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An Analytical Technique for the Simultaneous Determination of Apigenin and Luteolin in *Achillea magna* using Liquid Chromatography–Mass spectrometry

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Abstract

The objective is to create a liquid chromatography-mass spectrometry approach that is fast, precise, reliable, and effective. This method will be validated according to the requirements set by the International Council for Harmonisation (ICH). The purpose of this method is to simultaneously measure the levels of apigenin and luteolin in *Achillea magna*. The chromatographic separation was accomplished using a C8 column with dimensions of 150 x 4.6mm i.d. and a 5µ Hibar Lichrospher. The mobile phase consisted of a mixture of 0.1% formic acid and acetonitrile in a ratio of 20:80 v/v. The volumetric flow rate was 0.4 millilitres per minute. The apigenin and luteolin compounds exhibited retention times of 8.48 min and 8.01 min, respectively. Apigenin and luteolin demonstrated a linear relationship within the concentration range of 10-50 ng/ml. The technique was verified for criteria such as system appropriateness, specificity, linearity, accuracy, precision, limit of detection, and limit of quantification. The suggested technique was effectively used to simultaneously estimate the elements in *Achillea magna* and build a quantitative approach for determining both apigenin and luteolin from *Achillea magna*.

KEYWORDS: LC-MS method, Achillea *magna,* simultaneous estimation, flavonoids, apigenin, luteolin.

Introduction

There is a significant and widespread demand for pharmaceuticals obtained from plants. Many individuals hold the belief that plant-derived medicines are both safe and reliable, especially when contrasted to synthetic drugs that may have harmful consequences [1]. Ensuring the quality of herbal preparations or proprietary medicines is much more challenging compared to synthetic pharmaceuticals due to the intricate chemical composition of the constituents [2]. The objective of quality control for medicinal plant products is to guarantee their therapeutic effectiveness and detect any adulteration or unintentional blending in commercial batches. Ensuring the quality monitoring of plant products is a fundamental need. Conventional medicine is now starting to acknowledge the use of botanicals after they have been properly verified [3].

Flavonoids are a class of polyphenolic chemicals that are extensively found in many plants. Currently, there is knowledge of around 300 different types of flavonoids. Flavonoids, being a significant active component, have a notable role in diverse pharmacological activities such as anti-allergic, anti-inflammatory, and anti-oxidant actions [4,5,6].

Apigenin and luteolin have antioxidant properties and decrease the oxidation of low density lipoproteins [7]. Apigenin and luteolin are notable flavonoids recognised for their anti-inflammatory, anti-allergic, anti-thrombotic, hepatoprotective, anti-spasmodic, and anti-cancer properties [8]. Apigenin and luteolin are crucial nutrients because they have the capacity to enhance and regulate the permeability of blood vessel walls, particularly capillaries [9].

The literature study revealed a lack of studies on the concurrent determination of flavonoid components in *Achillea magna* utilising an LC-MS technology. The present work focused on the quantitative assessment of the key flavonoids that are typical for *Achillea magna*, which is relevant for current research.

The objective of this work was to create an LC-MS technique that is selective, fast, precise, and accurate for measuring the levels of apigenin and luteolin in *Achillea magna* simultaneously.

MATERIALS AND METHODS:

Materials and reagents

Apigenin and Luteolin, with purities of 97.8% and 98.1% respectively, were obtained from Natural Remedies Ltd. in Bangalore, India. Methanol and Acetonitrile were obtained from Qualigens fine chemicals, a supplier based in Mumbai, India, and were of high-performance liquid chromatography (HPLC) quality. All the reagents and chemicals used were of analytical and high-performance liquid chromatography (HPLC) quality. Milli Q RO system was used to get HPLC quality water.

Plant Material

In February 2020, the whole *Achillea magna* Linn. plant was gathered. The plant was taxonomically identified, verified, and validated by Dr. S. Rajan, a field botanist working in the Medicinal Plant Survey and Collection Unit of the Department of Ayush, which falls under the Ministry of Health and Family Welfare of the Government of India. The plants were harvested and subjected to dehydration in a tray drier at a temperature of 50°C for a duration of 48 hours. The desiccated samples were pulverised and used for the investigation.

