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RP-HPLC Technique for the Quantitative Determination of a Key Flavonoid in *Ginkgo biloba* (EGb 761) Extract

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Abstract: The complex and well-defined compound, was extracted from *Ginkgo biloba* (EGb 761) extract, prepared from the plant's green leaves. EGb 761 extract was subjected to chemical analysis and quality control. It is feasible to assess the amount of active principles in *Ginkgo biloba* extract and ensure that undesired elements have been removed by analysis. The section contains flavonoid compounds, including terpenoids and Ginkgo-flavone glycosides, which are exclusive to the ginkgo tree and have a distinctive structure (ginkgolides, bilobalide). Quercetin, a major flavonoid, has been determined to utilise a simple, new, affordable, time-effective, exact, and validated RP-HPLC approach employing an Infinity LC system with UV detection at 373 nm. To accomplish successful separation, a chromatic C-18 column (250 ×4.6 mm, 5µm particle size) was used with a mobile phase made up of methanol and acetonitrile (70:30 *v/v*) at a flow rate of 1 ml/min. The data obtained was analyzed using anachrome software. Linearity was found in the selected range (6-16µg/ml; R^2 value) with a retention time of 3.18 min and per cent recovery not less than 99±2%. The proposed method was also validated as per ICH Q2(R1) guidelines. Its LOD value was quantified to be 0.422 µg/ml and its LOQ value was 1.40 µg/ml indicating the efficacy, accuracy, sensitivity, and reproducibility of the developed method in the quantification of *Ginkgo biloba* extract in its various dosage forms, the % RSD of less than 2% for interday – intraday precision justifies the closeness of the theoretical and experimental values.

Keywords: *Ginkgo biloba* Extract, EGb 761 extract, RP-HPLC, Validated, Robustness, Spectrophotometer.

1. Introduction

Ginkgo biloba, is a tree variety that has been famous for ages for its neurological properties [1-4]. The extract prepared from various parts of the tree is renowned for treating multiple neuronal ailments like dementia, Alzheimer's, macular degeneration, hepatitis *etc*. The close agreement between theoretical and experimental values is supported by the LOD and LOQ values of 0.422 µg/ml and 1.40 µg/ml, respectively, which demonstrate the effectiveness, accuracy, sensitivity, and reproducibility of the developed method for quantifying *Ginkgo biloba* extract in its various dosage forms. It has a remarkable effect on the amyloid precursor protein (APP) by decreasing the levels of cholesterol, increasing the acceleration of acetylcholine release and decreasing the inflammatory responses due to the presence of flavonoids [5-6]. Quercetin is the major flavonoid in EGb 761 extract that has a free radical scavenging activity that reduces cognitive impairment due to ageing [7-8]. The present research paper aims to develop and validate a new RP-HPLC method for quercetin from EGb761 extract by following ICH Q(2)R1 guidelines. The accuracy, precision, linearity, LOD, and LOQ of the analytical parameters were established. The technique created was used to assess the amount of quercetin produced from EGb761 extract when administered orally *in-vitro*, *ex-vivo*, and *in-vivo* studies. It can be useful for evaluating quercetin in different dose forms.

2. Identification, Physicochemical and Spectral Characterization of EGb 761 Extract

EGb 761 extract was obtained from Tirupati Lifesciences, Paonta Sahib, Himachal Pradesh as a gift sample. The following criteria were used to determine the physicochemical characteristics of the obtained drug sample:

2.1 Qualitative and Quantitative Analyses of the EGb-761 Extract

Qualitative estimations were performed to analyze the presence of a few particular phytochemical groups in the selected test sample (EGb-761 extract). When the test sample was treated with a specific chemical reagent(s), it formed a complex mixture and attained a particular colour, which indicated the presence/absence of the aimed phytochemical in the test sample. Here, the standard extract of EGb-761 extract was analyzed by various biochemical tests to confirm the presence of the phenols, flavonoids, glycosides, alkaloids, saponins, triterpenoids, and tannins.

2.1.1 Identification Tests

2.1.1.1 Test for Phenols and Tannins

Polyphenols or phenolics are responsible for the colouration of fruits and are categorized as – phenolic acids, flavonoids, and polyphenols (flavanones, flavones, catechins, and xanthones). Tannins are also phenolic compounds of higher molecular weights, usually found in the outer layers of plant tissues like roots, bark, stem, etc. The presence/absence of polyphenols and tannins was confirmed by performing a ferric chloride test. For this assay, 50 mg of EGb 761 extract was dissolved in 5% ethanol (1 ml) and to this solution 3ml of 10%, ferric chloride solution was added. The dark green or green-brown colouration confirms the presence of phenolic or tannin compounds in the samples [9].

2.1.1.2 Tests for Flavonoids

This category of polyphenols is made up of a higher benzene ring structure. The qualitative test for flavonoids is commonly known as the Zn-HCl reduction test. To test the presence of flavonoid content in the extract, 50 mg of EGb 761 extract was dissolved in 5% ethanol (1ml) followed by the addition of 10% of zinc hydrochloride solution (2 ml) resulting in a dark brown, coloured solution confirming the presence of flavonoid group [9].

2.1.1.3 Tests for Glycosides

Glycosides are the water-soluble, crystalline carbon, hydrogen, and oxygen-containing phyto-constituents found in plant cells. The test to detect the same is known as Borntrager's test. The presence of glycosides was detected by taking the 50 mg of EGb 761 extract dissolved in a 5% ethanol (1 ml) solution followed by the addition of a pinch of magnesium flakes. Finally, concentrated hydrochloric acid was added drop-wise till a yellowish colour appeared confirming the presence of glycosides [10].

2.1.1.4 Test for Alkaloids

Alkaloids are one of the largest groups of secondary phytochemical compounds present in plants. These compounds are alkaline in reaction and have basic properties. It is also marked as a Dragendroff test. To assess this, 50 mg of EGb 761 extract dissolved in 5% ethanol (1 ml) was heated in a water bath with 2% hydrochloric acid (2 ml) for 5 minutes. Thereafter, a few drops of Dragendroff reagent were added to the extract solution and a yellowish-brown or dark orange colour indicates the presence of the group [9].

