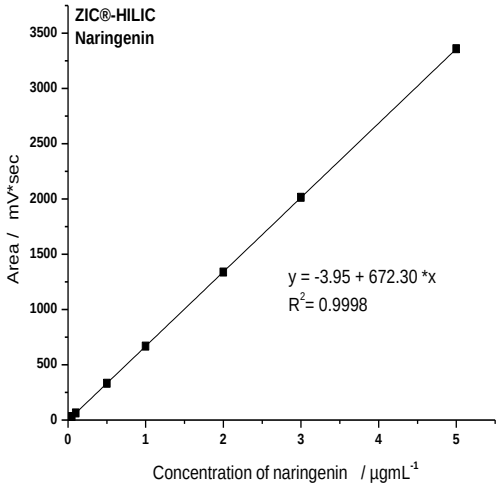

Figure 6: Standard curve of naringenin using the ZIC®-HILIC column

4. Method validation for determination naringenin

According to ICH guidelines, the performance of the method developed was tested by determining the precision and accuracy of the study linearity, the limit of detection (LOD) and Limit of quantification (LOQ) have been validated.

4.1 Linearity of naringenin

Naringenin is a (0.05 to 5 µgmL-1) concentration range the linearity analysis was shown to be linear. R2 was discovered to be a (0.9998) coefficient of determination. Results have shown that the naringenin concentration and its peak area are good correlated (Figure 6).

4.2 Statistical analysis of data

Accuracy and precision have been obtained by naringenin injection were replicated in (five replicates, n=5) in HPLC without changing the test protocol, and the results indicate that. RSD % for naringenin is less than 0.5 % (Table 1). On the same day and a during numerous days, to ensure accuracy and precision, the RSD% and recovery during the stationary phase are calculated (Table 2).

Table 1: Displays the findings of this analysis.

Parameter	Proposed method
Linearity (µg.mL^{-1})	0.05- 5
Regression equation	y= - 3.95 + 672.30* x
R^2	0.9998
LOD (µg.mL^{-1})	0.008
LOQ (µg.mL^{-1})	0.024

Table 2: Naringenin statistical precision and accuracy, on the same day and different days.

	Same-Day Analysis n=5				Day-to-Day Analysis n=5			
Taken (µg.mL^{-1})	**Found (µg.mL^{-1})**	**% Rec.**	**% Erel.**	**%RSD**	**Found (µg.mL^{-1})**	**% Rec.**	**% Erel.**	**%RSD**
2.00	1.992	99.60	- 0.40	0.27	1.996	99.80	- 0.20	0.31
2.50	2.492	99.68	-0.32	0.23	2.496	99.84	- 0.16	0.23
3.00	3.050	101.66	1.66	0.16	3.010	100.33	0.33	0.23

These findings were compared with results obtained by the Standard Method [33] to evaluate the competency and efficiency of the ZIC®-HILIC method. The results of the t-test and the variance F-test method (Table 3) have been used for statistical analyzes and represent 95% trust. The calculated T and F values did not exceed the theoretical value, which means that the accuracy of the determination of naringenin did not differ significantly in both methods.

Table 3: The comparison by examining t- and F-statistical tests of the proposed ZIC®-HILIC method with standard naringenin analytical method.

Methods	Naringenin	t-Test (theor.)	F-Test (theor.)
ZIC®-HILIC	99.60 99.68 101.66	0.5979 (2.7764)	4.0839 (19.000)
Standard method [31]	100.55 99.56 99.54		

5. Determination of naringenin in Royal Jelly and Pollen samples

The proposed method, using the ZIC®-HILIC column, was successfully used for the determination of naringenin in Royal Jelly and Pollen samples, and the findings of the analysis are shown in Table 4.

Table 4: The performance of naringenin was examined in Royal Jelly and Pollen.

Flavonoid	Royal Jelly mg/g*	Pollen mg/g*
Naringenin	0.0696 ± 0.005	0.756 ± 0.134

* Contents (mg/g) as mean + SD, are expresses (n = 5).

6. Conclusion

At the end of this study, the ZIC®-HILIC column was used for the separation and quantification of naringenin. And to obtain a complete and effective separation, were some requirements were controlled and studied through which we obtain a complete perception of the separation process, including the study of the effect of the acetonitrile ACN content on the separation of naringenin, and it was found that when the acetonitrile components were increased, the behaviour of this column reached the highest level of performance in naringenin separation. Similarly, by changing the separation conditions by changing the concentration of the buffer solution used and regulating the pH, which provided a full picture of the separation in this column by controlling the separation time and the results achieved, the complex behaviour in this column is the hydrophobic behaviour. This study concluded that naringenin extract was used in some basic applications, including some royal jelly and pollen as bee products, which indicates the presence of quantities of naringenin compounds in these natural products that were examined.

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