Preparation of standard solution

The apigenin and luteolin stock solutions were produced in methanol at a concentration of 1 mg/ml. 10 milligrammes of each apigenin and luteolin were placed into separate 10 millilitre volumetric flasks. Subsequently, 5 mL of methanol was introduced and subjected to sonication for a duration of 10 minutes until a transparent solution was achieved. The final solution was diluted to a volume of 10 mL using methanol. The stock solutions were kept in containers that are resistant to light. Apigenin and luteolin were produced in the mobile phase as aliquots for analysis.

Preparation of sample solution

A quantity of 50 milligrammes of each extract was measured and placed into a volumetric flask with a capacity of 50 millilitres. 10 mL of the mobile phase solution was added to the mixture and subjected to sonication for a duration of 10 minutes. The final solution was diluted to a volume of 50 mL using the mobile phase and then passed through a Whatmann filter paper No.42. Portions of the sample were produced in the mobile phase. The optimised chromatographic conditions were used to analyse both the standard and sample solutions, and the resulting chromatograms were recorded.

Instrumentation and LC-MS conditions

An Agilent 1290 infinite UHPLC-ESI-QTOF system was used to accomplish the concurrent measurement of apigenin and luteolin. The samples were separated using a Phenomenex C18 column with dimensions of 150 x 4.6mm i.d. and a particle size of 5µ. The drying process used high quality nitrogen gas at a flow rate of 5 L/min. The capillary voltage and probe temperature were set at 80 V and 350°C, respectively. The flow rate of the mobile phase under isocratic conditions was maintained at 0.4 ml/min. The temperature of the column oven was adjusted to 30°C. The mobile phase was a solution containing 0.1% formic acid and acetonitrile in a volumetric ratio of 20:80. The MS acquisition mode for apigenin was positive, whereas for luteolin it was negative. The process of data collecting included the use of Analyst software.

RESULTS AND DISCUSSION:

Method development and validation

The peak area was used to generate the calibration curve. The concentration of the analyte in the plant sample was determined using the equation ($y = mx + c$), where y

represents the peak area ratio. System suitability tests are used to guarantee the replicability of the equipment. The experiment included injecting a combination of a standard solution of apigenin and luteolin into six duplicates.

The LC-MS technique that was developed underwent validation in accordance with the requirements set by the International Conference on Harmonisation (ICH) [10] for validating analytical processes. The relative standard deviation (RSD) for apigenin and luteolin was determined to be 0.40% and 0.62% respectively. These values are considered acceptable as they are below the threshold of 5%.

After implementing the suggested approach, distinct peaks were achieved for both apigenin and luteolin (Figure 1). Apigenin and luteolin were detected in the extracts of Achillea plant. The quantitative analysis showed that luteolin was the most abundant compound in the ethanolic extract of *Achillea magna*, with a concentration of 26.3 mg/gm. In the aqueous ethanolic extract, luteolin was present at a concentration of 17.4 mg/gm. On the other hand, apigenin was found in lower amounts in the methanolic extract (15.6 mg/gm) and the aqueous ethanolic extract (19.2 mg/gm) of *Achillea magna*. The chromatograms of methanolic and aqueous ethanolic extracts, which include apigenin and luteolin, are shown in Figure 2 and 3, respectively.