2.1.1.5 Test for Saponins

Saponins are known to possess soap-like behaviour and on hydrolysis; another constituent aglycone/sapogenin is produced. These sapogenin compounds are either steroidal or triterpenoids in nature. For testing this compound, 50 mg of extract was mixed in 5% ethanol (1 ml), followed by the addition of water to make up the volume to 20 ml in a tightly capped falcon. The solution was mixed vigorously for 15 minutes and saponin presence was analyzed by any foaming/frothing layer formation [9].

2.1.2 Total Ash Value

The total ash value of a crude drug is what determines its grade and purity. Ash contains inorganic radicals such as phosphates, carbonates, calcium, potassium, magnesium, and sodium silicates. The "Total Ash Value" may change due to the inorganic components of crude drugs [9].

2.1.2.1 Determination of Total Ash Value

A tared silica crucible or a porcelain dish was weighed. In the dish/crucible, about 1g of the powdered drug was weighed. The retort stand's ring supported the dish, which was supported by a pipe-clay triangle. The burner was heated with a flame that was 2 cm high, and the dish was held at a height of about 7 cm above the flame until almost no fumes were formed. After that, the dish was lowered and heated more fiercely until all the carbon had been burnt off. To cool it, desiccators were utilised. Based on the air-dried sample of the crude drug, Eqn. 1 [10] was used to calculate the weight of the ash and the age of the total ash.

Calculation:

Therefore, 100g of the crude drug gives $100/y \times (z-x)$ g of the ash.

Where x = weight of empty petri dish; y = amount of drug taken; z = weight of the petri dish + ash.

Total Ash value of the sample= 100 (z-x) / y %.....Equation 1

2.1.3 Determination of Moisture (Loss on Drying)

The powdered drug was weighed and put into a small, flat porcelain dish with a weight of 1 gram. Then it was dried in the oven at 100°C or 105°C. It was controlled and cooled in a desiccator. To record weight loss as moisture, **Eqn. 2** was frequently utilised [10].

Calculation:

Loss on Drying = y-z / x %.....Equation 2

Where x = weight of the drug; Y = weight of the petri dish alone; z = weight of the Petri dish + drug.

2.2 Physicochemical and Spectral Characterization

2.2.1 Evaluation of organoleptic qualities

Organoleptic properties like colour and odour of the extract were evaluated.

2.2.2 Melting point determination

Using a capillary melting point apparatus, the melting point of *EGb 761* was measured.

2.2.3 Solubility

The solubility of the extract was evaluated in distilled water, phosphate buffer pH - 7.4, ethanol, acetone, n-octanol and ethyl acetate as reported protocols [11].

2.2.4 Spectral characterization

2.2.4.1 Fourier transform-infra-red absorption spectral analysis

Perkin Elmer's 8400 FT-IR/FIR Spectrometer was used to identify the EGb 761 extract. The IR spectra were scanned and recorded from 4000 cm^{-1} to 400 $cm^{-1.}$

2.2.4.2 DSC

DSC is used to study the drug's thermal behaviour (melting point) and to assess if the drug and its excipients are compatible. An atmosphere that was kept inert by nitrogen purging was used to run the DSC system. An appropriate amount of the sample (5 mg) was added to an aluminium pan before being tightly sealed. A reference was taken from an empty aluminium pan. The thermogram was recorded after samples were heated at a rate of 10°C per minute throughout a temperature range of 40–230°C [12].

3. Analytical Method Development

HPLC method was developed to separate, identify and quantify EGb 761 components (majorly quercetin) which are the basic flavonoids of standardized EGb 761 extract. In this study, a suitable method was developed using Quercetin, and all the calculations of % EE, % DR, and PK studies were considered by the AUC of Quercetin.

3.1 Isolation of Quercetin from EGb 761 Extract

The EGb 761 extract was extracted by Soxhlet with petroleum ether, followed by extractions with chloroform and methanol. The methanolic fraction was extracted with petroleum ether, diethyl ether, and ethyl acetate in that sequence using a separating funnel. The ethyl acetate fraction was concentrated for five hours and then hydrolyzed with 7% H_2SO_4. The hydrolyzed fraction was filtered and ethyl acetate was extracted using a separating funnel. It was then concentrated to produce crude Quercetin, which was then crystallised using diluted ethanol [13].

3.2 Instrumentation

An Agilent Laboratories HPLC system with model no. 1220, Infinity LC equipped with UV Detector along with a 20 µl loop rheodyne manual sample injector with switch (77251) was used for injecting the sample and carrying out the analysis using Anachrome software.

3.3 HPLC System

3.3.1 Chromatographic Conditions

Column: C-18

Mobile Phase: Methanol and Acetonitrile (70:30 *v/v*)

Flow Rate: 1ml per Minute

Run Time: 5 Minutes

Detection Wavelength: 373 nm

3.3.2 Mobile Phase Selection

To get an intense and sharp peak of quercetin without any interference various mobile phases were prepared in different ratios of the chosen solvents (methanol and ammonium acetate buffer). The mobile phases were prepared by combining solvents like acetonitrile and methanol in different- different ratios.

3.3.3 Identification of Peaks

A standard stock solution of 0.1mg/ml of quercetin was prepared in methanol. The further stock solution was diluted to give working concentrations of 6-16µg/ml. These standard solutions were injected to determine the retention time and peak area.

3.3.4 Preparation of Calibration Curve

The appropriate dilutions were made from the standard stock solution prepared above to obtain a concentration of 6 µg/ml, 8 µg/ml, 10 µg/ml, 12 µg/ml, 14 µg/ml and16 µg/ml. These different concentrations were injected into HPLC.

3.3.5 System Suitability

Analysis was done of 100% target concentration of quercetin solution by carrying out 6 replicate analyses. Chromatographic parameters like retention time, peak height, peak area, resolution between the peaks, and theoretical plates (tangent) of the column were determined.

3.3.6 Linearity

Linearity was tested by injecting a series of standard stock solutions/diluted aliquots of prepared stock solution spanning from six to eight varying concentrations of the expected/specified operating range utilizing mobile phase as the solvent. The graph to determine the linearity range was plotted as (concentration vs. peak area response) using Microsoft Excel.

3.3.7 Accuracy

At three distinct concentration levels by % age recovery, accuracy trials were conducted. The concentrations of EGb 761 extract added to the pre-analyzed sample solution were 80%, 100%, and 120%, respectively.

