Zwitterion Chromatography-Hydrophilic Interaction Chromatography for Separation and Quantitative of Rutin and Quercetin from Herbs and Bee Products

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(Received on 3rd June 2021, accepted in revised form 14th April 2021)

Summary: Quercetin and rutin have a number of useful pharmacological consequences and a possible medication of the future. The growth of cancer cells was observed in vitro and the production of tumors in experimental animals was decreased. In addition to preventing human cancer induction and progression. A set of four HILIC stationary phases, two home-made columns with different space chains between the ionic site groups, and two commercially stationary phases HALO®HILIC 2.7 and ZIC®–pHILIC for chromatographic separation and quantitation of quercetin and rutin in herbs and bee products. The separation mechanisms are based on hydrophobic and hydrophilic for the quercetin and rutin, respectively. The validated method was successfully applied in bee products and herbs. The results showed that the HILIC methods were simple and reliable and could be used in bee products and herbs to detect the content of quercetin and rutin.

Keywords: Quercetin, Hydrophilic interaction, Rutin, Herbs, Bee products.

Introduction

The pharmaceutical activities of quercetin and rutin include anti-tumor, antioxidants, anti-bacterial, analgesic, and anti-inflammatory. Our purpose is to define the chromatographic characteristics of stationary HILIC phases with different lengths of chain for study retention behaviour of quercetin and rutin. There is a tendency towards natural sources of food, including flavonoids, which are natural compounds found in many fruits and vegetables, herbs, tea, supplements, and wine. Some of the most popular flavonoids comprise quercetin and rutin a flavonol prolific in diverse food, wine, beer, herbs, supplement and tea, supplements and wine. Although there are multiple studies in the analysis of quercetin and rutin by RPLC [1-4], however, there are few studies to separate quercetin and rutin (Fig **1**) using HILIC columns with MS detection [5-8]. Rasheed and co-workers, who investigated the mechanisms of pharmaceuticals, nucleosides, amino acids and carboxylic acid separation and analysis using the ZIC-HILIC home-made columns, discussed the convergence of that research [9-17]. The first objective is separation and quantitative quercetin and rutin using HILIC columns with UV detection. In previous studies [18, 19] Rasheed and co-worker studied the effect of chain length between charges between its ZIC-HILIC home-made columns on the separation of carboxylic acids and ranitidine and found that the longer the chain between charges between its ZIC-HILIC columns, the greater the interaction between the analytes and stationary phases. In this study, the investigate the effect of the length of the chain between the two charges using ZIC-HILIC-1 and ZIC-HILIC-5. The study of this effect for the estimation of flavonoids has not been established before, so the second objective of this study will be. As well as studying the retention behaviour of flavonoids and using the ZIC-HILIC column set and compare that to the commercial columns. Bee products are one of the most important elements of a modern diet which are a great source of bioactive compounds it is for this reason that bee products have gained high appreciation from consumers [20]. Bee products like (pollen, propolis, and royal jelly) are regarded as a possible source of natural antioxidants that can mitigate the oxidative stress effects of various diseases [21]. Bee pollen was used in ancient China as a cosmetic agent which was used to help whiten the skin. The substances actually are used in the complementary and alternative medicine division apitherapy [22]. For a long time, propolis has been used as a folk medicine to treat many illnesses [23]. In this study, herbs were used such as (Ginger, Ginkgo, and Ginseng). Ginger is a medicinal plant whose root is widely used as a spice [24]. It is also commonly used in folk medicine due to its many health benefits in various diseases, including chronic diseases [25] such as diabetes, asthma [26], Alzheimer's disease [27], cardiovascular disease [28], and depression in these diseases, the advantage of ginger, in particular, derives from its antioxidant, antimicrobial and anti-inflammatory characteristics. For thousands of years,

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Ginkgo has been a tree growing in China [29]. Ginkgo, due to its effective and excellent biological operations [30]. Ginseng has a number of pharmacological advantages for the central nervous system and the cardiovascular system [31]. There is no analytical method for estimating quercetin and rutin using HILIC columns in bee products and medicinal plants (ginger, ginkgo, and ginseng), so this analysis will present a major challenge. The final objective is to submit a new method for the determination of quercetin and rutin in bee products and herbs.

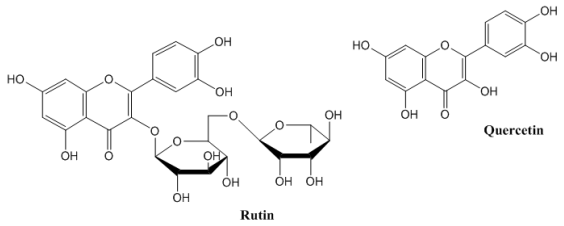

Fig. 1: Structures of quercetin and rutin.

