

# Simultaneous determination of rutin, isoquercetin, and quercetin flavonoids in *Nelumbo nucifera* by high-performance liquid chromatography method

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## Abstract

**Objective:** The present study was investigated to provide a documentary evidence for the determination of rutin, isoquercetin, and quercetin flavonoids from the flora of *Nelumbo nucifera* by reversed-phase high-performance liquid chromatography (RP-HPLC).

**Materials and Methods:** RP-HPLC analysis was performed by gradient elution with a low-pressure gradient using 0.5% acetic acid: acetonitrile as a mobile phase with a flow rate of 1.0 ml/min. The separation was done at 26°C using a Kinetex XB-C18 column as stationary phase and the detection wavelength at 356 nm. The proposed method was validated as per International Conference on Harmonisation guidelines with respect to specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ).

**Results:** The validated results were within the acceptable limits. In specificity, the retention time of rutin, isoquercetin, and quercetin peak in the sample was matched with the reference standard peak and showed good resolution. An excellent linearity was obtained with correlation coefficient (r) higher than 0.999. In precision, the repeatability and intermediate showed < 1.0% of % relative standard deviation of peak area percentage indicating high precision and accurate. The recovery rate for rutin, isoquercetin, and quercetin was between 99.85%–101.37%, 101.90%–103.24%, and 101.74%–106.73%, respectively. The lower LOD and LOQ of rutin, isoquercetin, and quercetin enable the detection and quantitation of these flavonoids in *N. nucifera* at low concentrations.

**Conclusion:** The developed analytical method is convenient for the determination of flavonoids content in herbal drugs.

**Keywords:** International Conference on Harmonisation, linearity, precision, recovery, limit of detection, limit of quantification

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## INTRODUCTION

Herbal drugs have become the main subject of attention and global importance since a decade and are used

for exploring botanical materials as medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge

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is important. Flavonoids are the secondary metabolites derived from plants having antioxidant and free radical scavenging properties.<sup>[1]</sup> Flavonoids play an important role in plants for normal growth, development, defense against infection, and injury.<sup>[2]</sup>

*Nelumbo nucifera* is a perennial aquatic plant belonging to family *Nelumbonaceae*. It is commonly known as Indian lotus, which is distributed throughout Asia.<sup>[3]</sup> Leaves, seeds, flower, and rhizome of *N. nucifera* are good source of nutritional and medicinal values. The main active phytoconstituents present in the *N. nucifera* contain flavonoids,<sup>[4]</sup> alkaloids,<sup>[5]</sup> steroids,<sup>[6]</sup> triterpenoids,<sup>[3]</sup> tannins,<sup>[7]</sup> glycosides,<sup>[8]</sup> and polyphenols.<sup>[8]</sup>

*N. nucifera* has been reported for antioxidant,<sup>[9]</sup> anticancer,<sup>[10]</sup> antiviral,<sup>[11]</sup> anti-obesity,<sup>[12]</sup> lipolytic,<sup>[13]</sup> hepatoprotective,<sup>[14]</sup> antidiabetic,<sup>[15,16]</sup> antidiarrheal,<sup>[17]</sup> antifungal,<sup>[18]</sup> antibacterial,<sup>[19]</sup> anti-ischemia,<sup>[20]</sup> anti-inflammatory,<sup>[15]</sup> and diuretic activities.<sup>[21]</sup>

## MATERIALS AND METHODS

### Materials

Rutin, quercetin, and isoquercetin were used as calibration standards. Rutin (94%) and isoquercetin (90%) were obtained from Sigma-Aldrich (India) and quercetin (99%) was obtained from S.D Fine-Chem Ltd. All reagents and solvents were analytical and high-performance liquid chromatography (HPLC) grade. The powdered floral material of *N. nucifera* was procured from Ess Kay Herbs, Lucknow, Uttar Pradesh, India.

### Preparation of the extract

Lotus flower extract (LFE) of *N. nucifera* was prepared by Soxhlet extraction of 100 g of powdered floral material using 70% ethanol at 80°C for about 5 h in successive batches. The first batch was extracted by adding 500 ml solvent for about 3 h, and further two successive extractions were done by adding 300 ml/batch for 1 h. After completion of extraction, the cooled liquid was concentrated by evaporating its liquid contents in rotary evaporator to remove the solvent residue to nil, with an approximate yield of 2%. The extract powder was used for further experiments.

