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Effect of Superficially Porous Particles on Chromatographic Analysis Time of Flavonoids

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Abstract

Due to superficial particles and remarkable packing technologies, the HALO-HILIC column outperformed regular porous columns in terms of analysis time. This study analyzes and identifies kaempferol and luteolin flavonoids in natural herbs. A fast, novel chromatographic method combining superficially porous silica particles packed in a column served as the separation tool for hydrophilic interaction liquid chromatography coupled with a UV detection system is being introduced in this work. Flavonoid separation and quantification took six minutes using only superficial porous particles in the HILIC column compared to hours using fully porous particles in the RP columns. The findings demonstrated that the HILIC mode might be utilized to determine kaempferol and luteolin levels in ginkgo plant samples. A commercial HALO-HILIC column was used to create the calibration curve, which had the following specifications: linear range (0.065-35 μgmL^{-1} for kaempferol and 0.065-9 μgmL^{-1} for luteolin), RSD% not exceeding 0.54%, the limit of quantification (0.036 μgmL^{-1} for kaempferol and 0.024 μgmL^{-1} for luteolin), and limit of determination (0.012 μgmL^{-1} for kaempferol and 0.008 μgmL^{-1} for luteolin).

Keywords: HALO-HILIC column, Kaempferol, Luteolin, Medicinal herbs, Superficial particles.

1. Introduction

Flavonoids and their derivatives are a significant group of natural compounds found inside cells or outside various plant organs (1). They are primarily sourced from foods like fruits, beverages, herbs, vegetables, and supplements. Natural flavonoids in fruits and vegetables help protect against oxidative stress and serve a nutritional role (2). They are incredibly potent antioxidants and may even be more effective than other now-recognized antioxidants, such as vitamins C and E (3-5). Flavonoids exhibit antioxidant action by preventing free radical synthesis and scavenging ROS, RNS, and other reactive species. Their antioxidant properties are due to the phenolic hydrogen, unique substitution patterns, and chemical composition (6, 7).

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Flavonoids comprised of two benzene rings (A and B) joined by a heterocyclic ring (C) through oxygen. Based on the relationship between the B and C rings, the configuration of the B ring, and the hydroxylation and glycosylation sequence of the three rings, flavonoids can be classified into various subclasses (8-10). Anthocyanidins (malvidin, pelargonidin, and peonidin), flavanones (hesperidin, naringenin, and naringin), isoflavones (genistein and daidzein), flavonols (kaempferol, fisetin, and quercetin), and flavones (apigenin, luteolin, and chrysin) are some of the subclasses (11-15). Kaempferol (3,4′,5,7-tetrahydroxyflavone) is a flavonoid structurally similar to estrogen, found in various fruits and vegetables like apples, grapes, tomatoes, and green tea (17-19). It has antioxidant, anti-inflammatory, antibacterial, cardiovascular, and neuroprotective properties. Due to its estrogen-like structure, kaempferol may help treat hormone-regulated tumors, including ovarian, breast, cervical, liver cancers, and leukemia (20-22). Luteolin (3′,4′,5,7-tetrahydroxyflavone), a common flavonoid in fruits, vegetables, and medicinal herbs, is used in Chinese traditional medicine to treat cancer, inflammation, and hypertension (23-26). It acts as an antioxidant or pro-oxidant with anti-inflammatory, anti-allergic, and anti-cancer properties. Recent studies suggest its role in cancer prevention (27-31). The separation and determination of flavonoids in natural sources have increased due to their chemical and biological importance. A common method for isolating and quantifying flavonoids is reversed phase-high-performance liquid chromatography, using various stationary phases and eluents. The mobile phase is often made up of combinations of water and methanol or acetonitrile combinations, while the stationary phases are typically C18 or C8 (32). Even when high-resolution columns are used, co-elution remains a significant issue in studying polyphenolic substances. A further drawback of the reversed-phase mode is the longest single run, which might last between 30 minutes and an hour (33). Separating the flavonoids that have been isolated from nuts and fruit was demonstrated by Harly et al., using a mobile phase consisting of a mixture of methanol, acetonitrile, and trifluoroacetic acid. In under 60 minutes, the authors separated 20 polyphenolic chemicals (34,35). Sakakibara et al., (36) developed a technique for separating and quantifying polyphenolic compounds in food, though it took up to 95 minutes per run. Lee et al., (37) analyzed chokeberry juice, with a single analysis taking 70 minutes. A gradient elution in another study took 65 minutes to determine the polyphenolic composition of Poligano purple carrots (38). Utilizing ultra-pressure liquid chromatography is one method of reducing the duration of a single analysis. This method's foundation is the employment of columns that are densely packed with particles with a diameter of less than 2 µm. In contrast to the usual approach, these findings create high pressure but are distinguished by improved resolution, sensitivity, and retention durations (39-42). Schwarz et al., (43) used UHPLC and traditional HPLC to separate phenolic components in brandy, with UHPLC completing the separation in 6.5 minutes, compared to 60 minutes with HPLC (44). The development of superficially porous particles offers similar efficiencies to UHPLC without high pressure requirements, allowing use with existing HPLC systems, providing financial advantages over UHPLC (45). The column used in this study, introduced in 2006, sparked renewed interest in particle design research. Additionally, hydrophilic interaction liquid chromatography (HILIC) is used as an alternative to RP and NP modes for fast separations (46). In 1990, Alpert first developed the concept of hydrophilic interaction liquid chromatography (47). HILIC typically incorporates polar stationary phases with eluents and many organic solvents (10, 48, 49). Jandera and Janas's study (50) identifies three HILIC