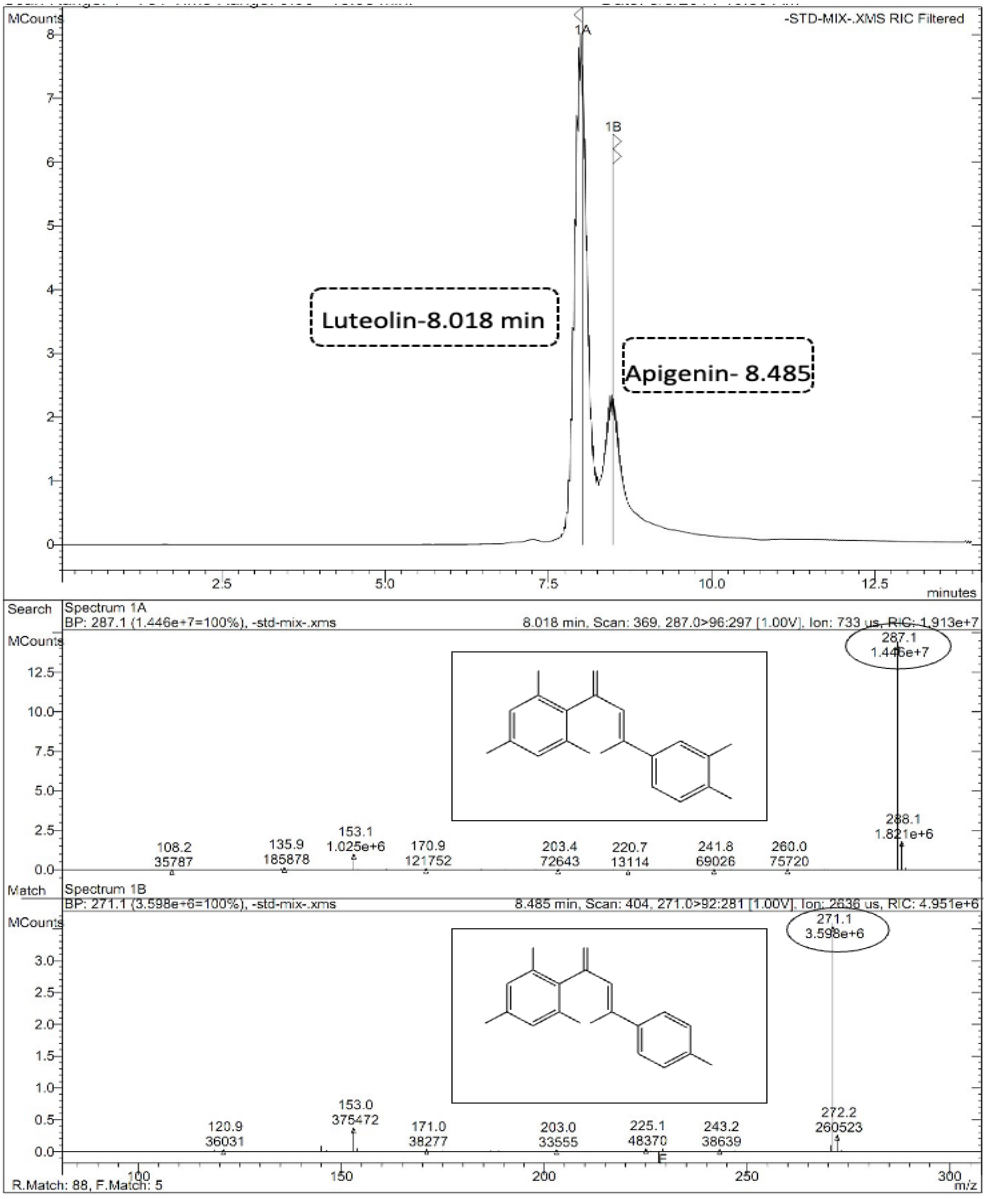

Fig 1: Typical LCMS Chromatogram of Apigenin and Luteolin standard solutions

