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"Analytical Method Development and Validation of High Performance Thin Layer Chromatographic Method for Simultaneous Estimation of Quercetin and Ferulic acid in Marketed Herbal Tablet Formulation"

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ABSTRACT

Quercetin is flavonoid which are naturally occurring in the human diet. In plant physiology, flavonoids have a long evolutionary history. They are known to control antifungal and bactericidal functions are two further biological functions that plants have exercise Ferulic acid (FA) is a phenolic acid that is found in abundance in fruits and vegetables. Several studies have revealed that FA works as an antioxidant by scavenging free radicals and boosting the cell stress response by up-regulating cytoprotective mechanisms in recent years**.**

Various organic solvents were tried in varied proportions then four were selected for mobile phase. The chromatographic condition suggested by the software were mobile phase of Toluene:Ethylacetate:formicacid:methanol in ratio of (7:2:0.9:0.3) and saturation time 15 min. Separation was achieved using 10.0 ×10.0 HPTLC plates silica gel 60 F254 GLP Manufacturer LOBA CHEMIE . CAMAG winCats software was used and densitometric scanning was accomplished at 366 nm.

Keywords: Quercetin,Ferulic acid, HPTLC,Validation, mobile phase, ICH

INTRODUCTION

Quercetin has been reported to inhibit the allergic and inflammatory responses of the immune system.Several studies have revealed that Ferulic acid works as an antioxidant by scavenging free radicals and boosting the cell stress response by up-regulating cytoprotective mechanisms in recent years.

High performance thin layer chromatography (HPTLC) is a preferred analytical tool for fingerprints and quantification of marker compounds in herbal drugs due to its simplicity, high sensitivity, accuracy and less expensive.

High Performance Thin Layer Chromatography (HPTLC) method equally suitable for qualitative and quantitative analytical tasks. HPTLC is superior to other analytical techniques in terms of total cost and time for analysis. It is an offline process in which various stages are carried out independently.

AIM

To Ḍevelope and Validate a High Performance Thin Layer Chromatography method for the Simultaneous Estimation of Quercetin and Ferulic acid in the Marketed herbal Tablet Formulation.

OBJECTIVES

- To Select Mobile phase and scanning wavelength
- To optimize mobile phase to obtain better resolution of peaks of above mentioned phytochemicals and lesser time of saturation
- To obtain separate and sharp peaks of phytochemicals without any interference
- To quantify Phytochemicals in the selected herbal marketed formulation
- To validate the method developed as per ICH guidelines.

RATIONALE AND SIGNIFICANCE

Haritaki *(Terminalia chebula)* contains markers as Quercetin and Ferulic acid respectively which are reported to produce immunomodulatory and antioxidant activity.

During the literature reviewing, it was found that Quercetin and ferulic acid not quantified simultaneously
Hence, the present aim to quantify the above mentioned phytochemicals in the tablet formulation by developing a simple and precise HPTLC method which can be reproducible.

PLAN OF WORK Literature review

Selection of phytochemicals

Procurement of herbal tablet

Procurement of standard phytochemicals

Selection of mobile phase

Optimization of the selected mobile phase

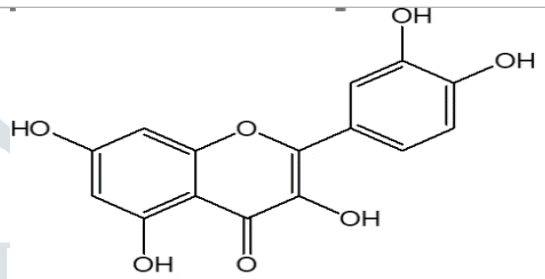
Selection of scanning wavelengths

Effective separation of selected phytochemicals in herbal tablet formulation

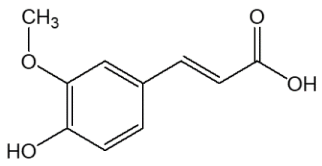
Quantification of phytochemicals in herbal tablet