Experimental

Materials

Acetic Acid (HOAc), Sodium acetate (NaOAc), Acetonitrile (ACN), Quercetin and Rutin were purchased from Sigma-Aldrich. All reagent solutions were prepared with the 0.1 µs / cm conductivity of Millipore water (Millipore system-USA). Millex® Syringe filters (0.45 µm-Merck, Germany) filtered the solution. Two groups will be taken, the first herbs such as ginger, ginseng, and ginkgo and the second bee products such as propolis, pollen, and royal jelly. These products were brought from the local markets in Iraq.

Chromatographic facilities and chromatographic conditions

The experimental study was carried out using a 766 IC auto-sampler and 709 IC Pump in 820 IC separation center (Metrohm AG, Herisau, Switzerland). The 820 IC outlet has been connected to a compact 844 UV / VIS IC. The pH measurements were performed on pH Orion 420 A (Thermo Scientific-USA). The IC Net 2.3 software (Metrohm AG, Herisau, Switzerland) was used to monitor the chromatogram and analyze the data. The use of elution was gradient. Eluent was a mixture of acetonitrile and acetate buffer. The eluent has been purified and degassed by using a 0.45 µm membrane filter. The flow rate of 0.75 ml/min was used. The analysis of flavonoids was carried out using the ultraviolet area at a wavelength of 350 nm. The chromatographic conditions are summarized in Table-1.

Table-1: The suggested method's chromatographic circumstances.

Chromatographic circumstances	
Detection	**350 nm**
Volume of the injection	**10 µL**
Flow rate	**0.75 ml/min**
Temperature	**35 ^{0}C**
Mobile phase	**ACN/NaOAc/ HOAc Buffer**

The stationary phases (ZIC-HILIC-1 and ZIC-HILIC-5, 100 mm × 4.6 mm I.D.) used for the separation of quercetin and rutin are home-made by grafted monomers of sulfobetaine (dimethyl(4-vinylbenzyl)ammonio)methanesulfonate and 5-methyl-5-(((4 vinylbenzyl)oxy)sulfonyl) hexan-1-aminium [12, 15] onto the polystyrene/divinylbenzene (PS/DVB) using PEEK columns (100 mm × 4 mm I.D.). The numbers 1 and 5 in ZIC-HILIC-1 and ZIC-HILIC-5 refer to methylene groups between the charged groups in sulfobetaine monomers. Raskop et al. [32] described the detailed procedure for the grafting reaction. The ZIC-HILIC-1 and ZIC-HILIC-5 columns have capacity 432 and 488 µeq g^{-1} respectively. The commercial columns ZIC®–pHILIC and HALO®HILIC 2.7 columns were obtained from Merck SeQuant and Advanced Materials Technology respectively (100 mm × 4.6 mm I.D. and 100 mm × 2.1 mm I.D.).

Preparation of stock solutions of flavonoids

By dissolving an accurately weighed amount of quercetin and rutin (10 mg) in 100 ml of eluent, a stock solution of quercetin and rutin were prepared (100 µg ml^{-1}). Aliquots (50-8000 µL) of a stock standard solution of quercetin (10-25 µg mL^{-1} quercetin) were transferred into 10 mL volumetric flasks. Aliquots (25-7000 µL) of a stock standard solution of rutin (20 µg mL^{-1} rutin) were transferred into 10 mL volumetric flasks.

Preparation of materials of herbs and bee products

For extraction of flavonoid compounds from the herbs (ginger, ginseng, and ginkgo) we took the herbs after draining , crushing, and grounding weighted (1g) of the herb sample and dissolved in 50 ml solvent of mixture (70: 30) methanol and water the resulting solution treated with ultrasound for 60 min, then after completion, the process will be repeated for 25 min. Then we puted in to a 350 x g centrifuge for 30 minutes than Filtered by 0.45 µm membrane filter (Millipore).

In order to remove the beeswax of bee products samples (propolis, pollen, and royal jelly), it was cut into small pieces by an operating scissors and diluted in 10 ml of water. The resulting solution was heated at 70 °C for 40 min in a thermostatic water bath system and filtered. This was then placed in a constant temperature drying

oven set at 80 °C until the weight was constant. The dried refined bee products samples were homogenized using an agate homogenizer and stored in sealed glass vials until analysis. In order to extract flavonoids compounds from bee products samples, 0.684 g of the dried refined bee products was immersed in 10 ml of 80 % methanol for 24 h, and the resulting solution was treated using ultrasound-assisted extraction procedure three times, each time for 20 min.