stationary phase subgroups: neutral (e.g., diol), charged (e.g., bare silica), and zwitterionic (e.g., sulfobetaine). The HILIC used in this study is charged bare silica with a fused core and porous shell, offering lower backpressure compared to sub-2-m particles in other columns (51). This study analyzes and identifies kaempferol and luteolin flavonoids in natural herbs.

2. Materials and Methods

2.1. Apparatus and chemicals

A quaternary pump, a column oven, and a UV-Vis detector were all parts of the HPLC apparatus (Metrohm AG, Germany) for the liquid chromatographic studies. The supplier provided acetonitrile (CH_3CN) and Formic acid (CH_2O_2) (HPLC grade) for analysis (Spelco). Sodium acetate is supplied from (Sigma-Aldrich). The water for the mobile phases was purified with a Milli-Q system (from Millipore). Standards for luteolin and kaempferol were provided by Carl Roth Gmbh (Karlsruhe, Germany). The HALO-HILIC column was purchased from (Advance Material Technology, USA).

2.2. Sample preparation

Ginkgo herb was drained, crushed, and ground before being weighed (1 g) and dissolved in 50 ml of acetonitrile and water (70:30 v/v) to extract flavonoids. The final product was then centrifuged for 30 minutes before being filtered through a 0.45 µm membrane filter after 60 minutes of ultrasonic treatment, 25 minutes of the operation, and 30 minutes of centrifugation.

2.3. Standard sample preparation

About 10 mg of each compound was dissolved in 100 mL of CH3CN to prepare a stock solution (100 mg/L) of kaempferol and luteolin. The solution was next diluted to the proper concentration ranges to create calibration curves and passed through a 0.45 µm filter.

2.4. Chromatographic conditions

Employing a HALO-HILIC (100 mm x 2.1 mm ID, 2.7 µm) to carry out the chromatographic separation of plant flavonoids; CH_3CN with CH_2O_2 content served as the comparable mobile phase A (organic portion of the mobile phase), while water with CH_2O_2 content served as the comparable mobile phase B (aqueous portion of the mobile phase). The linear gradient elution procedure was established at a 1.0 mL/min flow rate. The column was kept at a constant temperature of 30 ºC, and the injection volume was 10 µL.

3. Results and Discussion

3.1. Chromatographic separation

In contrast to columns filled with 5 µm porous silica particles, 2.7 µm superficially porous silica particles allow for speedy and efficient HILIC separations. Rapid separations and quantification of highly and moderately polar substances are possible because bare silica columns packed with 2.7 µm particles can be operated at conventional HPLC pressures, and flow rates equal those used in UPLC on RP columns (52). **Figure 1** demonstrates the chromatographic separation of kaempferol and luteolin in ginkgo herbs using a HALO-HILIC commercial column.