3.3.8 Precision

By choosing an aliquot concentration of 10 g/ml and injecting six replicates of samples both on the same day and on other days, method precision and inter-day precision were assessed.

3.3.9 LOD and LOQ Determination

Following ICH criteria, these values were derived using standard deviation.

3.3.10 Ruggedness/Robustness

Ruggedness was performed to check the method's reliability and capacity to produce consistent results after making deliberate variations in the process parameters. Hereby, the robustness study was conducted by injecting samples in replications six times and making slight modifications in the wavelength of the detector.

3.3.11 Repeatability

By examining the 10 µg/ml Quercetin-test solution a total of six times, the repeatability studies of the devised procedure were assessed. RSD was found to have a value of 2.

3.4 Extraction of Quercetin from the Plasma and Tissue Samples

A modified liquid-liquid extraction technique was employed. Rat plasma or tissue homogenate (100 µl) was combined with a 4:1 *v/v* solution of methanol and dimethyl sulfoxide before being vortexed for 60 seconds. Tetra butyl methyl ether was added to this mixture and centrifuged at 10,000 rpm for 5 minutes. The organic layer was then separated and evaporated in a turbo vap LV (Biotage USA) at 40°C while being surrounded by a nitrogen gas stream. Finally, 20 l of the reconstituted mobile phase was used for HPLC analysis after being reconstituted with 100 l of mobile phase from the dried residue.

4. Results and Discussion

4.1 Identification, Qualitative and Quantitative Tests of EGb 761 Extract

Qualitative estimations were performed to analyse the presence of a few particular phytochemical groups in the selected test sample (EGb 761 Extract). Here, the standard extract of *Gingko biloba* was analysed by the various biochemical tests to confirm the presence of the phenols, flavonoids, glycosides, alkaloids, saponins, triterpenoids and tannins and the results concluded the authenticity of the chosen extract.

4.1.1 Identification Tests

4.1.1.1 Test for phenols and Tannins

The dark green or green-brown colouration confirms the presence of phenolic or tannin compounds in the samples.

4.1.1.2 Tests for Flavonoids

50 mg of EGb 761 Extract was dissolved in ethanol 5% ethanol (1 ml) followed by the addition of 10% of zinc hydrochloride solution (2 ml) resulting in a dark brown, coloured solution confirming the presence of a flavonoid group.

4.1.1.3 Tests for Glycosides

The yellowish pink colour appears confirming the presence of glycosides.

4.1.1.4 Test for Alkaloids

Yellowish brown or dark orange colour indicates the presence of the group.

4.1.1.5 Test for Saponins

The solution was mixed vigorously for 15 minutes and saponins presence was analysed by any foaming/frothing layer formation.

The results of the above-stated tests are shown in **Figure 1**

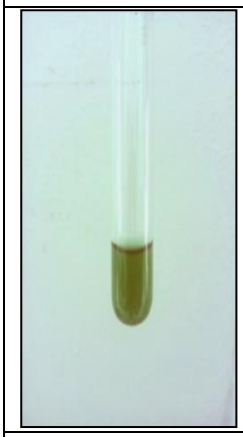
Phenolic compound	**Flavonoids**	**Glycosides**	**Alkaloids**	**Saponins**
$FeCl_3$ Test	**Zn-HCl reduction test**	**Bontrager Test**	**Dragendroff test**	**Foam Test**
				
Dark Green colour (+)	**Dark brown colour (+)**	**Pink color (+)**	**Orange color (+)**	**Foam present (+)**

Figure 1: Results of Identification tests of EGb 761 Extract.

4.1.2 Total Ash Value

The ash value of the drug was found in the standard limits of the pure drugs as shown in **Table 1.** Hence, the given drugs were claimed to be pure and were opted for further study.

Table 1: Ash value of EGb 761 Extract.

Drug	Specification	Result
EGb 761 Extract	NMT 10.0 %	4.84 ±0.8%

*****Value is presented as mean S.D. (n = 3).

4.1.3 Loss on Drying

Loss on drying was found to determine the volatile constituents in the drug and compared with the standard limits of drying and were found to be in the limits. The loss on drying values is enlisted in **Table 2** and was found within the specification.

Table 2: Loss of drying

Drug	Specification	Result
EGb 761 Extract	NMT 7.0 %	3.88±0.65 %

*Value is presented as mean S.D. (n = 3).

4.2 Physicochemical Characterization

4.2.1 Organoleptic Properties Evaluation

Table 3 shows the results of the organoleptic properties evaluated by the chosen extract.

Table 3: Properties of a drug.

Organoleptic properties	
Nature	Solid flowing powder
Colour	Brown Yellow
Taste / Odour	Characteristic taste odour

4.2.2 Melting Point Determination

By using capillary melting point equipment, the melting point of EGb 761 extract was measured and found to be 170°C, as opposed to the previously reported melting temperature of 150 – 170 °C.

4.2.3 Solubility Determination

The solubility of the drug in water was determined by the "Mechanical shaker method". It was found to be 3.5 ± 2.17 mg/ml while reported aqueous solubility of 4.08 mg/ml. Also, the Solubility of *Ginkgo biloba* extract in different media is shown in **Table 4.**

Table 4: Solubility of EGb 761 extract in different media.

Sr. No	Solvent	Solubility	Solubility definition
1	Distilled Water	+++++	Very soluble
2	Phosphate buffer pH - 7.4	++++	Freely soluble

3	Ethanol	++++	Freely Soluble
4	Acetone	+++	Sparingly Soluble
5	N-octanol	++	Slightly soluble
6	Ethyl acetate	+	Very slightly soluble

4.2.4 Spectral Characterization

4.2.4.1 Fourier transform infrared absorption spectral analysis (FT-IR)

The predominant peaks represent the main functional groups of pure showed characteristic peaks at 1734.84 cm^{-1} representing C=C and C=N and 1463 cm^{-1} representing C=O stretching. At the same time, C-OH (alcoholic and carboxylic acid) stretching appears at 1108 cm^{-1} with minor shifts (**Figure 2)**.

