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Hydrophilic Interaction Chromatographic Analysis Of Genistein In Herbs And Propolis

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ABSTRACT

The hydrophilic interaction chromatography (HILIC) coupled with UV detection was employed to study retention behaviour and separation of genistein. The genistein is a compound of the isoflavone family found in natural resources in various plants, including herbs, tea, bee products and legumes such as soybeans. A simple, fast and efficient HILIC-UV method was developed for the quantitative and qualitative purposes of genistein in fenugreek seeds, olive leaf, and propolis. The best separation was achieved using a gradient elution of acetonitrile and acetate buffer on ZIC-HILIC column from Merck SeQuant (100 mm×4.6 mm I.D., 5µm) at 35 °C. The genistein was identified with a UV detector at 260 nm. The result demonstrates that the linear range 0.05-5 µgmL-1, high precision (RSD% < 0.5%), and excellent validated values for both LOD (0.0047 µgmL-1) and LOQ (0.0142 µgmL-1).

Keywords: Genistein, propolis, fenugreek, flavonoids, olive leaf

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INTRODUCTION

Studies and research have provided proof of the prophylactic effect of different foods rich in Polyphenols against chronic diseases, including cancer [1], cardiovascular disease [2] and neurodegeneration [3]. From a chemical point of view, Polyphenols are a category of natural compounds with phenolic structural properties. Polyphenols are molecules that have at least one aromatic ring bound to one or more hydroxyl groups [4, 5]. These naturally occurring compounds include flavonoids [6]. Flavonoids are strong antioxidants found in natural resources in a variety of plants, including green leafy vegetables, herbs, tea, wine, onions and berries [7, 8]. Flavonoids can also be split into different sub-groups, such as Flavones, flavonols, flavanones, isoflavonoids, flavanols, and anthocyanidins. One of the most important flavonoids had been studied in this study; it is genistein (Figure 1). Genistein, as it is present in many plants, is a compound of a widespread family, a family called isoflavones [9]. Genistein is used it for anti-inflammatory, antioxidant, anticancer effects, and inflammatory diseases [10]. The studies have shown that genistein has been studied using HPLC and the RPLC C18 columns used. However, there have been few studies in genistein separation using hydrophilic interaction liquid chromatography (HILIC). A big success of HILIC is that HILIC complements reverse-phase fluid chromatography (RPLC) and is the best alternative to separate compounds not properly preserved in RPLC conditions in most cases [11]. For HILIC mode, a mixture of water and a mostly acetonitrile organic solvent (ACN) is used as a mobile phase with the use of a stationary polar phase [12]. It is well known that the effective groups in the columns of the C18 are hydrophobic and the separation mechanisms are clear and easy to explain. However, HILIC columns contain both hydrophilic and hydrophobic groups as well as ionic groups. Consequently, the separation mechanisms in the HILIC columns are complex, despite the extensive use of HILIC. It is worth noting that studies conducted by Rasheed and coworkers studied the mechanisms of separation and analysis of 2-deoxyuridine, amino acids, pharmaceutical and carboxylic acids using the HILIC columns [13-26]. During this study, the mechanism of genistein separation was studied using ZIC-HILIC column

with UV detector, and this is the first objective of this research. Another goal is to develop a new method for the determination of genistein in some important herbs, such as fenugreek and olive leaves, and one of the most important bee products is propolis. Plants have become a source of many active substances used around the world for the treatment of various diseases. One of these plants is fenugreek seeds. Fenugreek seeds is one of the most common yearly herbs used as a spice in food and medicine. Studies have shown that fenugreek seeds are a rich source of polyphenols, as they contain several compounds, the most important of which are flavonoids, such as apigenin, kaempferol, quercetin and genistein, which have been studied in this research. Fenugreek was used in numerous medications for control and treatment of certain diseases, such as diabetes [27]. Fenugreek has also been used in several patients to reduce cholesterol and triglycerides [28]. The second type of herb that has been studied in this research, which is no less important than fenugreek seeds, is olive leaf. Olive leaf contains amounts of monounsaturated fatty acids as well as ingredients such as phenolics, chlorophyll, and carotenoids [29]. This also includes various flavonoids, including quercetin, rutin and genistein. Bee products are one of the most important components of a contemporary diet which represents a major source of bioactive compounds; bee products have thus received a high level of consumer appreciation [30]. One of the most effective bee products is propolis, which has been used to treat many diseases as a common medicine because it is known as a very strong antioxidant [31].