DRUG PROFILE

QUERCETIN

Drug Name	Quercetin
Structure	
Molecular formula	$C_{15}H_{10}O_7$
Molecular Mass	302.238 g/mol
Solubility	It is very soluble in ether, methanol, soluble in ethanol, acetone
PKa	6.38
Category	Antioxidant, Anti- inflamatory
Melting point	316-320° C

FERULIC ACID

Drug Name	Ferulic acid
Structure	
Molecular formula	$C_{10}H_{10}O_4$
Molecular mass	194.8 g/mol
Solubility	Soluble in organic solvents such as Methanol, Ethanol
Pka	4.61
Category	Antioxidant
Melting point	167-172°C

MATERIALS

CHEMICALS	ROLE	OBTAINED FROM
Quercetin	Standard	Yucca enterprises
Ferulic acid	Standard	Yucca enterprises
Marketed formulation	Sample	Zandu
Precoated TLC plates	Stationary phase	Loba chemie
Methanol	Solvent	Qualigens
Toluene	Solvent	Qualigens
Ethyl acetate	Solvent	Qualigens
Glacial acetic acid	Solvent	Qualigens
Formic acid	Solvent	Fisher scientific
Hexane	Solvent	Qualigens
Iodine	Derivatizing agent	Modern chemicals
Aluminium chloride	Derivatizing agent	Modern chemicals

EQUIPMENTS

EQUIPMENTS	MODEL
Weighing balance	Shimadzu aux220 digital analytical balance
UV spectrometer	Shimadzu 1900
Sample applicator	Linomat 5 by camag
TLC scanner 3	camag
UV cabinet	camag
Twin trough chamber	camag
Oven	Star scientific
Ultrasonicator	Citizen
Syringe 10µl	Hamilton by camag

EXPERIMENTAL WORK

Preliminary testing of the standards

This testing has the most significance in this study as in this test the colour and texture is carefully observed and reported

Optimization of the solvents on thin layer chromatography

In order to achieve separation of the standards on the precoated TLC plates different neat solvents are tried such as formic acid, toluene, ethyl acetate, methanol were used, the separation was not achieved.Different ratio of these solvents were taken and spots were applied on the TLC to check the separation. In order to get a better resolution now these solvents were tried in various ratios final optimized ratio was obtained**.**

Selection of the wavelength for densitometric scanning

Various trials on HPTLC were taken to check the wavelength which gives optimum peak as per the literature survey 366 nm were wavelength reported for scanning of flavonoids and phenolic carboxylic compounds on HPTLC method development 366 nm wavelength was found to be favorable for scanning of the standards of Quercetin and ferulic acid

Method development on HPTLC

a) Preparation of standard solution

10 mg of Quercetin was weighed accurately and transferred to 10 ml volumetric flask to which methanol was added to make up the volume and then 1ml pippeted and transferred to 10 ml volumetric flask and make up volume with methanol and solution of 0.1mg/ml solution was obtained and further 1ml pipeted out and made volume upto 10 ml with methanol and solution of 0.01mg/ml solution was obtained of quercetin

10 mg of Ferulic acid was weighed accurately and transferred to 10ml volumetric flask to which methanol was added to make up volume and then 1ml pippeted and transferred to 10ml volumetric flask and make up volume with methanol and solution of 0.1mg/ml solution was obtained and further 1ml pipeted out and made volume upto 10 ml with methanol and solution of 0.01mg/ml solution was obtained of Ferulic acid

b) Preparation of sample solution

100mg of terminalia chebula tablet triturated and transferred to 10 ml volumetric flask then added methanol made final solution of 10mg/ml and sonicated for 15 min and methanol was added to make up volume.