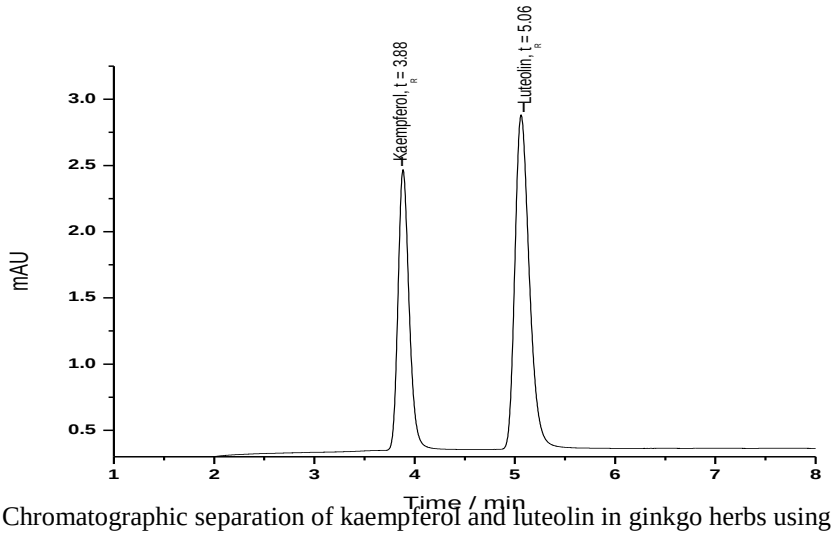

Figure 1. Chromatographic separation of kaempferol and luteolin in ginkgo herbs using a HALO-HILIC.

It clearly shows the fast run time of the current study (under 6 min) compared to other reported analysis times under the fully poured RP column. The main reason is the particle size and shape of the column used in this study. The effect of superficial particles on the chromatographic behavior best explain by the Van Deemter equation:

$$H = A + \frac{B}{u} + Cu \quad (1)$$

Where H is the plate height (as a measure of the resolving power of a column), u is the velocity; A is the Eddy diffusion (related to channeling through non-ideal packing, B is the longitudinal diffusion (eluting particles in the longitudinal direction resulting dispersion) and C the resistance to mass transfer (resistance to mass transfer coefficient of the analyte between mobile and stationary phase). A, B and C are directly connected to the parameters of the column. Both packing quality and particle size have an impact on eddy diffusion. Compared to fully porous particles, the size distribution of a superficial particle is substantially narrower. By decreasing Eddy diffusion, the distance between the particles in the column is lowered, and efficiency increases. Two significant factors impact A, the first associated with the typical particle packing structure, and the second on homogeneity within the column. A better packing of the spheres was achieved in the case of superficial particles due to the restricted particle distribution (53), as shown in **Figure 2**.

The B term describes axial molecule diffusion during the separation process. Because a core prevents a solute from diffusing through superficially porous particles, a silica column with superficial silica packing diffuses a solute less than one with total silica porosity. As a result, in a Van Deemter equation, the B term decreases in the core-shell superficial silica column, causing faster analysis time (54), as demonstrated in **Figure 3**.