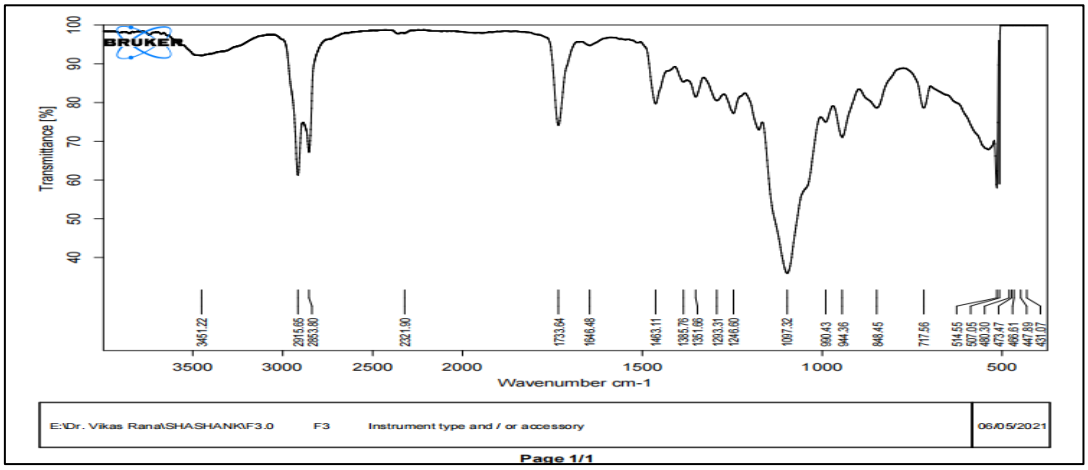


Figure 2: FT-IR Spectra of EGb 761 Extract

4.2.4.2 Differential scanning calorimetry (DSC)

DSC was employed to determine the melting point of EGb 761 extract between 50 and 250 °C. The drug's DSC thermogram (**Figure 3)**, showed a clear melting peak at 170.05°C, confirming its crystalline shape. The melting temperature measurement and the DSC experiment results showed high agreement.

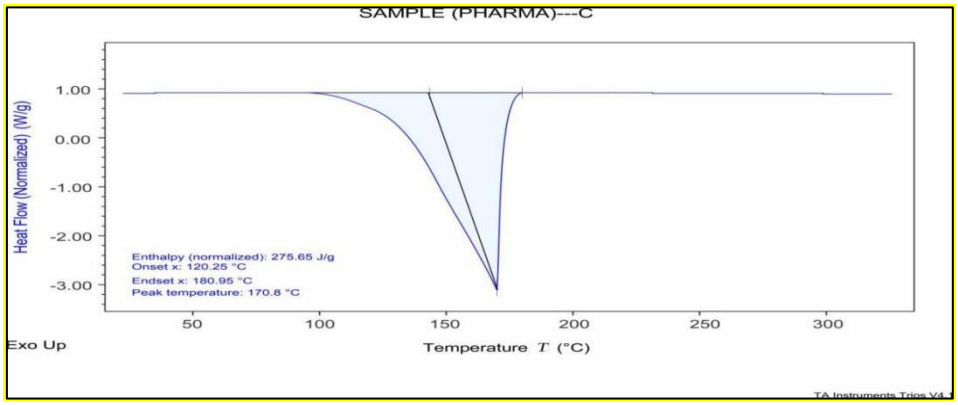


Figure 3: DSC Thermogram of EGb 761 Extract.

4.3 Analytical Method Development (RP-HPLC)

To determine quercetin from EGb 761 Extract, very few analytical procedures have been documented in the literature. As a result, the current study seeks to develop and verify a novel RP-HPLC technique for quercetin from EGb 761 extract by ICH-Q (2) R1 criteria. The accuracy, precision, linearity, LOD, and LOQ of the analytical parameters were established. The technique created was used to assess the amount of quercetin produced from intranasally administered EGb 761 extract *in-vitro*, *ex-vivo*, and *in-vivo*. It may be used to evaluate quercetin in different dose forms.

4.3.1 System suitability

Analysis was done of 100% target concentration of quercetin solution by carrying out 6 replicate analyses. Chromatographic parameters like retention time, peak height, peak area, resolution between the peaks and theoretical plates (tangent) of the column were determined. Also, the method was evaluated by analyzing these parameters as shown in **Table 5.** The standard chromatogram of EGb 761 Extract and Quercetin obtained at 373 nm is displayed in **Figure 4** and **Figure 5** respectively.

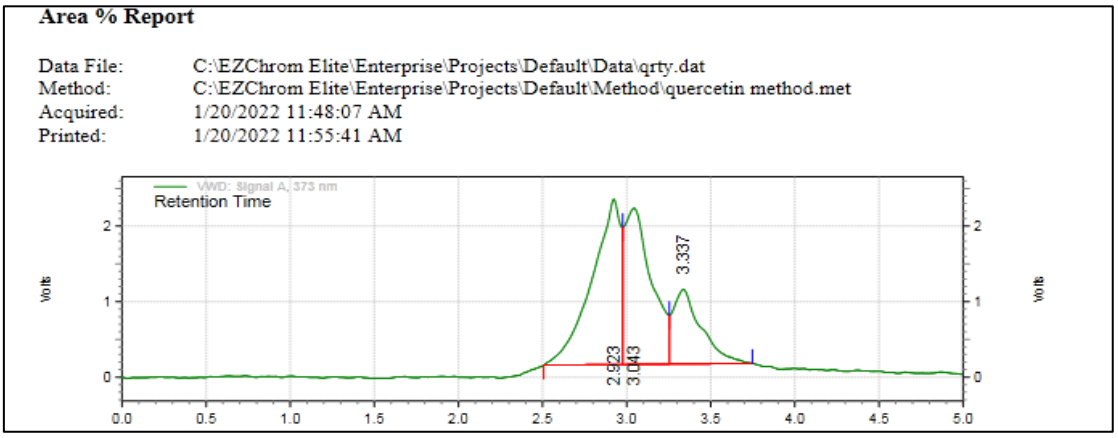


Figure 4: Standard chromatogram of EGb 761 Extract.