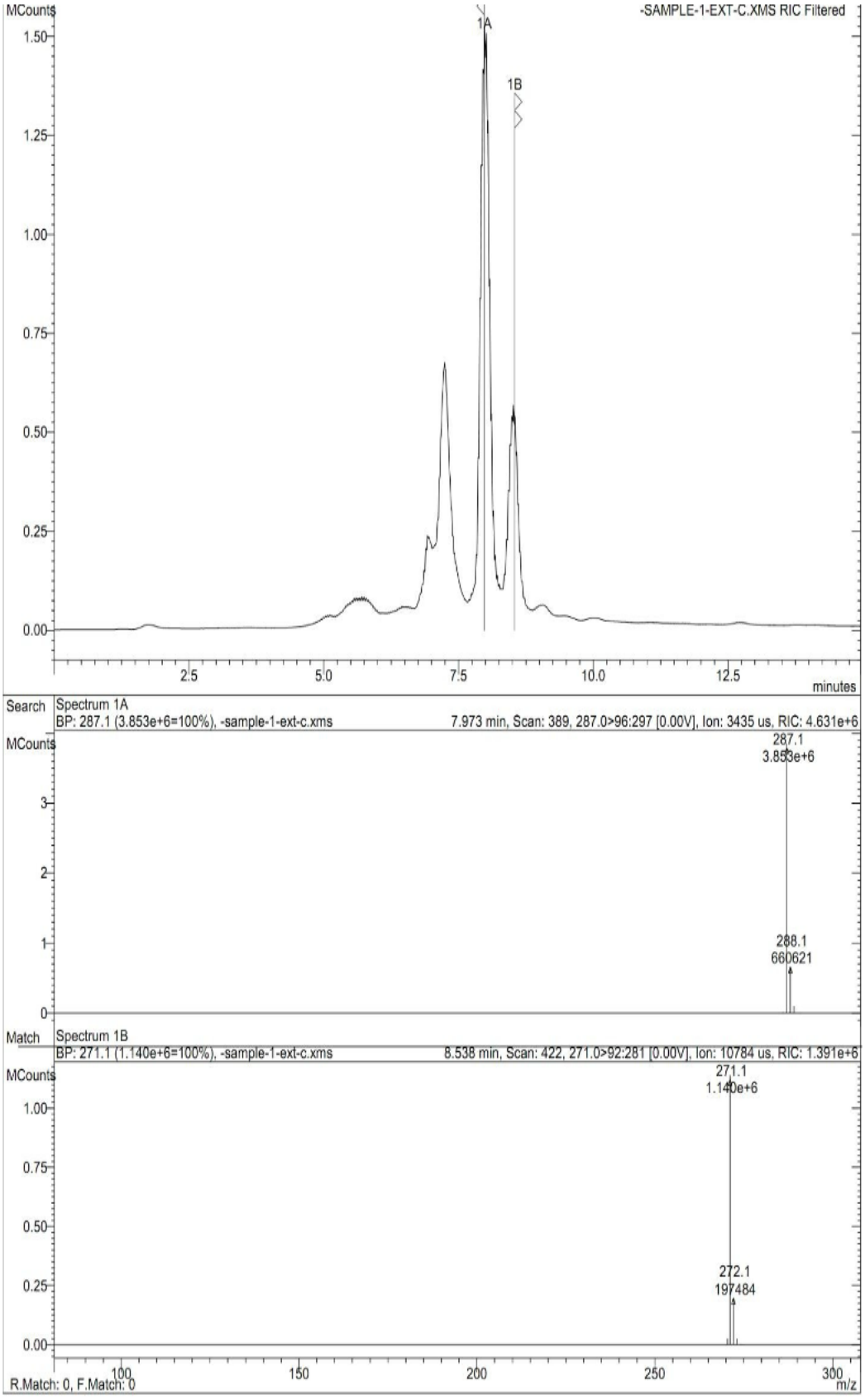

Fig 2: Typical LCMS Chromatogram of methanolic extract containing Apigenin and Luteolin