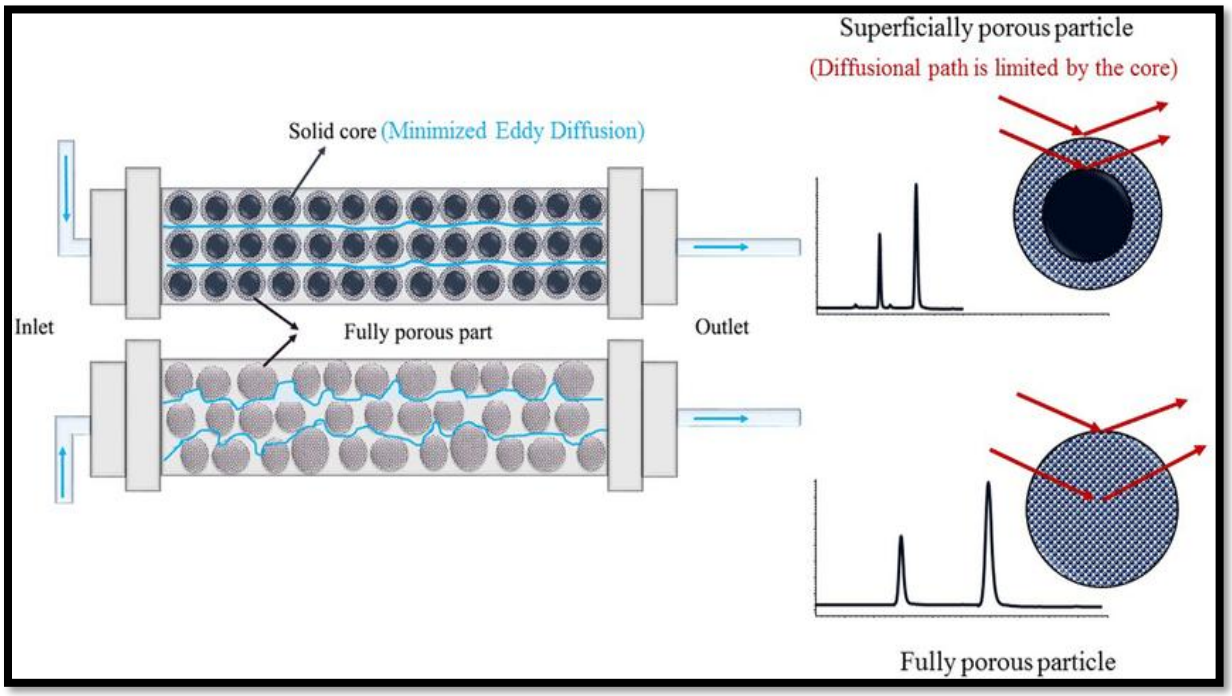

Figure 2. The contrast between fully porous particle distribution (traditional silica) and narrow particle distribution (superficial silica) is an example of the impact on peak efficiency and analysis time.

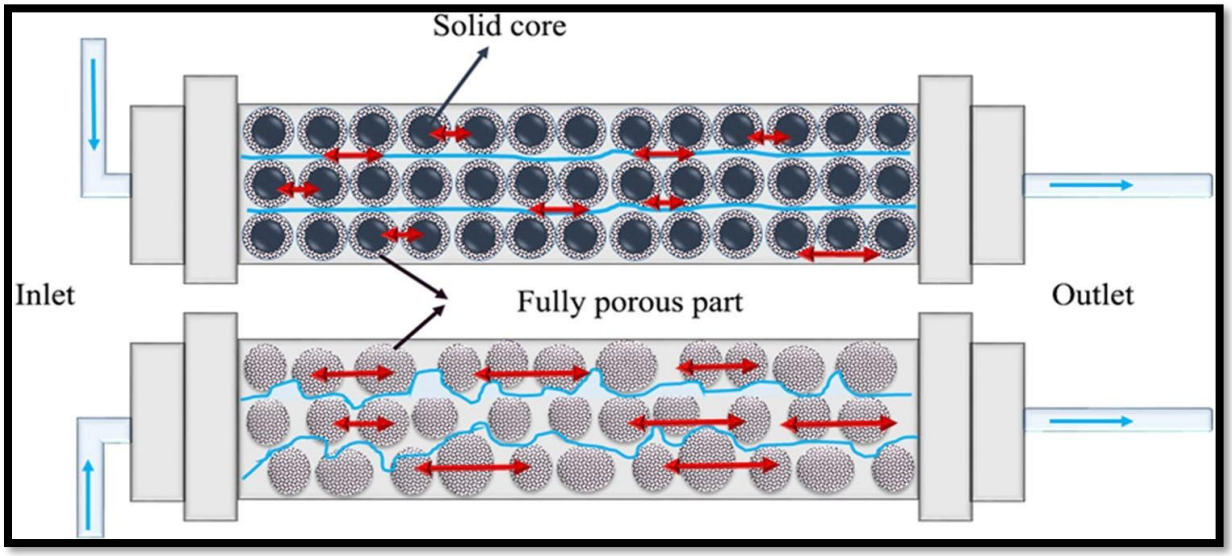

Figure 3. An illustration of the effect of the B term is provided by solid core particles preventing diffusion as a solute diffuses through a pore and outside of totally porous particles.

3.2. Optimization of chromatographic conditions

3.2.1. Mobile phase influence on the retention factor

The hydrophilicity factor plays a significant part in the behavioral differences between kaempferol and luteolin. Both kaempferol and luteolin exhibit RP activity for commercial columns (HALO-HILIC 2.7 µm), as shown in **Figure 4**, due to their log Pow values (2.188 and 2.403), respectively.