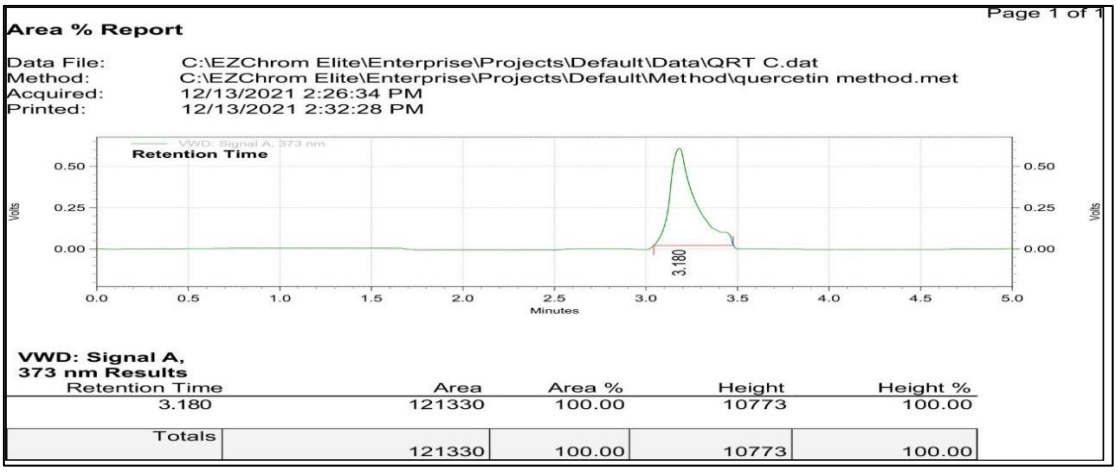


Figure 5: Chromatogram of Quercetin.

Table 5: System suitability parameters

Quercetin concentration	10µg/ml
Wavelength	373 nm
Column	C-18
Mobile phase	Methanol and Acetonitrile (70:30 v/v)
Run time	5 min
Retention Time	3.18 min
Area	2118767
Height	10773

4.3.2 Linearity

Linearity was determined by injecting a series of standard stock solutions/diluted aliquots of the prepared stock solution by using mobile phase as solvent ranging from six to eight different concentrations of the expected/specified working range. The graph to determine the linearity range was plotted as (Concentration vs... Peak Area Response) using Microsoft Excel as shown in **Figure 12.** Linearity for EGb 761 extract was observed in 6-16µg/ml **(Table 6)** concentrations and the chromatograms at different linearity points are shown in **Figure 6-11.**

Table 6: Linearity results of quercetin test solutions.

Concentration	**Peak area(± SD)**
6	77041 ± 55.012
8	109793 ± 58.045
10	121330 ± 132.613
12	150482 ± 103.522
14	178062 ± 159.675
16	219586 ± 147.696

*All values are expressed as Mean±S.D, (n=3)

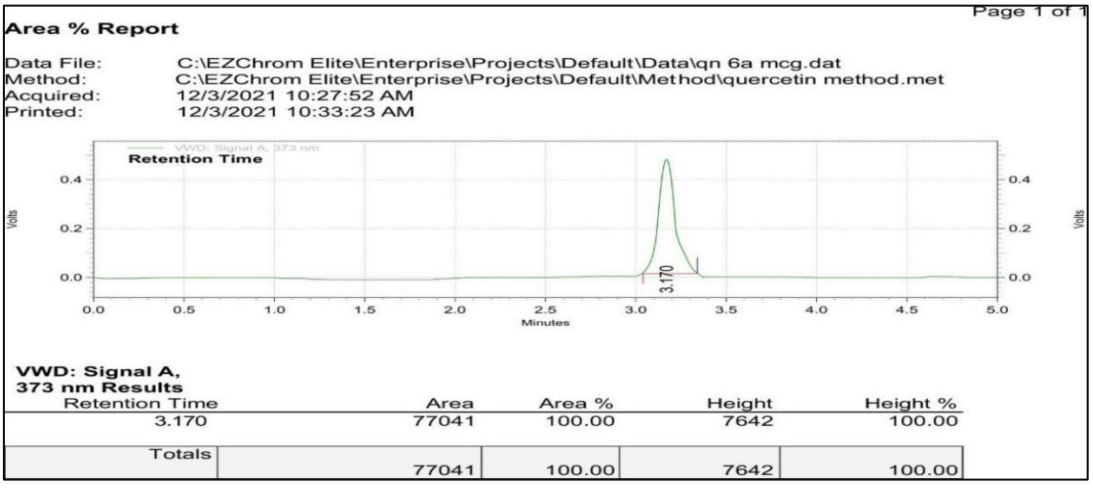


Figure 6: Linearity chromatograms of quercetin at a concentration (6µg/ml).

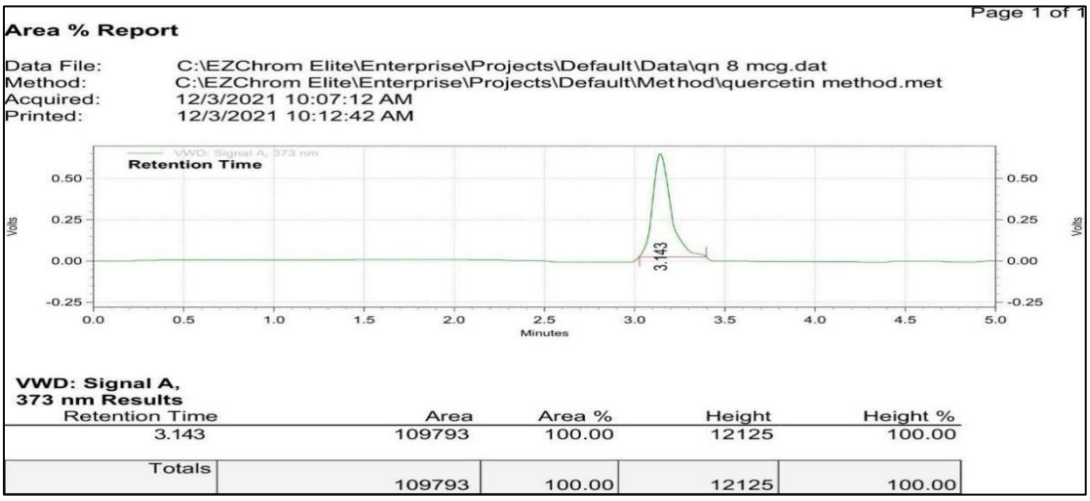


Figure 7: Linearity chromatograms of Quercetin at a concentration (8µg/ml).