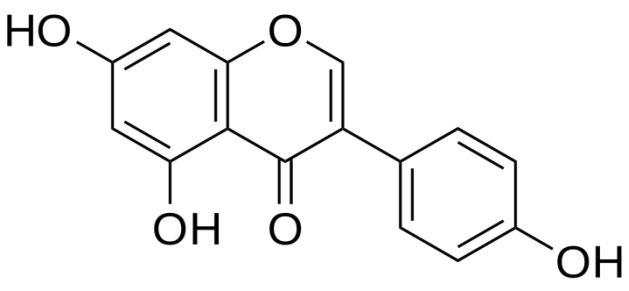

Figure 1: Chemical structure genistein.

MATERIALS AND METHODS

Reagents and materials

HPLC-grade acetonitrile, methanol, ethanol, acetic acid, sodium acetate and genistein have been purchased from Sigma-Aldrich. All reagent solutions were prepared with Millipore water at conductivity 0.1 µs/cm from System-USA Millipores. The solution was filtered by Millex® Syringe filters (0.22 µm).

Preparation of standard solutions

The genistein solution was prepared to get a stock solution of genistein (100 μgmL^{-1}), exactly dissolving the amount of genistein (0.01 g) in 100 ml of an eluent. The result was solved during the mobile phase and filtered through 0.22 µm (Millex® Syringe filters).

Plant materials and sample preparation

This study included two types of applications, the first, herbs such as (fenugreek seeds and olive leaf) and the second, propolis. These products have been brought from the local Iraqi market in 2019. After draining and crushing, 1 g of fenugreek seeds and olive leaf was taken, sample and dissolved in 50 ml solvent of mixture (70: 30) methanol and water the resulting solution treated with ultrasound for 40 min, then after completion, the process will be repeated for 20 min. The result is then taken to the 350 x g centrifuge for 40 minutes then filtered by 0.22 µm Millex® Syringe filters and then the result is kept in a dim bottle in preparation for the separation process by HPLC. To remove beeswax from propolis samples, the propolis was cut into small pieces and diluted in 30 mL of water, then the resulting solution was heated in a water bath at 60 ° C for 20 minutes, and then the solution was filtered to produce wax-free propolis. Approximately (0.7 g) was taken from the bee propolis, it was immersed in 60 ml of 70% ethanol and the resulting solution was treated three times every 30 minutes using ultrasound-assisted extraction. At 50 ° C, the mixed extract was evaporated to dryness and the remaining solution was filtered by 0.22 µm.

Equipment

Analyzes were administered using a high-performance liquid chromatographic system – HPLC. Including the L-6200 gradient on-line degasser with a 20 µL injection loop for the Merck Hitachi HPLC system, and the UV-visible L-4200. The results processed by the N2000 workstation photographic software are used to monitor and examine my chromatography. Separations were obtained on the commercial column ZIC-HILIC from Merck SeQuant (100 mm×4.6 mm I.D.). Ultrasound bath (Fisherbrand-CPXH-USA), centrifuge (Hermle-Germany) and pH 740 (WTW-Germany).

Chromatographic conditions for genistein separation

The mobile phase comprised of acetonitrile (ACN) and acetate buffer filtered through a 0.22 µm and degassed in an

ultrasonic bath before use. The UV-visible detector was set at a wavelength of 260 nm and the chromatogram was measured at 260 nm. Testing was conducted at a flow rate of 0.5 mL/min at 35 °C and the injection volume was 20 µL.

RESULTS AND DISCUSSION

Study separation mechanism

The chromatographic conditions illustrated in this study were obtained using ZIC-HILIC mode, in which genistein was chosen for HILIC retention mechanism as the flavonoid model. As a result, many experiments have been conducted, including the effect of the ACN content, the effect of the buffer concentration used, and the pH influence of eluent.

Study of ACN content impact

The choice of mobile phase composition has a strong and effective impact on retention in HILIC mode and, often when investigating such an influence, a large quantity of organic solvents is added in the ZIC-HILIC columns which may support compounds with low water solubility. The retention behaviour of the genistein was observed at pH 4.74-30 mM acetate buffer. The behaviour of genistein is reversed-phase (RP), continues to rise from 60% to 95%. Hydrophobicity of genistein is the reason for this behaviour, genistein behaviour is shown in Figure 2, which was caused by genistein log P_{OW} value (3.08) [32]. Log POW is a well-known measure of many of the compounds through which we know the behaviour of most of the compounds because each compound has a fixed value. And many pharmacists use these terms extensively and by understanding these terms we can understand the behaviour of many compounds, for example, flavonoids and pharmaceutical compounds.