Filtered the solution with the help of whatman filter paper and further used for analysis

c) Selected mobile phase

Toluene:Ethyl acetate:Formic acid:Methanol (7:2:0.9:0.3)

Saturation time 15 min

d) Iodine crystals for derivatization

Charged chamber with some crystals of iodine and placed developed dried plate in iodine vapours

Validation of the HPTLC method according to ICH guidelines

a) **Specificity**

Specificity of the method is the ability of the analytical method to assesment of analyte which is expected to be present unequivocally

b) **Linearity**

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of an analyte in samples within a given range.

Acceptance criteria of linearity Correlation Coefficient (r^2) should not be less than 0.999

c) **Accuracy**

The accuracy in analytical procedure means the closeness of the value that is accepted as true value or a reference value to the value that is obtained

The accuracy is carried out over 80%, 100% and 120% of the std solution prepared these solutions are prepared in triplicate and percentage recovery is calculated

Acceptance criteria

Mean recovery should be in the range of 98.00-102.00%

The Relative Standard Deviation should not be more than 2.0

d) **Precision**

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple Samplings of a homogenous sample The precision of an analytical method is usually expressed as a standard deviation or relative standard deviation. Precision is of two types, Repeatability and Intermediate precision. It is performed on an API sample.

Acceptance Criteria: % RSD = NMT 2% for test results

e) **Limit of detection**

The lowest conc. of the analyte in the sample that the method can detect but not necessarily quantify under the stated experimental conditions simply indicates that the sample is below or above a certain level.It may be calculated based on the standard deviation (SD) of the response and slope of the

curve(S). LOD= 3.3 (SD)/S

Where, SD= Standard deviation, S= Slope

f) **Limit of quantitation**

The limit of quantitation (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. It may be calculated based on the standard deviation (SD) of the response and slope of the curve(S). LOQ= 10 (SD)/S Where, SD= Standard deviation, S= slope

g) **Robustness**

The effect on results is investigated by introducing modest changes in mobile phase composition, volume, chamber saturation time, and slight changes in solvent migration distance.

%RSD should not more than 2

i)**Assay**

Assay is performed for quantitative estimation of phytochemicals from makerted herbal tablet.

%Assay = Area of sample X concentration of standard X purity of standard

Area of standard X concentration of sample

RESULTS AND DISCUSSION Organoleptic test for standard QUERCETIN

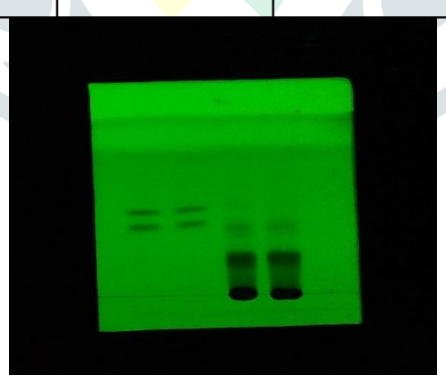
Parameters	**Observations**
Colour	Yellow
Appearance	Crystallline powder