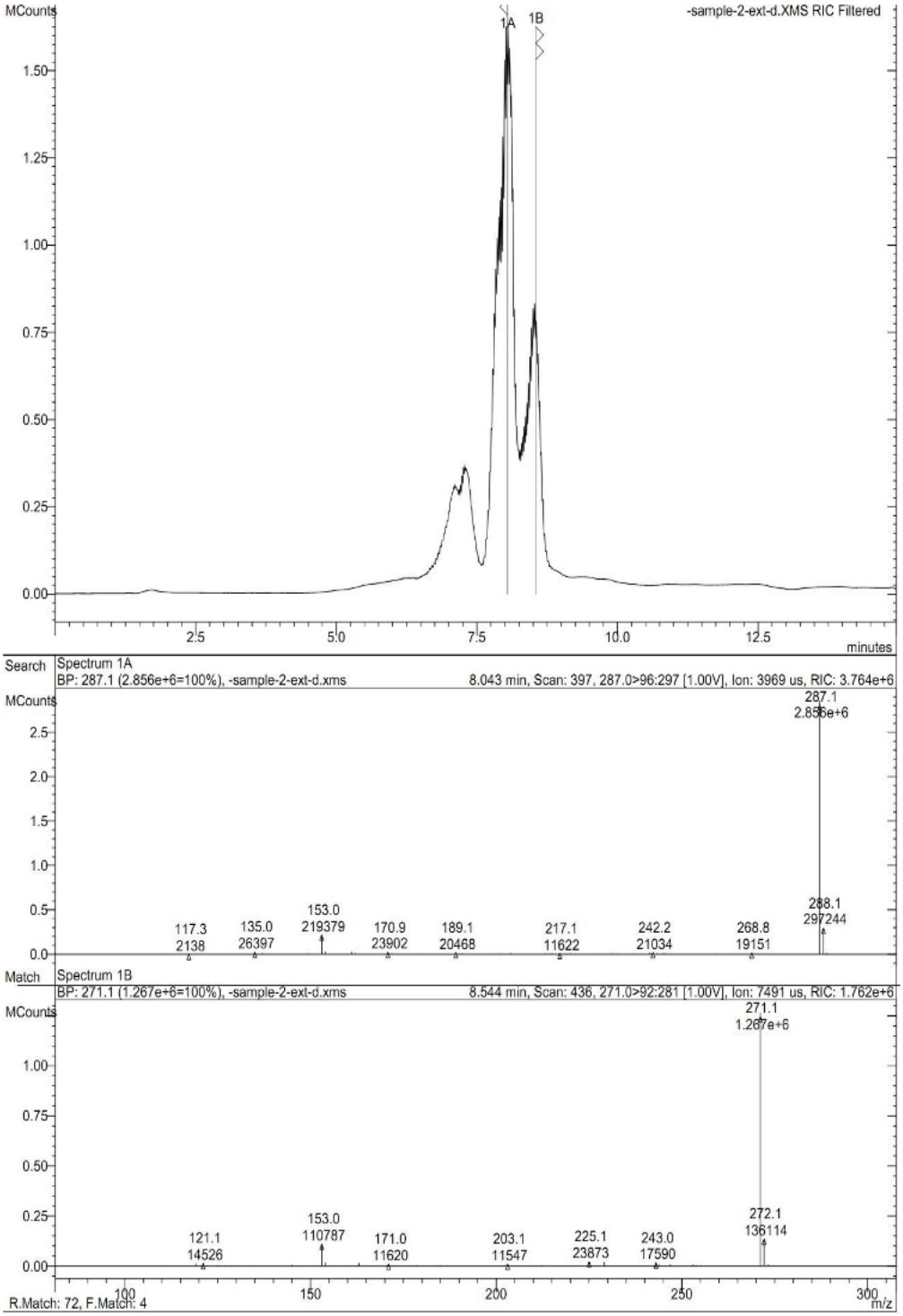

Fig 3: Typical LCMS Chromatogram of aqueous ethanolic extract containing Apigenin and Luteolin

Linearity and range

For the linearity research, we analysed five solutions with concentrations ranging from 10 to 50 ng/ml for apigenin and luteolin (Table 1). Every concentration was prepared and examined three times. The observed linearity within the specified range was satisfactory, as shown by the linear regression equations: Y=89.295x+2.572 for apigenin and Y=201.5x+25.762 for luteolin. Here, x represents the concentration of analytes in ng/ml, and Y represents the peak area. The correlation coefficient for apigenin was shown to be 0.9994, whereas for luteolin it was found to be 0.9996. The

findings suggest that the approach exhibits linearity within the range of concentrations examined (Figure 4).

Table 1: Linearity and range for apigenin and luteolin by LC-MS method

S.No.	Concentration of Apigenin and Luteolin (ng/ml)	Peak area	
		Apigenin	Luteolin
1	10	880	2050
2	20	1782	3994
3	30	2716	6126
4	40	3616	8225
5	50	4418	9969