The combined extract was evaporated to dryness in a rotary evaporator at 65 °C, and the residue was reconstituted with 100 ml of mobile phase, filtered through a 0.45 syringe filter and injected into HPLC system.

Method Validation

All methods have been validated in compliance with the ICH Harmonized Tripartite Guideline [33], including the accuracy, precision, linear range, detection limit, and quantification limit (Tables 2-4). The dilution of the stock solution with eluent in a different concentration of quercetin and rutin has created a variety of stock solutions.

Table-2: Linearity, detection limit, quantification limit and R^2 for quantitative determination of Quercetin and Rutin

Parameter		ZIC-HILIC-1	ZIC-HILIC-5	ZIC®–pHILIC	HALO®HILIC
Linearity (µg.ml^{-1})	Quercetin	0.5-20	0.1-15	0.05-7	0.05-18
	Rutin	0.005-10	0.01-12	0.05-3	0.05-14
R^2	Quercetin	0.9991	0.9994	0.9996	0.9996
	Rutin	0.9996	0.9990	0.9996	0.9998
LOD (µg.ml^{-1})	Quercetin	0.0340	0.0260	0.0160	0.0220
	Rutin	0.0021	0.0035	0.0200	0.0140
LOQ (µg.ml^{-1})	Quercetin	0.1130	0.0880	0.0550	0.0700
	Rutin	0.0073	0.0122	0.0730	0.0490

Table-3: The analytical accuracy and precision of quercetin on the same day and on daily.

	Same-Day Analysis n=5				Day-to-Day Analysis n=5			
Quercetin Taken (µg.ml^{-1})	Quercetin Found (µg.ml^{-1})	% Rec.	% Erel.	%RSD	Quercetin Found (µg.ml^{-1})	% Rec.	% Erel.	%RSD
ZIC-HILIC-1								
2	2.050	102.50	2.50	1.23	2.030	101.50	1.50	1.44
4	4.070	101.75	1.75	1.05	4.060	101.50	1.50	1.26
5	5.020	100.40	0.40	0.93	5.030	100.60	0.60	1.13
ZIC-HILIC-5								
2	2.030	101.50	1.50	1.06	2.010	100.50	0.50	1.22
4	4.020	100.50	0.50	0.97	4.030	100.75	0.75	1.16
5	5.010	100.20	0.20	0.90	5.040	100.80	0.80	1.03
ZIC®–pHILIC								
2	1.950	97.50	- 2.50	0.75	1.970	98.50	- 1.50	0.82
4	3.930	98.25	-1.75	0.63	3.980	99.50	- 0.50	0.79
5	4.960	99.20	- 0.80	0.55	4.940	98.80	- 1.20	0.62
HALO®HILIC								
2	2.010	100.5	0.50	0.88	2.030	101.50	1.50	0.93
4	3.970	99.25	- 0.75	0.71	4.010	100.25	0.25	0.82
5	4.977	99.54	- 0.46	0.55	4.985	99.70	- 0.30	0.67

Table-4: The analytical accuracy and precision of rutin on the same day and on daily.

	Same-Day Analysis n=5				Day-to-Day Analysis n=5			
Rutin Taken (µg.ml^{-1})	Rutin Found (µg.ml^{-1})	% Rec.	% Erel.	%RSD	Rutin Found (µg.ml^{-1})	% Rec.	% Erel.	%RSD
ZIC-HILIC-1								
1	0.980	98.00	- 2.00	0.73	0.980	98.00	- 2.00	0.81
2	1.970	98.50	- 1.50	0.55	1.970	98.50	- 1.50	0.66
3	2.992	99.73	- 0.27	0.41	2.993	99.33	- 0.67	0.60
ZIC-HILIC-5								
1	1.020	102.00	2.00	0.65	1.010	101.00	1.00	0.68
2	2.020	101.00	1.00	0.49	2.040	102.00	2.00	0.53
3	2.993	99.76	- 0.24	0.40	3.005	100.16	0.16	0.45
ZIC®–pHILIC								
1	0.990	99.00	- 1.0	0.48	1.010	101.00	1.00	0.56
2	2.010	98.25	- 1.75	0.34	2.020	101.00	1.00	0.38
3	2.985	99.50	- 0.50	0.28	2.996	99.86	- 0.14	0.31
HALO®HILIC								
1	1.010	101.00	1.00	0.66	1.010	101.00	1.00	0.78
2	1.990	99.50	- 0.50	0.41	2.010	100.50	0.50	0.50
3	3.007	100.23	0.23	0.36	3.010	100.33	0.33	0.43

Linearity

In the same conditions, three injections from each concentration were analyzed. The linear regression analysis has been employed to assess the linearity of the least square calibration curve strategy (Table-2).