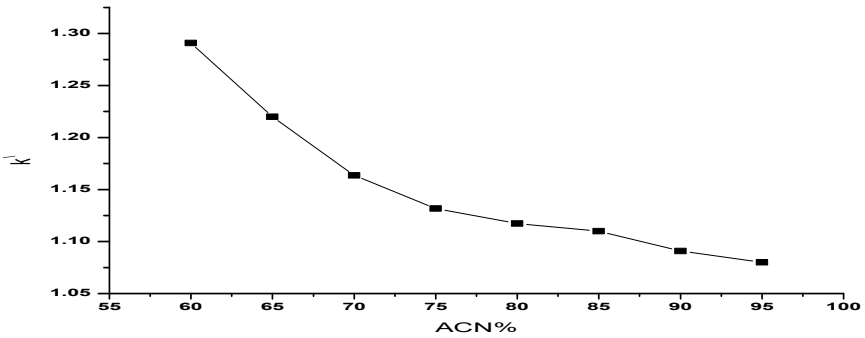

Figure 2: Retention behaviour of genistein as an ACN content variation.

Study the salt concentration impact

The effect of the acetate buffer concentration on genistein retention was achieved while maintaining a constant ACN percentage at 85% and pH at 4.75 while changing the acetate buffer concentration (20-80 mM) in the mobile phase. The results are presented in (Figure 3). The purpose of salts being added to the mobile process is to monitor the interaction between charged analytes and the stationary phase. It was found that when the concentration salt increases, it will have an effective effect by reducing the electrostatic interactions of the charged analytes on ZIC-HILIC columns. It is known that the increase in electrostatic attraction leads to a decrease in retention while, in the direction of repulsion of static electricity, it leads to an increase in retention. Since the salts used in HILIC are considered to be ammonium acetate, formate, and bicarbonate, sodium acetate was used in this study because of its strong solubility in the mobile phase with high levels of organic solvents.

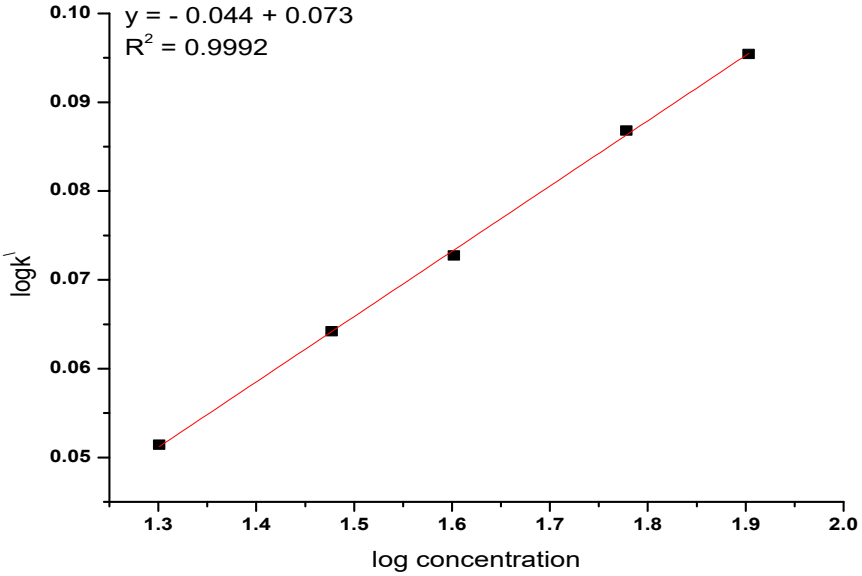

Figure 3: Retention behaviour of genistein as a function of salt concentration

Study of eluent pH impact

When studying genistein retention in the ZIC-HILIC columns of it is preferable to establish pH. This important influence is studied to resolve high electrostatic attraction between analyses and stationary HILIC material of charged states. The eluent pH should be changed to completely separate genistein in HILIC mode. The pH increased from 3 to 5.5 at a constant buffer concentration of 30 mM and 85% of ACN. The retention factor of genistein increases as seen in Figure 4. The reason for this is due to the deprotonation of the hydroxyl group in Genistein. This is in consideration of the physicochemical data to be expected of Genistein. The pKa value from just fewer than 6.55. Increasing the pH of the mobile phase to 5.5 causes it certainly has recognizable deprotonation of the analytes [34].