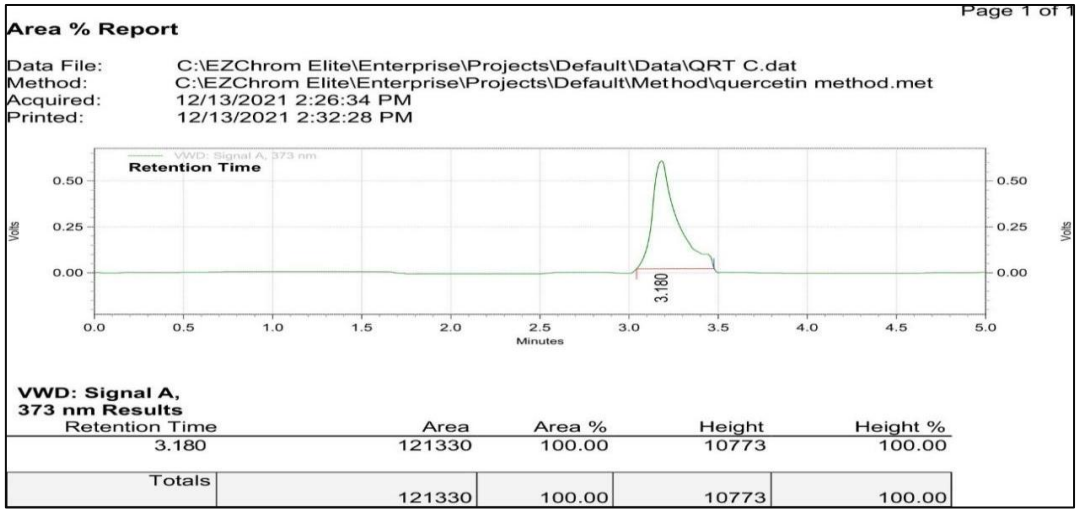


Figure 8: Linearity chromatograms of Quercetin at a concentration (10µg/ml).

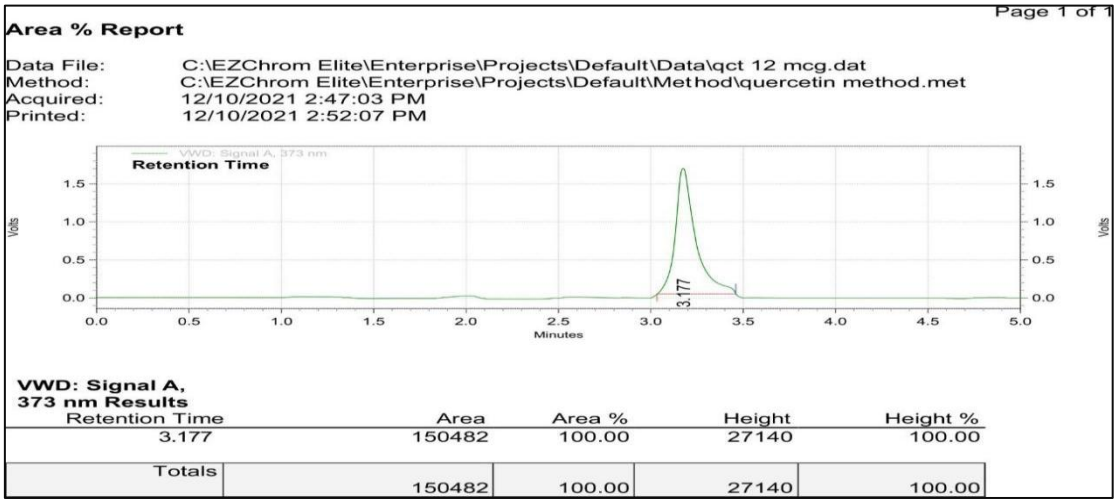


Figure 9: Linearity chromatograms of Quercetin at a concentration (12µg/ml).

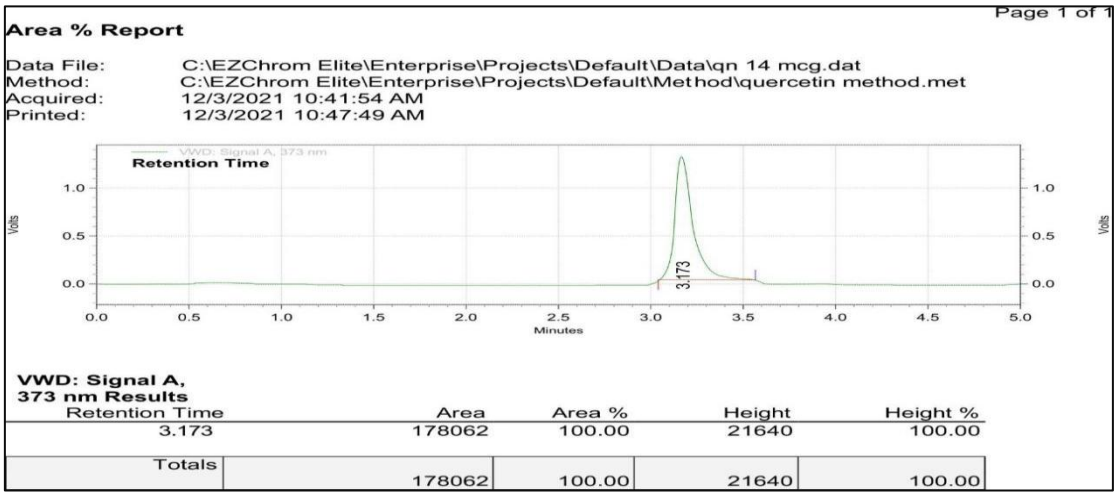


Figure 10: Linearity chromatograms of Quercetin at a concentration (14µg/ml).