Accuracy

The precision of the methods proposed was calculated by three concentrations of recovery experiments (2, 4 and 5 μgml^{-1}), with three replicates (n=3) being injected at each concentration. The recoveries are monitored on the same day and on different days (Table-4).

Precision

The relative standard deviation (RSD %) measurements of three concentrations (2, 4 and 5 μgml^{-1}) were used to determine the accuracy of the proposed methods. The values of RSD% are monitored on the same day and multiple days by five replicates (n =5) for each concentration were injected (Table-4).

Results and discussion

Optimization separation of flavonoid:

Quercetin and rutin have been selected in two columns of the home-made column and two commercial columns (ZIC®–pHILIC and HALO®HILIC 2.7) as a flavonoid model for evaluating HILIC retention mechanism with ACN acetate buffers. The chromatograms of quercetin and rutin exhibit in Fig. 2 under condition 90% ACN and 30 mM acetate buffers (pH 5.5). An evaluation of the characteristics of the ZIC-HILIC exchangers and thus the separation process shall be made of the ACN, pH and eluent concentrations. It is worth noting that the retention of quercetin and rutin in the column ZIC-HILIC-5 displayed the highest retention compared with the behavior of quercetin and rutin in the columns ZIC-HILIC-1, ZIC®–pHILIC and HALO®HILIC 2.7. This is most likely the clarification of the methylene group between charged groups in ZIC-HILIH-1 and ZIC-HILIH-5 columns between the charged groups and core material in ZIC®–pHILIC and HALO®HILIC 2.7.