### Instrumentation and chromatographic conditions

HPLC was performed on a Shimadzu LC-2030 C Prominence-i (Japan) system equipped with a quaternary low-pressure gradient solvent delivery LC-2030 pump with a high-pressure switching valves, online LC-2030 degasser unit, a high-sensitivity LC-2030 ultraviolet (UV) detector, high-speed drive LC-2030 autosampler with a 100 µl loop

and it accommodates 216 samples at a time with direct access rack system and large-capacity column oven. The system controlled and data analyzed by laboratory solution system software. A gradient elution was carried out in Kinetex XB-C18 column (100 Å, 100 mm × 4.6 mm, 2.6 µm pore size). The mobile phase consists of two different solutions 0.5% acetic acid and acetonitrile which act as solution A and solution B. All solutions were degassed and filtered through 0.45 µm pore size filter. The gradient elution initial conditions were 18% of eluent B with linear gradient to 18.5% from 0.01 to 7 min, followed by linear gradient to 35% of eluent B at 9 min and this proportion being maintained for 2 min. The mobile phase composition returned to the initial condition at 10 min and allowed to run for another 5 min before the injection of another sample. The total runtime of each sample is 15 min. The flow rate was 1.0 ml/min and the sample injection volume was 5 µl. The column was maintained at 26°C throughout analysis and the UV detector was set at 356 nm. Seventy percentage methanol used as a diluent for assay by HPLC analysis and the total LC runtime was 15 min. The instrument was calibrated and qualified before the analysis. Using these chromatographic conditions, it was possible to confirm the retention time (RT) of rutin, isoquercetin, and quercetin by injection of corresponding standards separately.

### Preparation of standard solution

Accurately weighed appropriate amounts of rutin, isoquercetin, and quercetin reference standards were dissolved in 70% methanol in a 25 ml volumetric flask to obtain a stock solution. The concentration of rutin, isoquercetin, and quercetin in the solution was 220, 80, and 220 ppm, respectively. Working standard solutions were obtained by diluting the 2.0 ml standard stock solution to 10 ml volumetric flask and made up with 70% methanol to get a final concentration of 44, 16, and 44 ppm for rutin, isoquercetin, and quercetin.

### Preparation of sample solution

The dried LFE was prepared in 70% methanol to achieve the final concentration of 400 ppm. Before analysis, the solutions were filtered through 0.20 µm nylon membrane filters.

### Preparation of spiked sample solution

Three different volumes (1, 2, 3 ml) of standard stock solution were added to the sample solution (400 ppm) separately. The standard stock solution was spiked into the samples to determine recovery. Before analysis, the solutions were filtered through 0.20 µm nylon membrane filters.

### Validation of the method

The validation of the developed method was done according to the International Conference on Harmonisation (ICH) guidelines.<sup>[22]</sup> The method is validated for specificity, linearity, repeatability, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and robustness.

### Specificity

Specificity is the ability of a method to discriminate between the study analytes and other components in the sample. In this study, the specificity was demonstrated by running a procedure blank, standard, and sample. The chromatographic parameters such as column efficiency and peak symmetry were done according to the ICH guidelines.

### Linearity

Linearity was determined by different known concentrations of rutin, isoquercetin, and quercetin standard solution in triplicate by diluting the standard stock solution. The standard solutions were injected and the peak area was measured. The concentration of 44, 16, and 44 ppm for rutin, isoquercetin, and quercetin standards in the solution was considered to be 100%. Calibration curve of 50%, 75%, 100%, 125%, and 150% was constructed for rutin, isoquercetin, and quercetin by plotting peak areas against concentration and linear regression equations. The correlation coefficient was also computed.

### Precision

Precision is a measure of the reproducibility of the whole analytical method. Precision was determined by studying the repeatability and intermediate precision. The repeatability was determined at a minimum of three different concentrations (75%, 100%, and 125%) of rutin, isoquercetin, and quercetin standards per six replicates. According to ICH Guidelines the intermediate precision variations have to be studied for different analysts only. The precision was expressed as percentage relative standard deviation (% RSD).

### Accuracy

Accuracy is a measure of closeness of test results obtained by a method to the true value. The accuracy of the method was tested by performing the recovery studies at three different levels of standard stock solution added to the samples. The standard stock solution was spiked into the samples to determine recovery. Three different volumes (1, 2, 3 ml) of standard stock solution were added to the sample solution (400 ppm). Triplicate injections were made with all the spiked samples.

$$\% \text{ of recovery} = (b - a)/c \times 100$$

Where, “a” is the amount of drug found in the sample before addition of standard drug.

“b” is the amount of drug found after addition of standard drug.

“c” is the amount of standard drug added.

### Limit of detection and limit of quantification

Detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated under the stated experimental conditions. Quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with accuracy. LOD and LOQ were determined based on the signal-to-noise ratio response. For LOD, it should not be not <3. For LOQ, it should not be not <10.