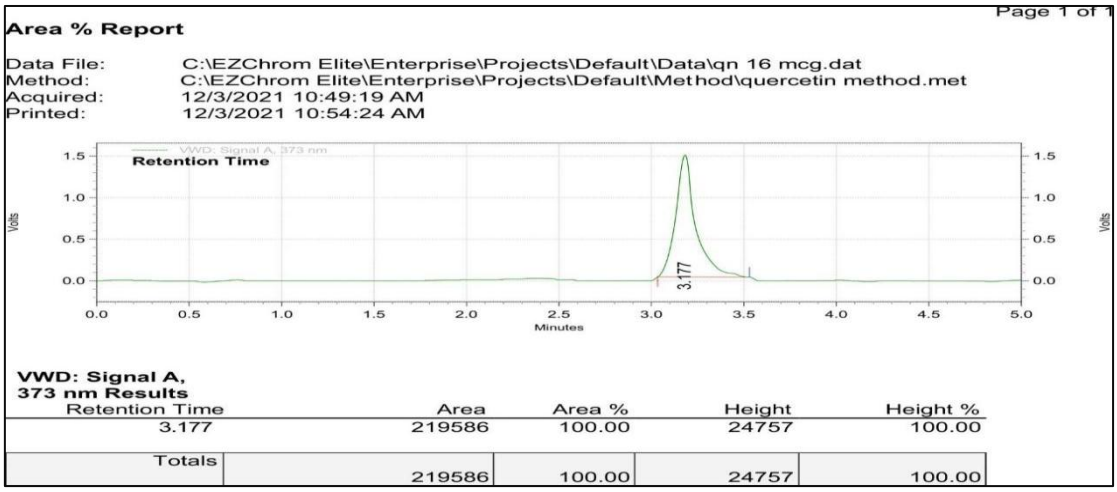
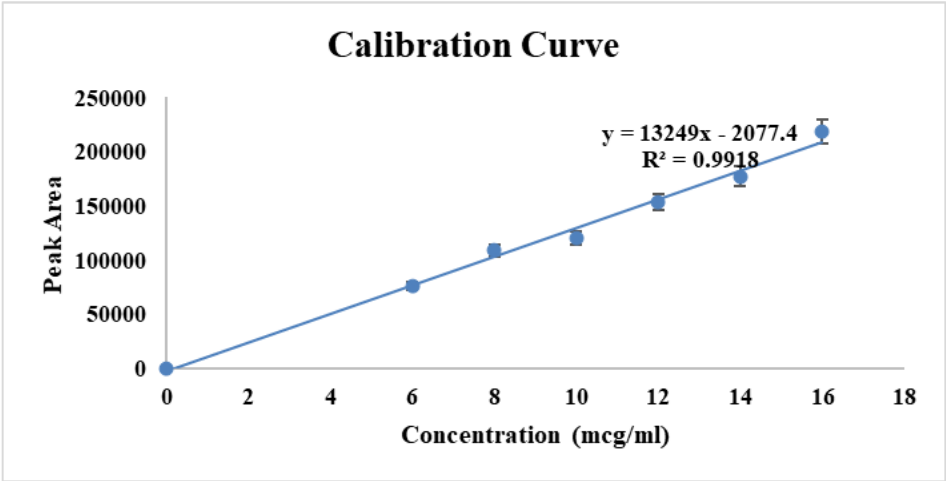


Figure 11: Linearity chromatograms of Quercetin at a concentration (16µg/ml).



***All the readings are the three replicates of six readings**

Figure 12: Standard Calibration curve of EGb 761 extract.

Acceptance Criteria: The value of the correlation coefficient (R^2) should not be less than 0.98.

4.3.3 Accuracy

Accuracy studies were carried out at three different concentration levels by %age recovery as shown in **Table 7.** To the pre-analyzed sample solution of EGb, 761 sample solutions were added at 80%, 100% and 120% respectively.

Table 7: Accuracy results of quercetin test solutions.

Amount Present (µg/ml)	**Amount Added (µg/ml)**	**Area**	**Amount Found (µg/ml ±SD)**	**Recovery (%)**	**% RSD**
10	8	231123	17.645	98.02	0.05
10	10	242661	19.415	97.07	0.04
10	12	282661	21.434	97.42	0.03

*All values are expressed as mean ± SD where (n=3)

Acceptance Criteria: The mean recovery should not be less than 99 ± 2 %

4.3.4 Precision

Method precision and inter-day precision were carried out by selecting the concentration of aliquot as 10µg/ml concentration and six replicates of samples were injected during a single day and different days and results are mentioned below as can be seen in **Table 8.**

Table 8: Precision results -quercetin test solution (10µg/ml).

	Intra Day precision		**Inter Day Precision**	
	RT	**Area for conc. 10 µg/ml**	**RT**	**Area for conc. 10 µg/ml**
1	3.17	121330	3.177	120298
2	3.177	121224	3.17	121358
3	3.173	120421	3.163	122251
4	3.173	122563	3.173	120991
5	3.163	122521	3.17	120432
6	3.177	122185	3.153	121338
Mean	3.172167	121707.3333	3.167667	121111.3333
±SD	0.005231	854.7548576	0.008524	713.9393999
%RSD	0.164913	0.702303497	0.269109	0.589490166

*All values are expressed as mean ± SD where (n=3)

Acceptance Criteria: %age RSD for (C.V.) of the determinations should not be more than 2.

4.3.5 LOD and LOQ determination

These values were calculated by standard deviation and results are shown in **Table 9**.

Table 9: LOD and LOQ results Of Quercetin Test Solution (10µg/ml)

Aliquote Concentration	**SD**	**%RSD**	**LOD µg/ml**	**LOQ µg/ml**
10 µg/ml	1863.61	1.52	0.422	1.40

*All values are expressed as mean ± SD where (n=3)

4.3.6 Ruggedness/Robustness

Ruggedness was performed to check the method's reliability and capacity to produce consistent results after making deliberate variations in the process parameters. Hereby, the robustness study was conducted by injecting samples in a replicate six times and making slight modifications in the wavelength of the detector. The results of ruggedness were expressed as % age relative standard deviation as expressed in **Table 10.**

Table 10: Ruggedness results of quercetin test solution (10µg/ml).

S.No	Wavelength	RT	Area
1	372	3.03	996575
2	373	3.12	1005331
3	374	3.13	985538
Mean		3.09	995814.7
SD		0.06	9918.382
%RSD		1.94	0.996007

*All values are expressed as mean ± SD, where (n=3)

Acceptance Criteria: % age RSD for (C.V.) of the determinations should not be more than 2.

4.3.7 Repeatability

By examining the 10 µg/ml EGb 761-test solution a total of six times, the repeatability studies of the devised procedure were assessed. As may be seen in **Table 11**, the value of % RSD was confirmed to be 2.