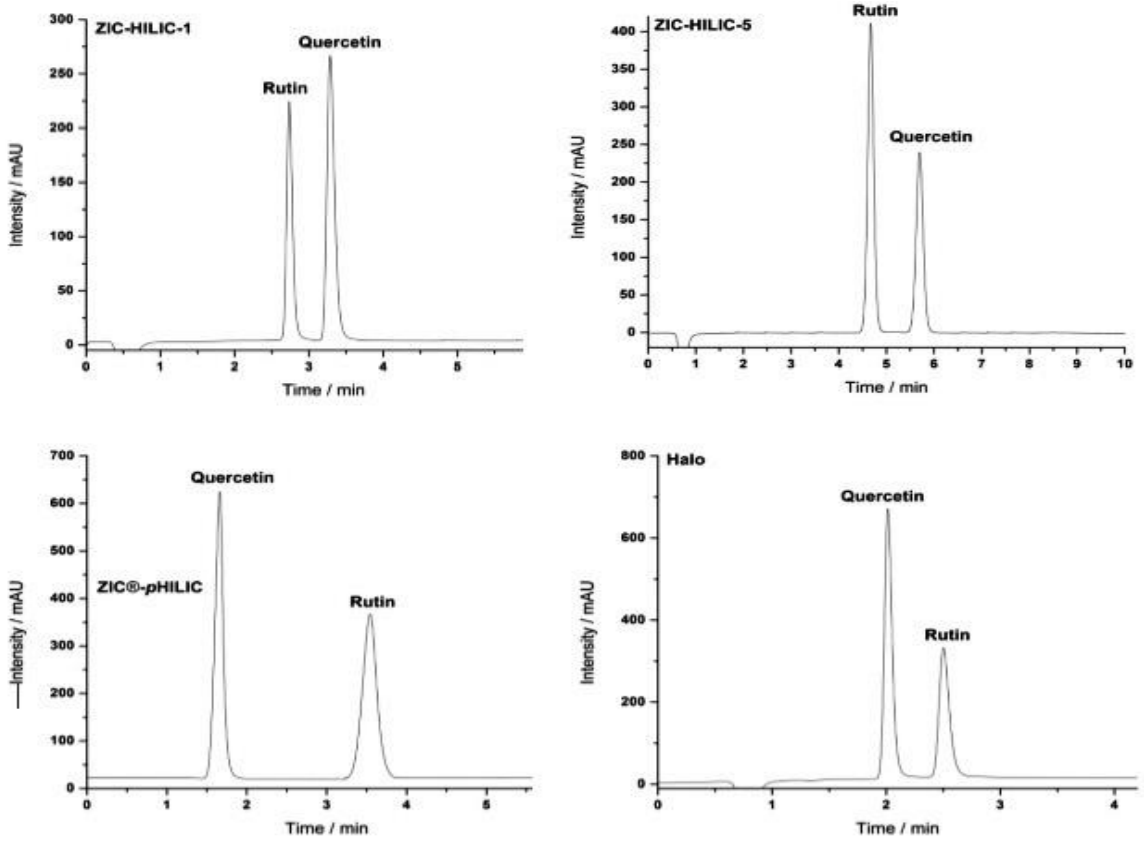

Fig. 2: Chromatograms for the separations of quercetin and rutin.

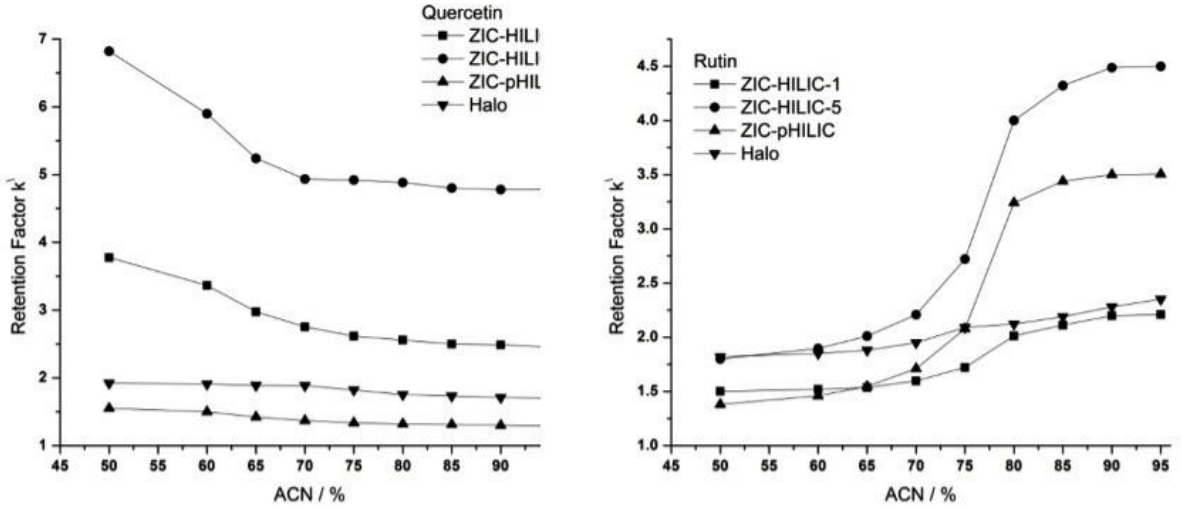

Fig. 3: Effect of ACN content on quercetin and rutin retention.