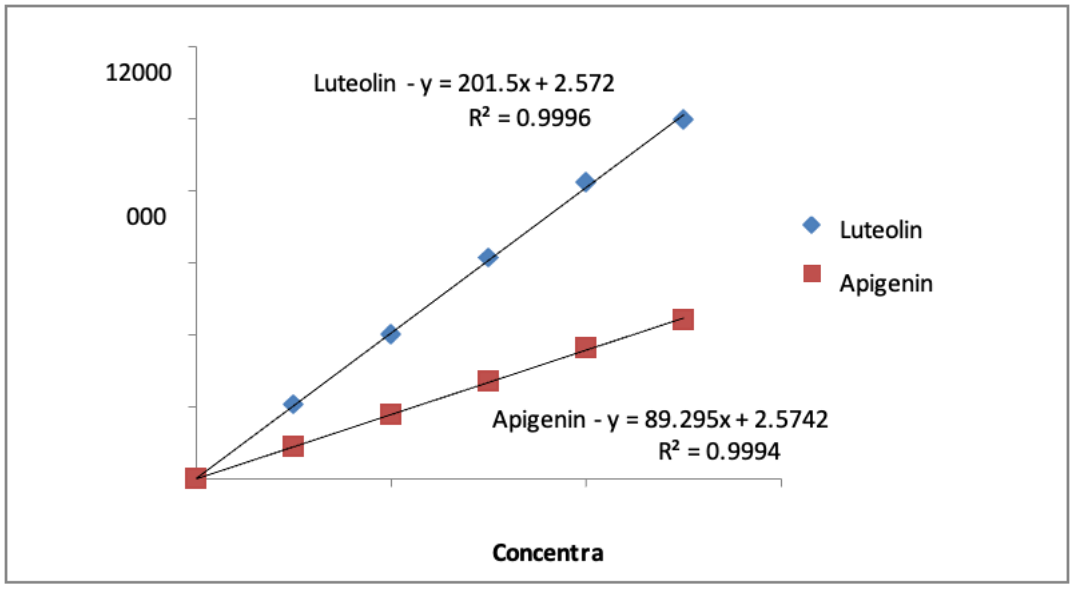

Figure 4: LC-MS Calibration curve of apigenin and luteolin

Accuracy

Three solutions were prepared with concentrations of 10, 20, and 30 ng/ml, respectively. The apigenin recovery range was determined to be between 99.82% and 100.14%, while the luteolin recovery range was discovered to be between 99.76% and 100.5%. The relative standard deviation for apigenin varied from 0.12% to 0.40%, whereas for luteolin it ranged from 0.12% to 0.62%. The accuracy data is shown in Table 2.

Table 2: Recovery and accuracy data for the estimation of apigenin and luteolin by LC-MS method

Compounds	Recovery		
	Amount Added (ng/ml)	Recovery (%)	RSD (%)
Apigenin	10 20 30	100.14 99.99 99.82	0.14 0.12 0.40
Luteolin	10 20 30	100.5 100.2 99.76	0.12 0.34 0.62

Precision

The study of repeatability included the calculation of the relative standard deviation (RSD) for six measurements conducted on the same day and under identical experimental circumstances. The relative standard deviation of apigenin and luteolin determinations in the working standard solution was computed and shown in Table 3. Intermediate precision studies include assessing the variability in analytical results when a technique is employed in multiple labs or on different days. The relative standard deviation (RSD) values for apigenin and luteolin were 1.66% and 1.79% respectively.

Table 3: Precision studies for apigenin and luteolin by LC-MS method

Compound	Conc. (ng/ml)	N	Inter day		Intra day	
			Mean	% RSD	Mean	% RSD
Apigenin	20	6	1654.5	1.66	1562.6	1.15
Luteolin	20	6	3850.3	1.79	3859	0.54

Limit of detection and quantification

LOD values were determined using the following formulae. The limit of detection (LOD) is calculated as 3.3 times the standard deviation (SD) divided by the slope (S) of the calibration curve. The limit of quantification (LOQ) is calculated as 10 times the SD divided by S. The limit of detection (LOD) for apigenin was 3 ng/ml, while the LOD for luteolin was 1 ng/ml. The limit of quantification (LOQ) for apigenin was 9 ng/ml, and for luteolin it was 3 ng/ml. These values are provided in Table 4.

Table 4: System suitability studies and validation for the estimation of Apigenin and Luteolin by LC-MS method

S. No.	Parameters	Apigenin	Luteolin
1	Linearity range	10-50 ng/ml	10-50 ng/ml
2	Regression equation (Y=mx+c)	Y= 89.295x+2.572	Y= 201.5x+25.762
3	Correlation coefficient	R^2=0.9994	R^2=0.9996
4	Asymmetric factor	1.2	1.0
5	LOD (ng/ml)	3	1
6	LOQ (ng/ml)	9	3

Conclusions:

A LC-MS technique has been developed and validated according to ICH recommendations to assess accuracy, precision, linearity, limit of detection, and limit of quantification for the simultaneous estimation of apigenin and luteolin. Thus, it can be inferred that the LC-MS approach that was developed is fast, accurate, and exact. It is also selective, allowing for the simultaneous quantitative detection of apigenin and luteolin from the herb extract of *Achillea magna*.

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