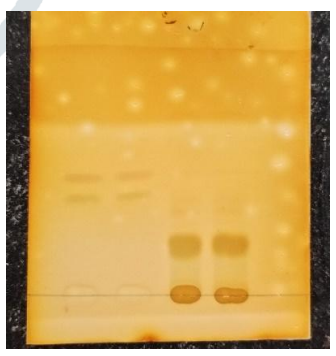
FERULIC ACID

Parameters	Observations
Colour	Pale white
Appearance	Crystalline powder

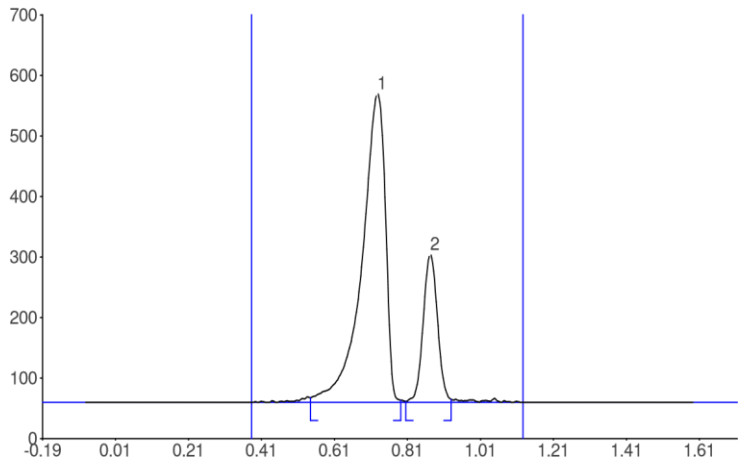
❖ Optimisation of mobile phase to develop the method

SR.NO	MOBILE PHASE	RATIO	REMARK
1	Toluene:Ethyl acetate	(9.3:0.7)	spots not resolved
2	Toluene:Ethylacetate:Methanol	(5:3:1)	Rf values not matched
3	Toluene:Ethylacetate:Methanol	(6:4.2:0.8)	Spots are spreading and not resolved well
4	Ethylacetate:Hexane	(6:4)	Matching Rf of quercetin but not of ferulic acid as compared with sample
5	Toluene:Ethylacetate:Formic acid:Methanol	(5:3:0.4:1.6)	Well resolving compounds but Rf values not matched
6	Toluene:Ethylacetate:Hexane	(5:3:2)	Slight difference in Rf values
7	Ethyl acetate:hexane:Gaa	(6:4:0.4)	Not resolving spots
8	Toluene:Ethylacetate:Formic acid:Methanol	(7:2:0.7:0.3)	Nearly matching Rf values and well resolved sharp peaks
9	Toluene:Ethylacetate:Formic acid:Methanol	(7:2:0.9:0.3)	Developed method with Rf 50±0.5 Quercetin Rf 76±0.5 ferulic acid





Developed TLC plates and visualized under 366nm and 254nm **Dervatized TLC plate with iodine vapours**



Chromatographic separation of Quercetin and Ferulic acid

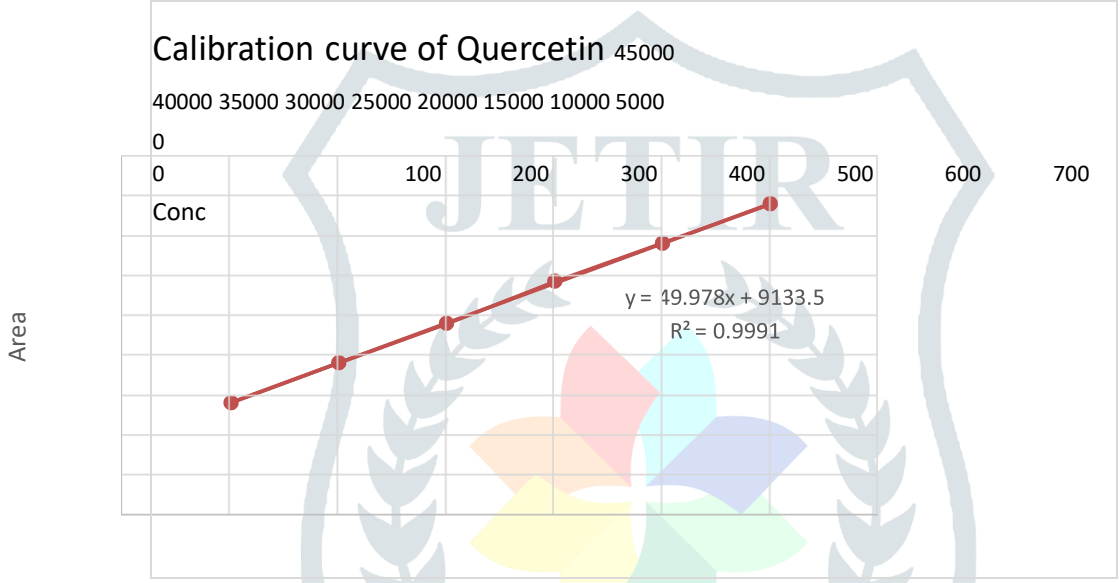
Validation of the HPTLC method

a) **Linearity**

The linearity carried out by making a solutions of the standard substances Quercetin and Ferulic acid for range selection. The typical solution for Quercetin and Ferulic acid in this suggested study activity has a strength of 100 to 600 µg/ml