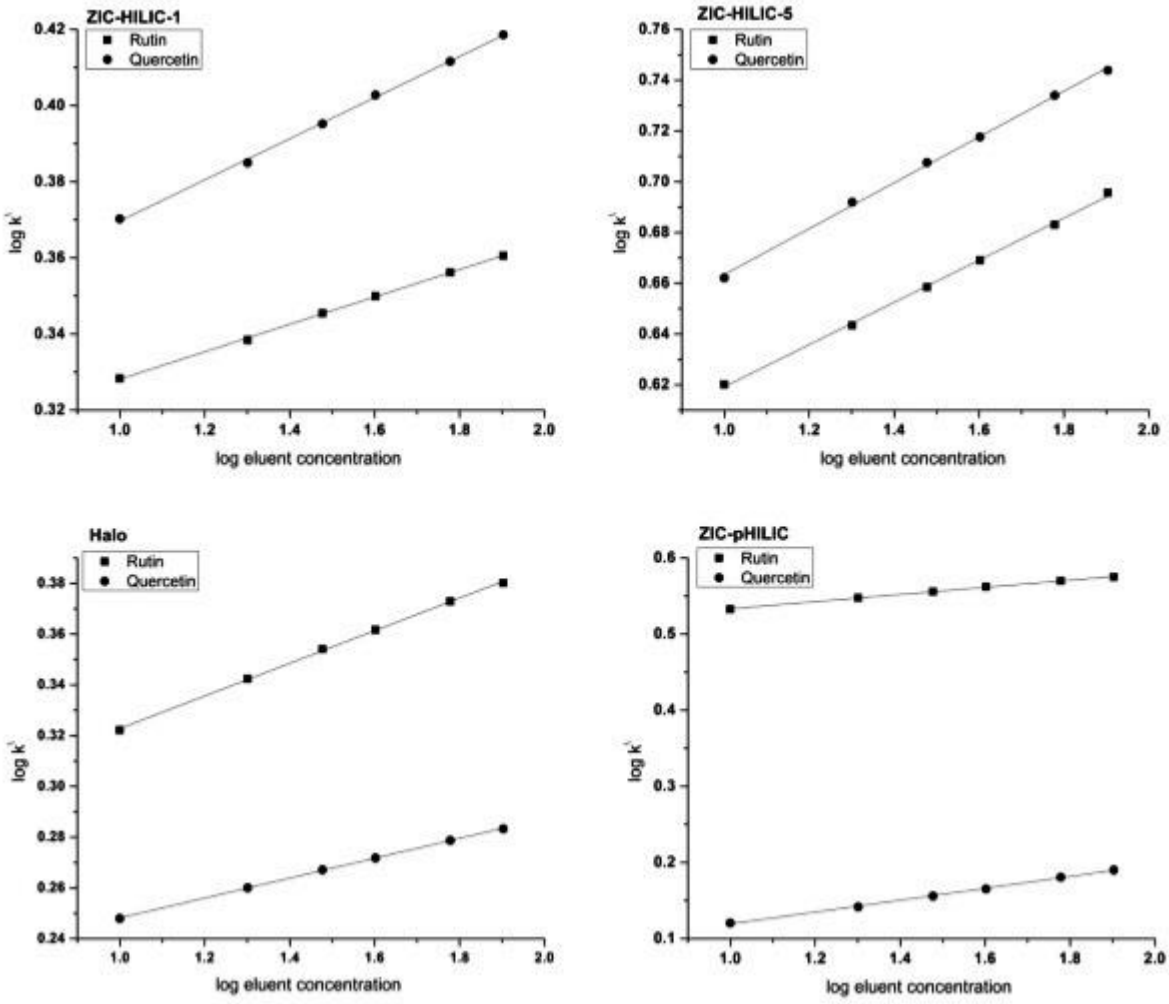

Fig. 4: Effect of eluent concentration on quercetin and rutin retention.

ACN Content effect on quercetin and rutin retention

The effect of the eluent ACN content on the retention activity of the quercetin and rutin tested was studied at 5.5 pH 35 mM *(in aqueous part)* NaOAc / HOAc. Quercetin and rutin have RP and HILIC behavior, with the percentage of ACN eluent increasing from 60% to 95%. The explanation for this behavior is quercetin and rutin hydrophilicity. The RP and HILIC conduct of quercetin and rutin for all columns is illustrated (Fig 3). This is attributed to the log P_{OW} of quercetin and rutin (2.16 and -0.87) [34], respectively.

Eluent concentration effect on quercetin and rutin retention

Salts are normally applied to the eluent in order to control the electrostatic interactions between solute and exchangers. In its concentration of 10-80 mM (pH 5.5) at 90% ACN in the eluent the effect of the NaOAc / HOAc buffer in the eluent was investigated on the retention behavior of quercetin and rutin. The results are presented in (Fig 4). Increasing NaOAc / HOAc buffer concentration in the eluent increases the retention factor of quercetin and rutin for all columns. The complexity of the separations prevents individual consideration of the interplaying separation mechanisms. However, it can at least be shown that the hydrophilicity of the analytes and electrostatic influences known from the ZIC play a role. In addition, the quality of the phase separation between the mobile and pseudo-stationary phase also seems to have an impact on the chromatographic separations. The separation of flavonoids is considered to be mainly based on the formation of a pseudo-stationary water layer on the column stationary phase between which fast partitioning occurs with the rich organic solvent mobile phase [35, 36].

Eluent pH effect on quercetin and rutin retention

A change in eluent pH is the next improvement of eluent composition, which can be implemented. The eluent pH must be changed to complete the separation of the quercetin and rutin image in HILIC mode. At a constant buffer concentration of 35 mM and 90% ACN, the pH increased from 3 to 5.5. The retention factor of quercetin and rutin increasing as shown in Fig 5. The reason for this is due to the deprotonation of the hydroxy group in quercetin and rutin. This is in consideration of the physicochemical data to be expected of the two quercetin and rutin. The pKa values range from just fewer than 6.38 [34].

Calibration graph

The calibration graphs quercetin and rutin are generated by plotting the quercetin and rutin concentrations contrary to the peak area and showing the four stationary phases range concentration (Fig 6).