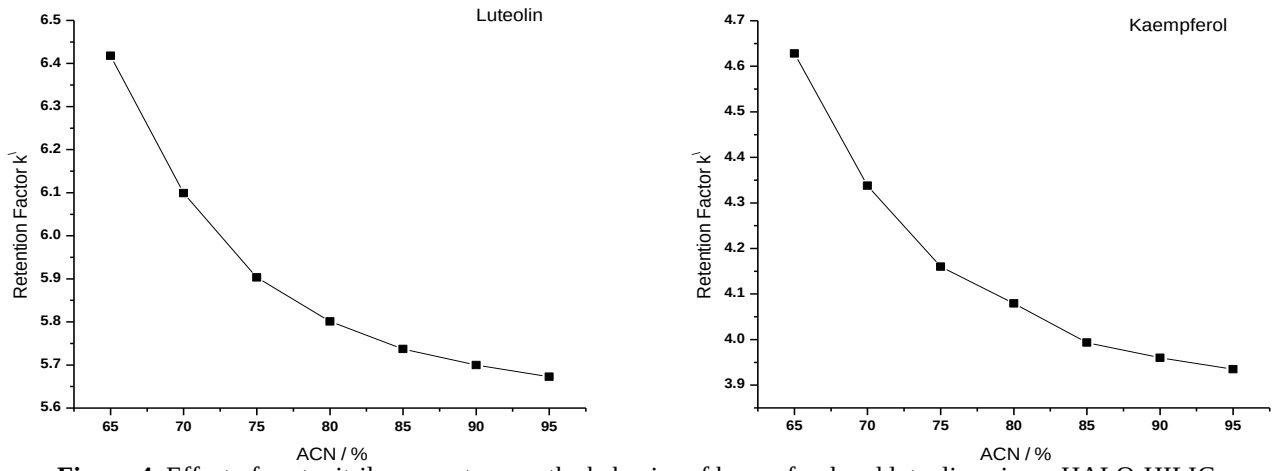

Figure 4. Effect of acetonitrile percentage on the behavior of kaempferol and luteolin using a HALO-HILIC.

3.2.2. Eluent concentration effect on the retention factor

With a rise in eluent concentration, solute behavior has become more hydrophilic, which has resulted in the deactivation of intermolecular ion pairs. Even if CH_3CN exists, this method improves the linearization of the functional column groups. Surprisingly, when the quantity of CH_2O_2 is increased from 10-100 mM while retaining CH_3CN at 90% and pH at 5.5, kaempferol and luteolin exhibit enhanced retention factors (**Figure 5**). But at least the hydrophilicity of the analytes may be demonstrated. Additionally, the chromatographic separations are influenced by how well the mobile and pseudo-stationary phases are separated. Creating a pseudo-stationary water layer on the column is believed to be the primary basis for separating kaempferol and luteolin.

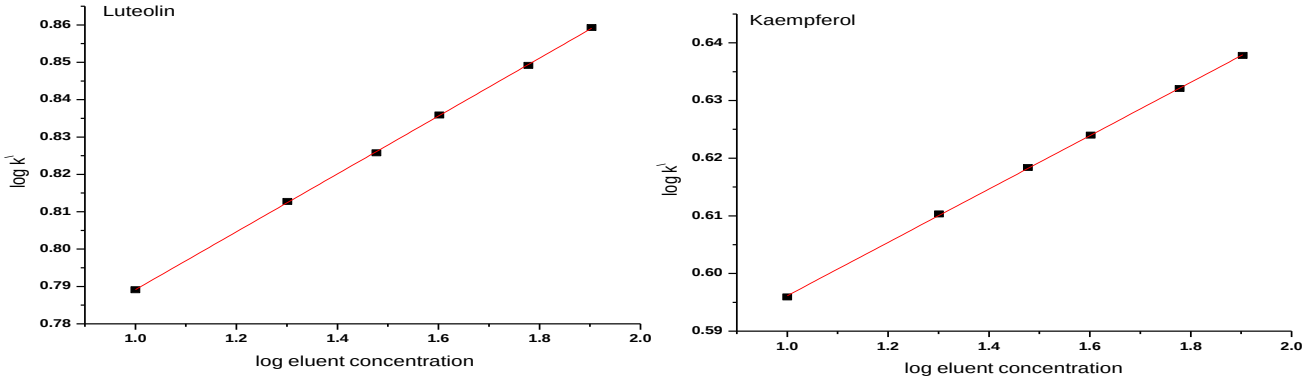

Figure 5. Effect of eluent concentration on the behavior of kaempferol and luteolin using a HALO-HILIC.