### Statistical analysis

Results were expressed as mean  $\pm$  SD and % RSD. The data were submitted to statistical analysis using excel software.

## RESULTS

### Specificity

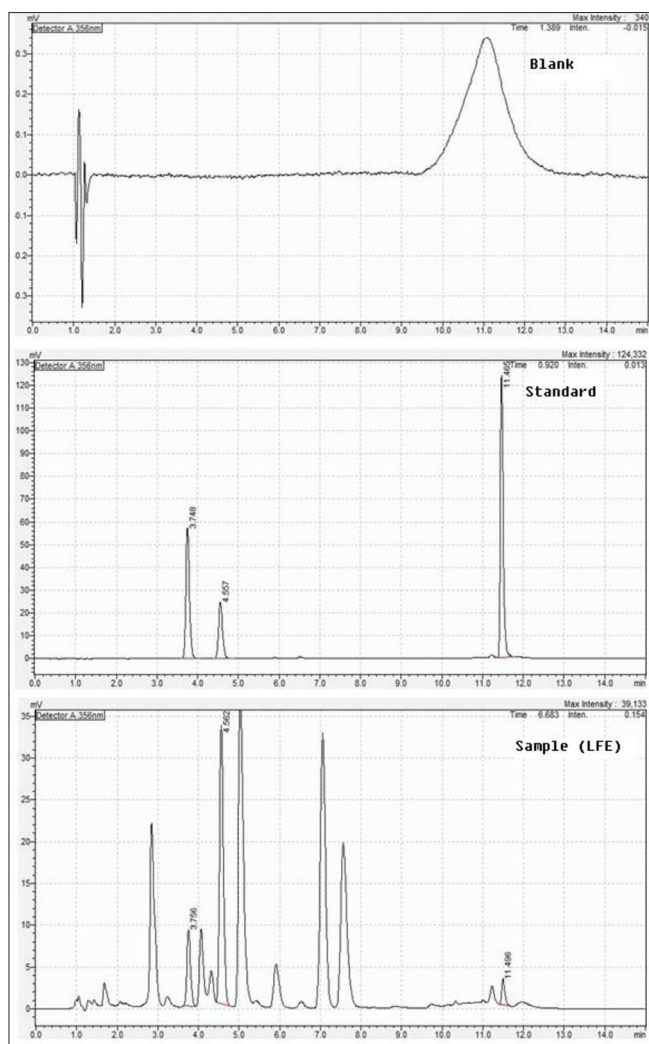
Specificity was determined by comparing the chromatogram obtained from blank, standard, and sample solutions are summarized in Figure 1. Good separation between the peaks of rutin, isoquercetin, and quercetin was achieved. The RT of rutin, isoquercetin, and quercetin reference standards and sample (LFE) peak were found to be at 3.748, 4.557, 11.465 and 3.756, 4.562, 11.496, respectively.

### Linearity

Linearity was evaluated by the peak area against concentration of rutin, isoquercetin, and quercetin standards which is summarized in Figure 2. The calibration plots were linear in the range 50%, 75%, 100%, 125%, and 150%, and the correlation coefficient (r) of all the three rutin, isoquercetin, and quercetin compounds is 0.999.

### Precision

Precision was evaluated on the basis of the % RSD value. The data pertaining to repeatability (intra) and intermediate (inter-day) precision was summarized in the Table 1. In repeatability, the % RSD of peak area at three different levels (75%, 100%, and 125%) of rutin was found to be 0.053%, 0.121%, and 0.140%, isoquercetin: 0.048%, 0.084%, and 0.121%, and quercetin: 0.019%, 0.091%, and 0.110%, respectively. In intermediate precision, the % RSD of peak area at three different levels (75%, 100%, and 125%) of rutin was found to



**Figure 1:** High-performance liquid chromatograms of blank, standard and sample. The retention time of rutin, isoquercetin and quercetin reference standard and sample (LFE) peak were found to be at 3.748, 4.557, 11.465 and 3.756, 4.562, 11.496 respectively

be 0.090%, 0.081%, and 0.122%, isoquercetin: 0.098%, 0.176%, and 0.157%, and quercetin: 0.082%, 0.098%, and 0.113%, respectively.