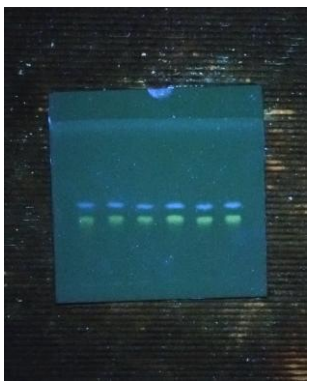
Linearity of Quercetin

Quercetin	
Concentration	**Area**
100	14124.8
200	19098.6
300	24034.4
400	29340.5
500	44120.7
600	49034.6

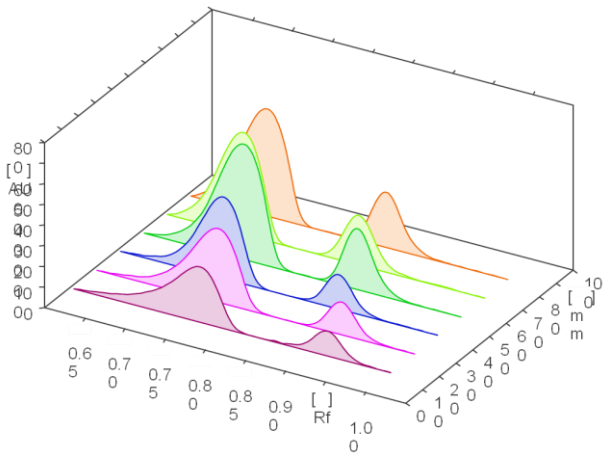


Linearity of Ferulic acid

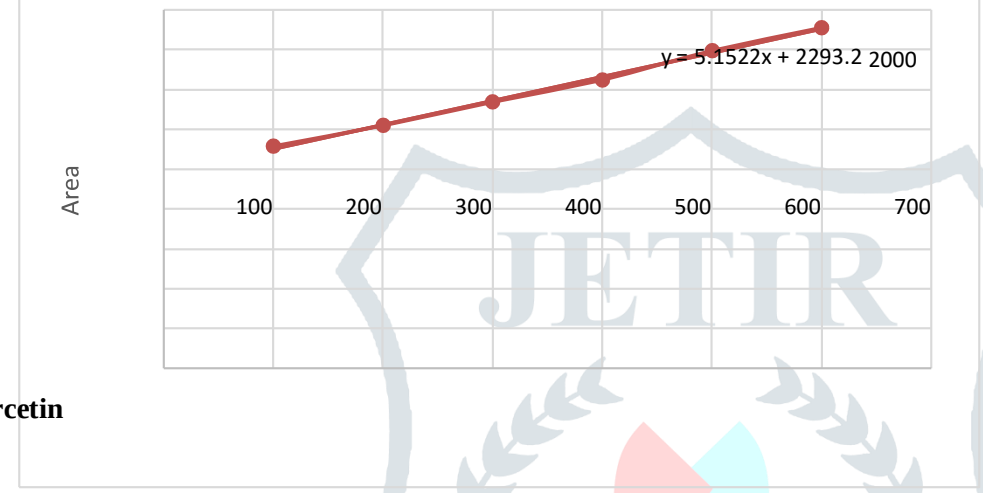
Ferulic acid	
Concentration	**Area**
100	2790.8
200	3050.6
300	3345.7
400	3623.6
500	3989.4
600	4278.5