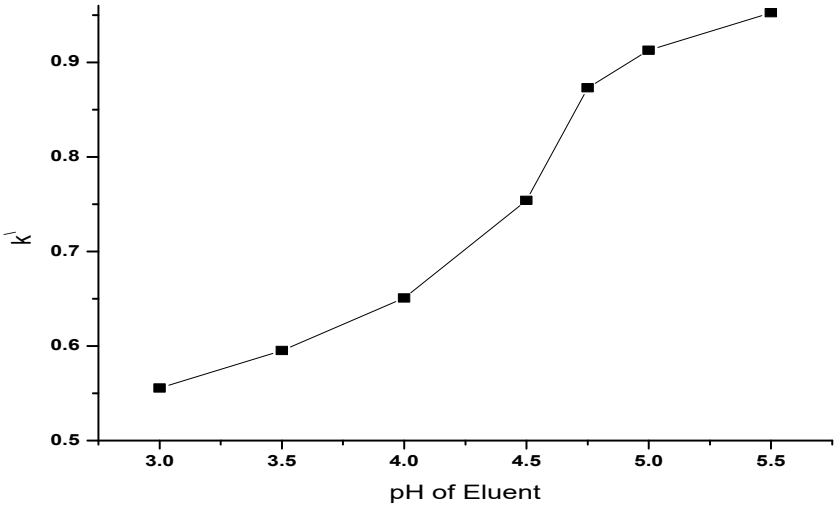

Figure 4: Genistein retention behaviour in difference eluent pH.

Optimization of the proposed method for genistein separation

The effect on the acetonitrile content, the concentration of an acetate buffer and the buffer pH was determined following

analysis of the genistein separation mechanism. The best condition for genistein separation was 85% acetonitrile and acetate buffer 30 mM-pH 4.75. Genistein chromatogram separation as shown in Figure 5.

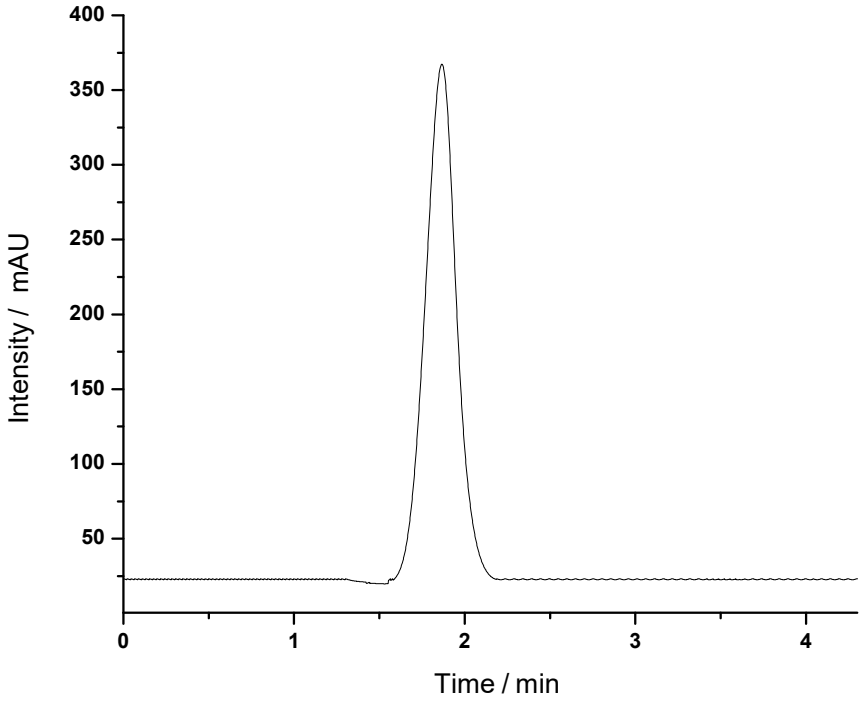

Figure 5: Chromatogram for the separations genistein.