3.2.3. Effect of pH on the retention factor

For the separation of kaempferol and luteolin in HILIC mode to be successful, the eluent pH must be altered. The pH increased from 3 to 5.5 using acetic acid/sodium acetate buffer while the eluent concentration was constant at 35 mM and 90% CH_3CN. **Figure 6** demonstrates the rising retention factor of kaempferol and luteolin. The hydroxyl group in the examined flavonoids has been deprotonated in light of the physicochemical information that can be predicted for both flavonoids, which have a pKa value of 7.3 and 7.82, respectively. The analytes' deprotonation is unquestionably discernible when the mobile phase's pH is raised to 5, causing an increase in the electrostatic interaction between the stationary phase and the analyte and increasing the retention factor.

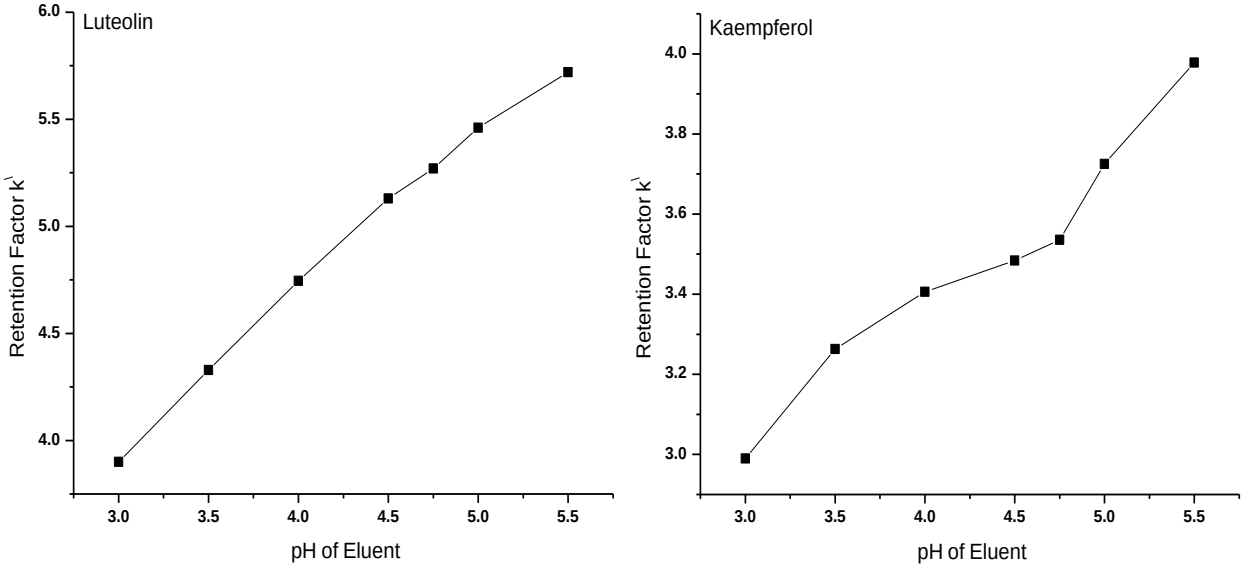

Figure 6. Effect of pH on the behavior of kaempferol and luteolin using a HALO-HILIC.

3.3. Linearity and range

In this work, the suggested method's linearity was examined by graphing peak areas against the concentrations of kaempferol and luteolin. The peak area concerning the concentrations of the respective flavonoids was linear in a concentration range of (0.065-35 µgmL^{-1} for kaempferol) and (0.065-9 µgmL^{-1} for luteolin); **Figure 7** shows the Calibration Curve of Kaempferol and Luteolin. **Table 1** shows the linearity of kaempferol and luteolin, regression equation, and calibration statistics. The results show that the peak area and concentration are strongly correlated.

Table 1. Linearity, regression, LOD, and LOQ.

Parameter	Flavonoids	HALO-HILIC column
Linearity (µgmL^{-1})	Kaempferol	0.065-35
	Luteolin	0.065-9
R^2	Kaempferol	0.9997
	Luteolin	0.9999
LOD (µgmL^{-1})	Kaempferol	0.012
	Luteolin	0.008
LOQ (µgmL^{-1})	Kaempferol	0.036
	Luteolin	0.024