Developed linearity plate



Linearity 3D plot



Accuracy

Accuracy of Quercetin

Quercetin							
Sr.no	**Conc. Level**	**constant amount µg**	**Added amount µg**	**Area**	**Mean Area**	**Recovered Conc.**	**Recovery %**
1	80%	200	160	28775.41	28783.0	358.66	99.62
		200	160	28790.12			
		200	160	28783.56			
2	100%	200	200	32198.13	32203.1	401.28	100.32
		200	200	32202.04			
		200	200	32209.30			

3	120%	200	240	35418.13	35414.5	441.29	100.29
		200	240	35435.45			
		200	240	35390.19			

Accuracy of Ferulic acid

Ferulic acid							
Sr.no	**Conc. Level**	**constant amount µg**	**added amount µg**	**Area**	**Mean Area**	**Recovered Conc.**	**% Recovery**
1	80%	200	160	3984.15	3970.20	356.07	98.90
		200	160	3975.02			
		200	160	3951.45			
2	100%	200	200	4420.65	4430.30	397.30	99.32
		200	200	4455.08			
		200	200	4415.15			
3	120%	200	240	4885.35	4903.52	439.77	99.88
		200	240	4915.13			
		200	240	4910.09			

Acceptance criteria: % recovery should be between 98%-100%

Conclusion

Since% recovery of both the drugs have been found within the range it is concluded that method developed is accurate

c) Precision

Intra and inter day precision of Quercetin

QUERCETIN

Mean peak area	Amount (ng/spot)	Intra-day	%RSD	Inter-day Mean peak area	%RSD		
200		19054.84	0.62	300	18976.45	0.70	24076.72	1.21
24182.98	1.73				29221.38	0.95	
400		29183.38	0.78				

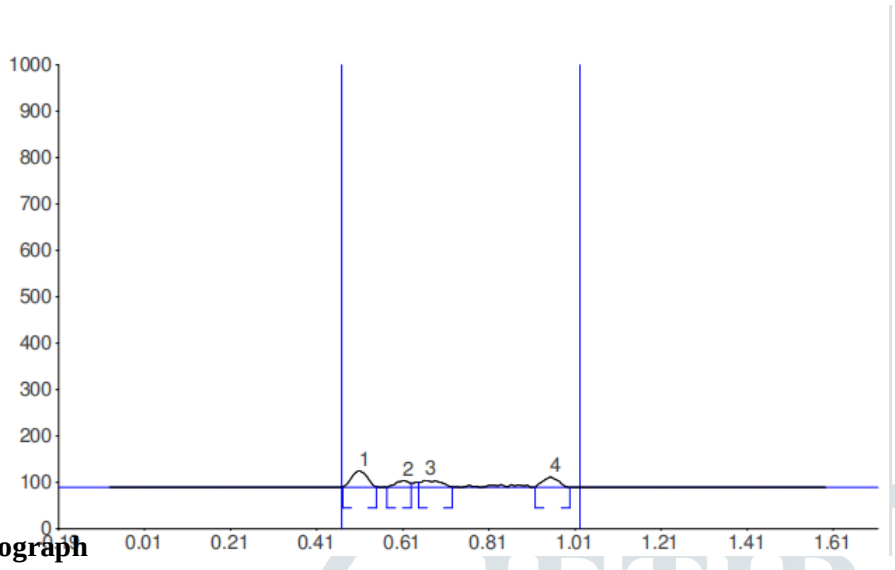
Intra and inter day precision of Ferulic acid

FERULIC ACID

Amount (ng/spot)	Intra-day		Inter-day	
Mean peak area		%RSD	Mean peak area	%RSD
200	3121.26	1.80	300 3056.21	1.85
3277.51	1.47			
3305.14	1.26		3646.31	1.57
400	3623.73	1.85		