Validation of the developed method for determination genistein

Following ICH guidelines [33], the method was developed. Linearity, accuracy, precision, the limit of detection (LOD) and Limit of quantification (LOQ) have been validated.

Linearity of genistein

Genistein is a 0.05 to 5 μgmL^{-1} concentration range was found to be linear in the linearity analysis. R2 was found to be a 0.9997 correlation coefficient. Results showed that the concentration of the genistein and its peak area are well correlated (Figure 6).

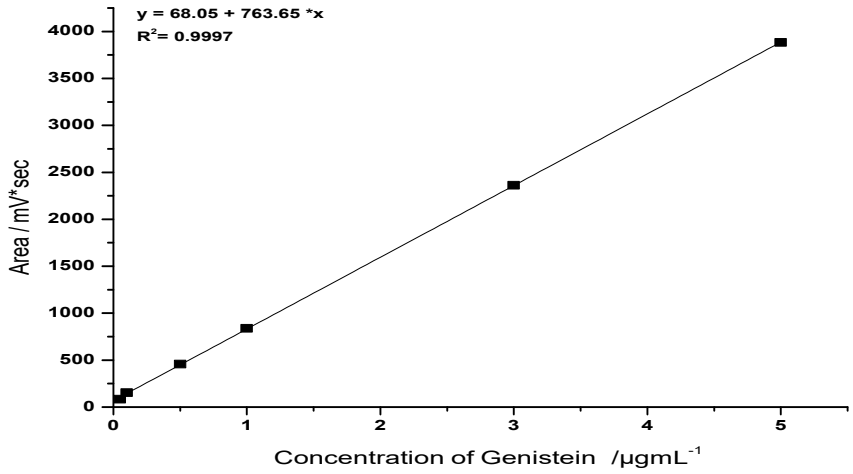

Figure 6: Standard curve of genistein using the ZIC-HILIC column.

Statistical analysis of data

The precision and accuracy investigate were conducted by injecting a genistein solution (five replicate, n=5) into HPLC without altering the test protocol, and the results show that the percentage RSD for genistein is less than 0.5% (Table 1). The low RSD value showed the approach to be correct. The accuracy was calculated by the %recoveries of the concentrations of genistein (1, 2, and 3 μgmL^{-1}). The recovery value is close to 100%, and this indicates the high accuracy of the proposed method. The LOD and LOQ of the proposed method were determined based on the standard deviation of the response and the slope. LOD and LOQ of genistein were found to be 0.0047, 0.0142 μgmL^{-1} respectively.

Table 1: The evidence for studying precision and accuracy, on the same day and different days.

	Same-Day Analysis n=5				**Day-to-Day Analysis n=5**			
Taken (μgmL^{-1})	**Found (μgmL^{-1})**	**% Rec.**	**% Erel.**	**%RSD**	**Found (μgmL^{-1})**	**% Rec.**	**% Erel.**	**%RSD**
1	0.99	99.00	-1.00	0.15	0.99	99.00	-1.00	0.17
2	1.99	99.50	-0.50	0.23	2.01	100.50	0.50	0.21
3	3.02	100.66	0.66	0.17	3.01	100.33	0.33	0.20

Genistein determination in herbs and propolis samples

This suggested method was successfully used for the evaluation of genistein in herbs (fenugreek seeds, olive leaves) and propolis. The results presented in Table 2. It turns out that these herbs, such as (fenugreek seeds, olive leaf) and propolis, are a good source of polyphenol compounds and this is what the results have shown.

Table 3: The performance of genistein in herbs and propolis has been investigated.

Flavonoid	**fenugreek seeds mg/g***	**olive leaf mg/g***	**propolis mg/g***
Genistein	0.0696 ± 0.005	0.0978 ± 0.004	0.756 ± 0.134

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

CONCLUSIONS

Through what was presented in this study, the use of HILIC mode to separate and determine genistein. The HILIC method developed for the detection of genistein in the bulk, herbal and propolis samples was precise, clear, sensitive, reliable and accurate. With good accuracy and precision, the process has a good linear dynamic range. This study concluded by applying the extract of genistein in some important applications, including some important herbs, which are fenugreek and olive leaf, showing the presence of quantities of the genistein compound in these herbs that have been studied. Likewise, propolis was used as one of the bee products and it was found that it contained quantities of genistein compound.

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