### Accuracy

The recovery of the compounds rutin, isoquercetin, and quercetin was determined by spiking the LFE with known amounts of standard stock solution. Recovery of each substance was obtained from the calculated amount found and original amount. The results are presented in Table 2. The average percent recoveries at three different levels (50%, 100%, and 150% spiked sample) of rutin were found to be 100.71%, 101.37%, and 99.85%, isoquercetin: 102.91%, 103.24%, and 101.90%, and quercetin: 106.73%, 104.03%, and 101.74%, respectively. The average % RSD at three different levels (50%, 100%, and 150% spiked sample) of rutin was found to be 0.08%, 0.08%, and 0.06%,

**Table 1: Repeatability (intra) and intermediate (inter-day) precision study for the developed method for rutin, isoquercetin, and quercetin**

Reference standard	Mean area %±SD	RSD (%)
Repeatability (intra-day precision)		
Rutin 75%	31.102±0.016	0.053
Isoquercetin 75%	14.946±0.007	0.048
Quercetin 75%	53.953±0.036	0.019
Rutin 100%	30.927±0.037	0.121
Isoquercetin 100%	14.933±0.013	0.084
Quercetin 100%	54.141±0.049	0.091
Rutin 125%	30.835±0.043	0.140
Isoquercetin 125%	14.893±0.018	0.121
Quercetin 125%	54.271±0.060	0.110
Intermediate (inter-day) precision		
Rutin 75%	31.748±0.029	0.090
Isoquercetin 75%	15.277±0.015	0.098
Quercetin 75%	52.975±0.044	0.082
Rutin 100%	31.546±0.026	0.081
Isoquercetin 100%	15.270±0.027	0.176
Quercetin 100%	53.184±0.052	0.098
Rutin 125%	31.379±0.038	0.122
Isoquercetin 125%	15.215±0.024	0.157
Quercetin 125%	53.405±0.060	0.113

Values are expressed as mean ± SD (n=6). SD: Standard deviation, RSD: Relative standard deviation

isoquercetin: 0.15%, 0.01%, and 0.01%, and quercetin: 0.21%, 0.13%, and 0.04%, respectively.

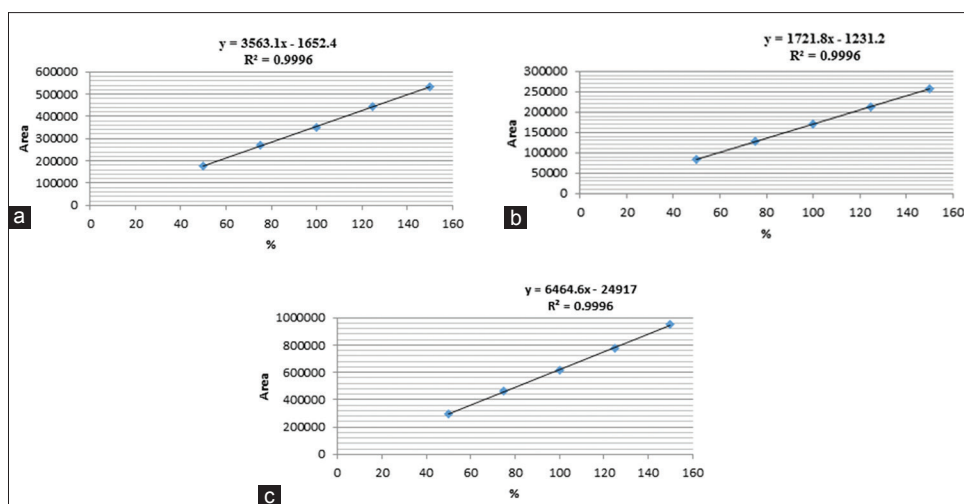
### Limit of detection and limit of quantification

LOD at signal-to-noise ratio (3:1) with the smallest concentration of rutin, isoquercetin, and quercetin standard that gives a measurable response was found to be 0.38, 0.97, and 0.21 ppm, respectively. The LOQs at signal-to-noise ratio (10:1) of rutin, isoquercetin, and quercetin were found to be 1.26, 2.94, and 0.60 ppm, respectively.

### DISCUSSION

Standardization of herbal medicines is fraught with many challenges. In the present study, we have developed a simple, optimized, and validated HPLC method for the standardization of *N. nucifera*. Flavonoids are the active antioxidant components in the flora of *N. nucifera*. Flavonoids belong to a group of natural substances with variable phenolic structure and are found in the fruits, vegetables, grains, bark roots, stem, flowers, tea, and wine. *N. nucifera* which contains biologically effective flavonoids including rutin, quercetin, and isoquercetin exhibits pharmacological activities. Rutin is a phenolic compound with glycosidic linkage. Isoquercetin is a flavonoid, a type of chemical compound. It is the 3-O-glucoside of quercetin. It is comparatively much more bioavailable than quercetin aglycone, the commonly available form as a supplement. Quercetin is a major representative of the flavonol subclass. It is an aglycone, meaning that it lacks a glycoside side chain. The rutin, isoquercetin, and quercetin flavonoids