As showed in tables intraday and inter day precision of both drugs having %RSD within 2 so method is precise

d) Specificity



Specificity chromatograph

Developed TLC plate

As observed in figures the peaks that of no 1 and 3 of quercetin and ferulic acid have peaks without any interference, thus method was found to be specific

e) Limit of detection

It may be calculated based on the standard deviation (SD) of the response and slope the curve(s)

LOD= 3.3 (SD)/S

Where, SD= Standard deviation of intercept of Quercetin= 287.17

LOD of Quercetin = 18.96ng

Where, SD= Standard deviation of Ferulic acid =69.34

LOD of Ferulic acid= 76.27ng

f) Limit of quantitation

It may be calculated based on the standard deviation (SD) of the response and slope of the curve(S).

LOQ= 10 (SD)/S

Where, SD= Standard deviation of Quercetin= 287.17

The LOQ of Quercetin = 57.46 ng

Where, SD= Standard deviation of Ferulic acid=69.34

The LOQ of Ferulic acid = 231.13ng

g) Robustness

Robustness of Quercetin

QUERCETIN		
Parameters	**100µg/ml (%RSD)**	**200µg/ml (%RSD)**
Toluene:ethyl acetate:formic acid:methanol (6.8:2:0.9:0.5)	0.732	1.59
Toluene:ethyl acetate:formic acid:methanol (7:2:0.9:0.3)	0.94	0.78
Toluene:ethyl acetate:formic acid:methanol (7.2:2:0.7:0.3)	1.823	1.66
Duration of saturation time(min)		
13	1.2	0.67
15	0.56	0.94
17	1.74	1.44

Robustness of Ferulic acid

FERULIC ACID		
Parameters	**100µg/ml (%RSD)**	**200µg/ml (%RSD)**
Toluene:ethyl acetate:formic acid:methanol (6.8:2:0.9:0.5)	1.261	1.307
Toluene:ethyl acetate:formic acid:methanol (7:2:0.9:0.3)	0.84	0.69
Toluene:ethyl acetate:formic acid:methanol (7.2:2:0.7:0.3)	1.60	1.84
Duration of saturation time(min)		
13	1.34	1.78
15	0.86	0.64
17	1.94	1.34

As shown in above tables %RSD of peak areas of Quercetin and Ferulic acid are within the acceptance range so it shows that developed methos is robust.

i)**Assay**

%Assay = Area of sample X concentration of standard X purity of standard

Area of standard X concentration of sample

Results:

1) Quercetin = 11.88% 2) Ferulic acid = 6.9%

SUMMARY AND CONCLUSION

SUMMARY

- The aim of the study was to develop and validate the method for simultaneous quantitative estimation of phytochemicals that is Quercetin and Ferulic acid was achieved
- The method development through mobile phase optimization and other chromatographic conditions helped in achieving the desired resolution for simultaneous estimation
- The method was validated for the ICH parameters such as specificity, accuracy for which percent recovery for both the standards were obtained in 98%-100%, precision for %RSD was obtained less than 2%, limit of detection for Quercetin and Ferulic acid was obtained 18.96µg and 76.27µg respectively and limit of quantitation for Quercetin and Ferulic acid was obtained 57.46µg and 231.13µg
- The quantitative estimation of phytochemicals in marketed formulation achieved and Quercetin and Ferulic acid found 11.88% and 6.9% respectively.

CONCLUSION

From the studies conducted it is clear that a easier, cost effective,simpler, accurate, precise and specific method could be developed and validated for various phytochemicals and thus quantitative estimation of these phytochemicals in marketed formulation

FUTURE SCOPE

- Quality by design approach can be use to optimize the method development
- Method for analytical quantitative estimation of phytochemicals in the marketed formulation could be achieved
- Such method can be developed for various phytochemicals commercialized to assess the purity

Such method once developed and validated on HPTLC can be used for routine assessment of phytochemicals and their formulation

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