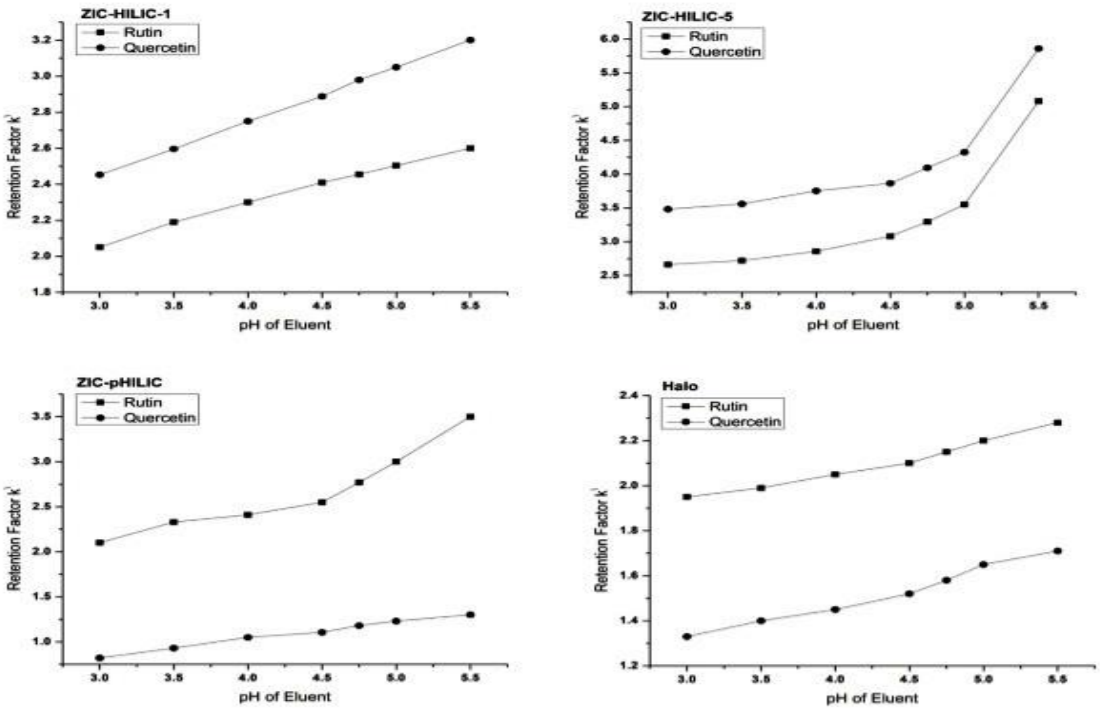

Fig. 5: Effect of eluent pH on quercetin and rutin retention.

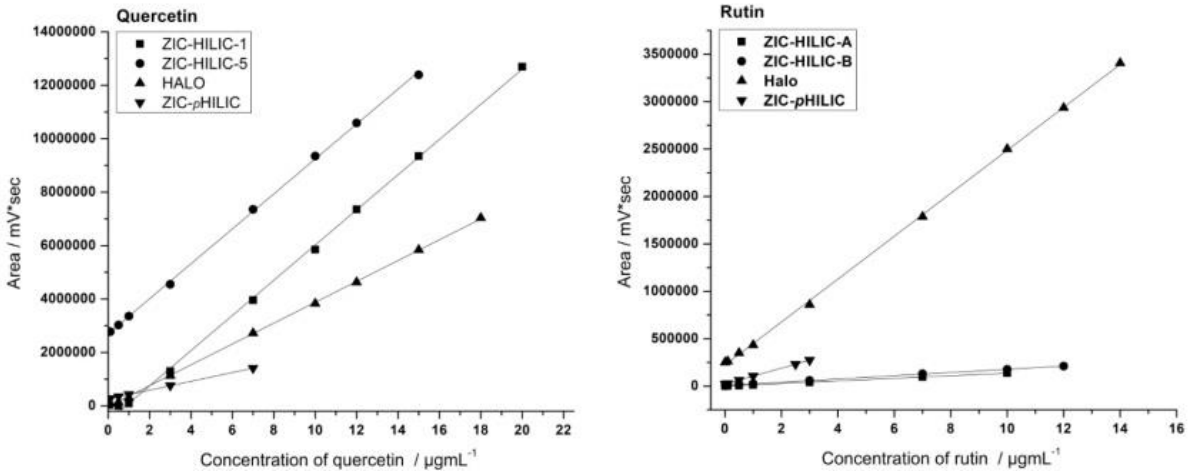

Fig. 6: Calibration graphs for quercetin and rutin using ZIC-HILIC-1, ZIC-HILIC-5, and HALO® HILIC 2.7 and ZIC®–pHILIC columns.