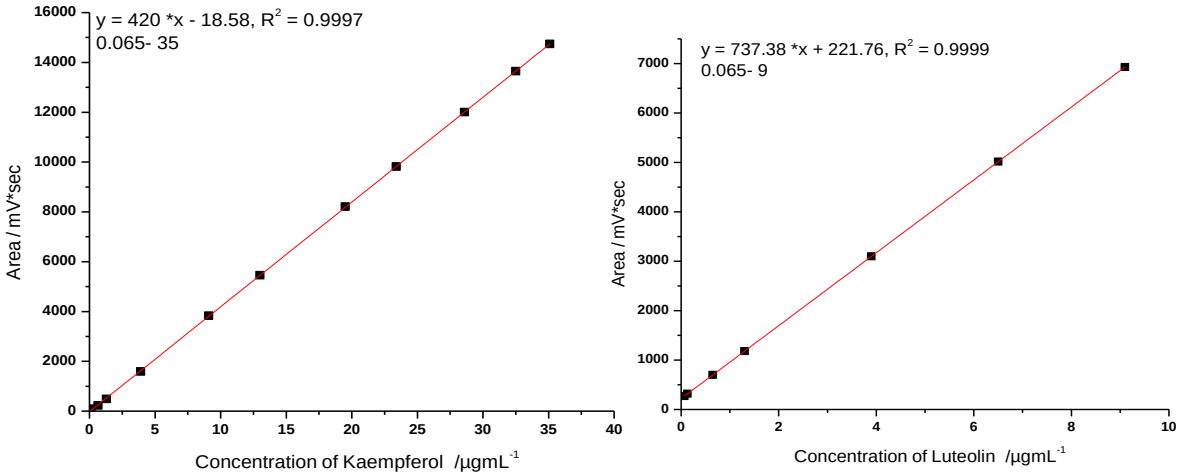

Figure 7. Calibration curves of standard solutions of kaempferol and luteolin.

3.4. Statistical analysis

Measurements of accuracy, RSD%, and recovery percentage were made on both the same day and different days. This method's effectiveness in **Table 2** is explained by its high recovery values.

Table 2. Accuracy and precision.

Flavonoids	Taken µg/mL	HALO-HILIC			
		Inter-Day		Intera-Day	
		Rec. (%)	RSD (%)	Rec. (%)	RSD (%)
Kaempfero	3	100.60	0.28	101.00	0.36
	4	99.00	0.41	99.50	0.45
	5	100.60	0.33	100.20	0.37
Luteolin	3	99.20	0.52	99.00	0.39
	4	100.57	0.44	100.71	0.54
	5	100.50	0.19	100.12	0.29

The results of the t-test and variance ratio F-test methods were used in statistical analyses with a 95% confidence level, as shown in **Table 3**.

Table 3. compares the proposed methods 2D with a standard procedure for flavonoid analysis by examining t- and F-statistical tests**.**

Flavonoids	Taken µg/mL	Proposed method	Standard method (55)	t-Test (Theor.)	F-Test (Theor.)
Kaempferol	3	100.60	98.67	0.9085 (2.7764)	1.5790 (19.000)
	4	99.00	100.13		
	5	99.00	99.55		
Luteolin	3	99.20	100.22	0.4122 (2.7764)	1.9718 (19.000)
	4	100.57	99.34		
	5	100.50	99.21		

These results were compared with those obtained using the standard procedure to assess the effectiveness and competency of the HALO-HILIC approach compared to the standard method (24). The estimated T and F values were within the theoretical values, indicating little difference in the two approaches' flavonoid quantification accuracy.

3.5. Kaempferol and Luteolin content in ginkgo samples

The investigation findings are reported in **Table 4** and show that the proposed approach, which used the HALO- HILIC column, successfully identified and quantified kaempferol and luteolin in black and ginkgo herbs samples.

Table 4. Flavonoids contents in ginkgo samples.

	Flavonoids	
Herbs sample	**Kaempferol (mg/g[a])**	**Luteolin (mg/g[a])**
Ginkgo	0.325 ± 0.065	0.245 ± 0.021

4. Conclusion

The newly developed approach was successfully used to establish the concentrations of kaempferol and luteolin in ginkgo plants. The study also covered the method of collecting and examining the components of these herbs. The analytical process demonstrated great precision, sensitivity, reproducibility, feasibility, and practicability of these technological advancements. This is the outcome of using the HALO-HILIC column. It addressed problems that caused the separation process to be slow when utilizing conventional columns. Based on the method created, the newly established optimization was made.

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Conflict of Interest

There is no conflict of interest in this research

Funding

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Ethical Clearance

The local Scientific Committee at the College of Science/ University of Baghdad approved this study.

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