**Figure 2:** Calibration curve for (a) Rutin, (b) Isoquercetin, and (c) Quercetin, by plotting peak area against concentration and linear regression equation

**Table 2: Recovery study for the developed method for rutin, isoquercetin, and quercetin**

Recovery study of rutin, isoquercetin, and quercetin						
	Volume of reference standard stock solution added (mL)	b	a	c	Percentage recovery	Percentage of RSD
<b>Rutin</b>						
50% spiked sample	1.0	234,100	56,790	176,055	100.71	0.08
100% spiked sample	2.0	412,479	223,476	350,887	101.37	0.08
150% spiked sample	3.0	591,003	16,366	535,027	99.85	0.06
<b>Isoquercetin</b>						
50% spiked sample	1.0	310,802	56,790	84,856	102.91	0.15
100% spiked sample	2.0	398,153	223,476	169,190	103.24	0.01
150% spiked sample	3.0	486,577	16,366	258,201	101.90	0.01
<b>Quercetin</b>						
50% spiked sample	1.0	335,869	56,790	299,365	106.73	0.21
100% spiked sample	2.0	655,466	223,476	614,340	104.03	0.13
150% spiked sample	3.0	982,677	16,366	949,795	101.74	0.04

Values are expressed as mean, (n=3). RSD: Relative standard deviation

were selected for quantification, and these are responsible for physiological action of the plant.

Validation of analytical procedure is the process of demonstrating that analytical procedure is suitable for their intended use and that they support the identity, strength, quality, purity, and potency of the drug substances and drug products. Results obtained from method validation can be used to judge the quality, reliability, and consistency of results.

A novel HPLC method for quantitative determination of three flavonoids: rutin, isoquercetin, and quercetin from floral part of *N. nucifera* was developed and validated. In HPLC method, the optimization of the mobile phase and stationary phases giving that rutin, isoquercetin, and quercetin peaks simultaneously detected using the gradient elution method. The mobile phase was chosen after several trials with 0.5% acetic acid and acetonitrile in various proportions giving the satisfactory resolution and the shortest analysis time.

### Specificity

Specificity of an analytical method is its ability to measure accurately an analyte in the presence of interference in the sample matrix. The RT of the standard and sample peak was same so that the method was specific. The chromatographic peaks of rutin, isoquercetin, and quercetin were well separated at different RT with the good resolution of 4.47 and 41.64 for rutin-isoquercetin and isoquercetin-quercetin, respectively. The theoretical plates (United States Pharmacopeia) 7571 for rutin, 9233 for isoquercetin, and 104102 for quercetin were observed. The tailing factor was found to be 1.24, 1.22, and 1.39 for rutin, isoquercetin, and quercetin, respectively, which indicated column efficiency is satisfactory.

### Linearity

Linearity of an analytical procedure is the ability for showing the response of the analyte which is proportional to the analytical concentration within a given range. The R<sup>2</sup>

values for rutin, isoquercetin, and quercetin were  $>0.990$ . This indicates that the good fitting of the curve and method is good linearity corresponds to peak area on concentrations. The minimum acceptable correlation coefficient is 0.990.<sup>[22,23]</sup>

### Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed condition. Repeatability expresses the precision under the same operating conditions over a short interval of time. Intermediate precision is the same procedure performed by a second operator within the laboratory. The repeatability and intermediate precisions of the % RSD of peak area at three different levels (75%, 100%, and 125%) of rutin, isoquercetin, and quercetin were found to be  $<1.0\%$ , which shows high precision of the method.

### Accuracy

The accuracy to be assessed using a minimum of nine determinations over a minimum of three concentration levels covering the specified range. The recovery rate for rutin, isoquercetin, and quercetin was between 99.85%–101.37%, 101.90%–103.24%, and 101.74%–106.73%, respectively. The low percentage relative SD value (%RSD  $<1$ ) shows the high accuracy of the method. Therefore, this HPLC method can be regarded as selective.

### Limit of detection and limit of quantification

The low LOD and LOQ values indicate that the method provides adequate sensitivity.

### CONCLUSION

Here, we have developed and validated the new reversed-phase-HPLC method which is simple, rapid, and reliable and is recommended for routine quality control analysis of rutin, isoquercetin, and quercetin from the flora of *N. nucifera*. Therefore, the method was proved to be suitable for flavonoids determination in various alcoholic extracts of *N. nucifera*. Further investigations are required to standardization of individual phytoconstituents of *N. nucifera*.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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