Table-5: The comparison of the proposed methods ZIC-HILIC-A, ZIC-HILIC-B, and ZIC®-*p*HILIC and HALO®HILIC with comparison method for Quercetin and rutin analysis by examining t- and F-statistical tests.

Methods	Quercetin	Rutin	t-Test(theor.)	F-Test(theor.)
ZIC-HILIC-1	**102.50**	**98.00**	**0.0844***	**4.4371***
	101.75	**98.50**	**0.1366****	**2.1073****
	100.40	**99.73**	**(2.7764)**	**(19.000)**
ZIC-HILIC-5	**101.50**	**102.00**	**0.2067***	**1.8153***
	100.50	**101.00**	**0.2405****	**3.3477****
	100.20	**99.76**	**(2.7764)**	**(19.000)**
ZIC®–pHILIC	**97.50**	**99.00**	**0.0424***	**2.8438***
	98.25	**98.25**	**0.1236****	**1.0523****
	99.20	**99.50**	**(2.7764)**	**(19.000)**
HALO®HILIC	**100.5**	**101.00**	**0.6504***	**1.6770***
	99.25	**99.50**	**0.5761****	**1.4958****
	99.54	**100.23**	**(2.7764)**	**(19.000)**
Comparison method [37]	**100.55**	**99.83**		
	99.56	**100.55**		
	99.54	**99.33**		

** For Quercetin*
*** For Rutin*

Table-6: The quality of flavonoids examined in bee products and herb extracts.

	Herbs samples		
Name of flavonoid	**Ginger mg/g**	**Ginseng mg/g**	**Ginkgo mg/g**
Quercetin	**4.285 ± 0.260**	**207.45 ± 15.33**	**3.185 ± 0.202**
Rutin	**2.300 ± 0.08**	**2.850 ± 0.06**	**2.330 ± 0.133**
	Bee products samples		
	Propolis mg/g	**Pollen mg/g**	**Royal jelly mg/g**
Quercetin	**0.125 ± 0.09**	**7.035 ± 0.15**	**0.051 ± 0.06**
Rutin	**0.077 ± 0.05**	**6.253 ± 0.16**	**0.083 ± 0.07**

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

Statistical analysis

The HILIC mode is used for statistical results (Table 2) of quercetin and rutin calibration graphs. Table-3 and 4 indicate accuracy and precision are measured for the %RSD and % recovery on the same day and different days for four stationary phases. The standard deviation is relatively small and the high recovery value suggests accuracy in the four proposed methods. In order to assess the competence and efficiency of the ZIC-HILIC-1, ZIC-HILIC-5, and ZIC®-*p*HILIC and HALO®HILIC methods, these findings were compared with the results obtained by the comparison method [37]. Statistical analyses were performed with the findings of the four methods t-test and variance ratio F-test (Table-5), which were 95% confidence. The t and F values calculated did not exceed the theoretical values, which mean that both methods do not differ significantly in the precision of the quercetin and rutin determination.

Determination of quercetin and rutin in herbs and bee products

The suggested methods using four stationary phases were successful application to the quantitation of quercetin and rutin in two groups' herbs and bee products (Table-6). For the study of the bioactive compounds in extracts of common herbs (ginger, ginseng, and ginkgo) and bee products (propolis, pollen, and royal jelly), the ability of HILIC separation was demonstrated. The eluent was used with the 35 mM (pH 5.5) NaOAc / HOAc buffer contained 90% acetonitrile.

Conclusions

HILIC methods for evaluating quercetin and rutin in bee products and herbal extracts were developed in this report. The stationary phase ZIC-HILIC-5 with long-chain length exhibits more interaction with quercetin and rutin contrast with ZIC-HILIC-1, HALO®HILIC 2.7 and ZIC®–pHILIC columns. There are two explanations for this, first the methylene groups between the ionic site groups in the stationary phase, like in the three columns (ZIC-HILIC-1, ZIC-HILIC-5, and ZIC®–pHILIC). The second explanatory argument that the core material (backbone) of stationary phases, which that the PS/DVB as a backbone in ZIC-HILIC-1 and ZIC-HILIC-5 whereas silica and methacrylate as a core material in HALO®HILIC 2.7 and ZIC®–pHILIC columns respectively. Quercetin and rutin demonstrate hydrophobic and hydrophilic retention with HILIC columns.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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