Table 11: Repeatability result for quercetin-test solutions.

Conc (µg/ml)	Avg. conc. (n=6)	Std.dev	%RSD
1863.61	1.52	0.422	1.40

*All values are expressed as mean ± SD where (n=3)

The results of the parameters were validated as given in **Table 12.**

Table 12: Summary of validation parameters.

Validation parameter	Results
Absorption maxima (nm)	373 nm
Regression equation	y = 13249x - 2077.4
Value of R^2	0.9918
Slope (m)	13249
Intercept (c)	2077.4
Linearity range (µg/ml)	6-16 µg/ml
Inter-day precision results: (Retention Time)%RSD (Peak Area)%RSD	 0.269 0.589
Intra-day precision results: (Retention Time)%RSD	 0.165

(Peak Area)%RSD	0.702
LOD value	0.422
LOQ value	1.40
Ruggedness %RSD (Retention Time) %RSD (Peak Area)	 1.94 0.996007
Repeatability SD %RSD	 0.422 1.40

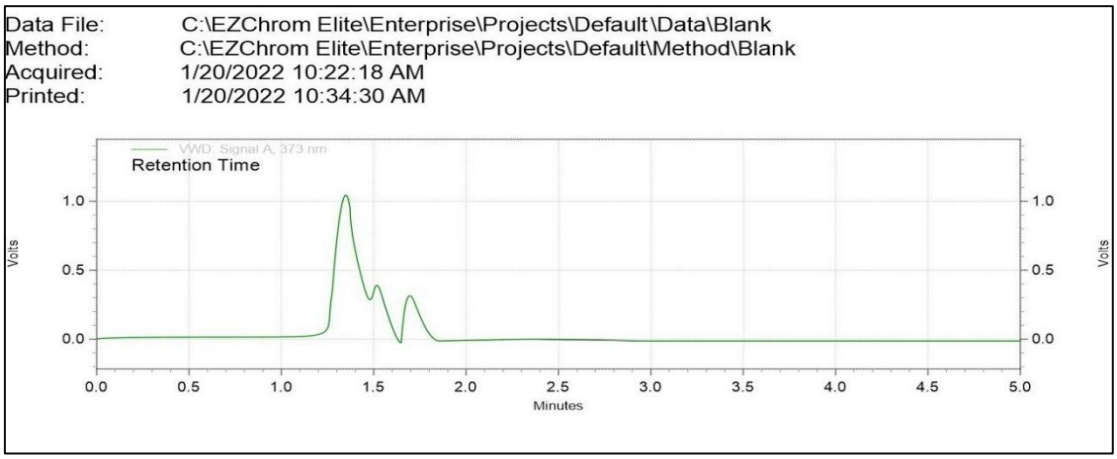


Figure 13: Chromatogram of blank plasma.

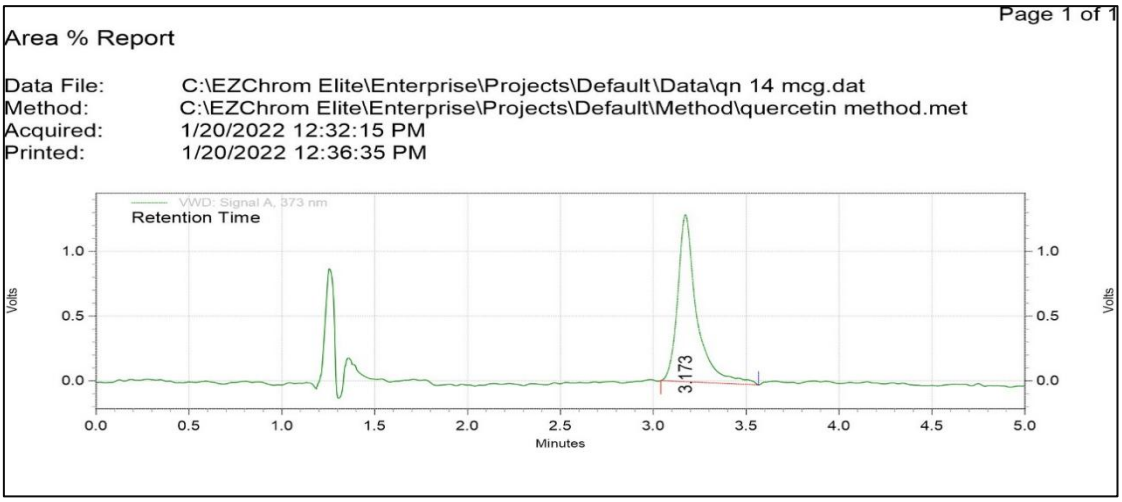


Figure 14: Chromatogram of blank plasma and quercetin.

5. Conclusion

In conclusion, the provided analytical method shows excellent linearity, precision, sensitivity, ruggedness, and repeatability. The regression equation ($y = 13249x - 2077.4$) with a high R^2 value of 0.9918 suggests a strong linear relationship between the concentration (x) and the response (y) in the analyzed system. This indicates that the calibration curve is well-fitted and reliable for quantification. Furthermore, the linearity range of 6-16 µg/ml indicates the concentration range over which the analytical method can accurately quantify the

compound. This is a reasonably broad range, which is favorable for analytical applications. In addition, the low %RSD values for retention time (0.269% & 0.165%) and peak area (0.589% & 0.702%) indicate good precision and repeatability of the method when the analysis is performed on different days and the same day respectively.

The limit of detection (LOD) value of 0.422 and the limit of quantification (LOQ) value of 1.40 are indicative of the sensitivity of the analytical method. These values suggest that the method can detect and quantify the compound at very low concentrations, which is often desirable in analytical chemistry. Additionally, the ruggedness of the method, as indicated by the %RSD values for retention time (1.94%) and peak area (0.996007%), suggests that the method is robust and can provide consistent results even when slight variations in experimental conditions occur. Moreover, the low standard deviation (SD) and %RSD values for repeatability (0.422% and 1.40%, respectively) demonstrate that the method can be reproduced with high accuracy and precision under the same conditions. These results indicate that the method is suitable for the accurate quantification of the compound of interest within the specified concentration range and can be relied upon for quality control and research purposes.

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It was declared to be none.

Conflict of interest

It was declared to be none.

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