Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 8
Open Access

Simultaneous Quantification of Luteolin and Curcumin in Herbal Supplements Using RP-HPLC for Addressing Nutritional Deficiencies.

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Cite this paper as: Dr. Karavadi Thejomoorthy, Deshpande Padmanabh Bhagwan, Sheela Shivaji Thorat, Dr. Mausami Chandrakantbhai Vaghela, Sneha B. Surkar, Junmoni Nath, Richa Saxena (2024), Simultaneous Quantification of Luteolin and Curcumin in Herbal Supplements Using RP-HPLC for Addressing Nutritional Deficiencies.. *Frontiers in Health Informatics*, *Vol.13, No.8*, 2385-2394

Abstract

Background: Medicinal plants are rich sources of bioactive compounds, primarily secondary metabolites, contributing to their therapeutic and nutritional properties. Moringa oleifera and Curcuma longa are widely recognised for their high nutritional value, with luteolin (a flavonoid) and curcumin (a polyphenol) as their primary active constituents. These compounds possess antioxidant and anti-inflammatory properties, effectively addressing nutritional deficiencies.

Objective: This study aimed to develop and validate a Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) method for simultaneously estimating luteolin and curcumin in herbal formulations used for nutritional supplementation.

Methods: Herbal tablet and granule formulations containing Moringa oleifera and Curcuma longa extracts were prepared. The RP-HPLC method was optimized and validated according to ICH guidelines, evaluating parameters such as precision, accuracy, linearity, and robustness. The formulations' physicochemical parameters were also assessed.

Results: The developed RP-HPLC method demonstrated excellent precision, accuracy, and linearity for simultaneously estimating luteolin and curcumin. The herbal formulations exhibited acceptable physicochemical properties, and the presence of luteolin and curcumin in significant quantities was confirmed.

Conclusion: The validated RP-HPLC method provides a reliable and robust approach for the quality control and standardization of herbal formulations containing luteolin and curcumin. These formulations show potential as effective nutritional supplements, suitable for all age groups, supporting their use in addressing nutritional deficiencies.

.**Keywords:** Malnutrition, *Moringa oleifera*, *Curcuma longa*, RP-HPLC, Analytical method validation

Introduction

Nutritional deficiencies are a global health concern, leading to various chronic diseases and impaired physiological functions [1]. The increasing interest in natural and plant-based remedies has highlighted the importance of medicinal plants as a source of bioactive compounds with both therapeutic and nutritional benefits [2]. These plants owe their medicinal properties to secondary metabolites, such as flavonoids, polyphenols, and other phytochemicals, which exhibit antioxidant, anti-inflammatory, and immunomodulatory activities [3]. Moringa oleifera (commonly known as the "drumstick tree" or "miracle tree") and Curcuma longa (turmeric) are two widely studied medicinal plants recognized for their exceptional nutritional and medicinal properties [4]. Moringa oleifera is a rich source of vitamins, minerals, and antioxidants, with luteolin being one of its significant bioactive components [5]. Similarly, Curcuma longa contains curcumin, a polyphenolic compound with potent antioxidant and anti-inflammatory properties [6]. Combining these plants in herbal formulations offers a promising approach to combat nutritional deficiencies while promoting overall health [7]. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) is a widely used analytical technique for estimating bioactive compounds due to its accuracy, precision, and sensitivity [8]. However, the simultaneous quantification of luteolin and curcumin in complex herbal formulations remains challenging [9]. The present study focuses on developing and validating an RP-HPLC method for the simultaneous estimation of luteolin and curcumin in herbal formulations. This work aims to establish a standardized analytical approach for quality control and highlight these formulations' potential as effective nutritional supplements [10].

Material and Methods

Materials

The Moringa leaf powder was purchased from Shashwat herbals Pvt. Ltd. PVP K30, isopropyl alcohol, talc, and magnesium stearate were purchased from chemdyes ltd. Luteolin marker was given as gift sample by Dr. Mamta Shah (L M College of pharmacy) and curcumin was purchased from Dutt enterprise, Nadiad. Acetonitrile and Methanol (Merck) used of HPLC grade.

Methods

Two herbal formulations were developed. A herbal tablet from *Moringa oleifera* leaf powder and herbal granules from *Moringa oleifera* leaf powder, *Curcuma longa* powder Both the formulations were prepared by using 3^2 factorial design and optimized formula were selected for HPLC analysis.

Selection of Analytical wavelength

For selection of wavelength, working standards of Luteolin (10 µg/ml) and Betainine (10 µg/ml) were prepared using methanol as solvent. They were scanned in UV range of 200 nm – 800 nm and overlapped.

Instrumentation

The present RP-HPLC method was developed sing an Agilent Technologies Infinity 1200. Separation was achieved on HYPERSIL ODS $C1_8$ (250 mm x 4.6 mm) column at ambient temperature in isocratic mode with mixture of Acetonitrile: Methanol: 1.5% v/v acetic acid (45:30:25 v/v) at flow rate of 1ml/min.

2.3 Preparation of standard solutions

Preparation of standard working solution of Luteolin and Curcumin (For selection of analytical wavelength) For the preparation of standard stock solution of Luteolin, accurately weigh 10 mg of Luteolin in 100 ml volumetric flask and dilute it with methanol up to the mark (100 µg/ml). Take 1 ml from above solution and further dilute it with mobile phase in 10 ml volumetric flask (10µg/ml). In similar way 10 mg of Curcumin was weighed and diluted to 100 ml with methanol (100 µg/ml) and was further diluted with mobile phase to give final concentration of 10 µg/ml. Preparation of stock solution (For chromatographic development)

A stock solution of the mixture was prepared by diluting accurately weighed 10 mg each of Luteolin and Curcumin into 100 ml volumetric flask using methanol (100 µg/ml). 10 µl from above prepared solution was further diluted using mobile phase into 10 ml volumetric flask which contains final concentration of 100 ng/ml of Luteolin and Curcumin each.

Standard solution

Tablet: 1 prepared tablet was crushed and transferred into 10 ml volumetric flask and volume make up with

methanol. Solution was sonicated under heating conditions and filtered through 0.45 µm filter paper. Further, 1 ml from the filtrate was transferred to 10 ml volumetric flask and volume make up with mobile phase. The prepared final solution was injected in HPLC in developed chromatographic conditions.

Syrup: 5 ml prepared syrup was transferred into 10 ml volumetric flask and volume make up with methanol. Solution was sonicated under heating conditions and filtered through 0.45 µm filter paper. Further, 1 ml from the filtrate was transferred to 10 ml volumetric flask and volume make up with mobile phase. The prepared final solution was injected in HPLC in developed chromatographic conditions.

Statistical Analysis:

The samples were analyzed in triplicates for and results were averaged. Within day and between days, the accuracy samples were analysed six times and the results were averaged. The extracts from different parts of the plants were analyzed in triplicates and their results were presented as mean ± standard deviation (SD).

System Suitability Parameters

Solutions of Luteolin and Curcumin (Q+B, 100+100 µg/ml) was injected 3 times for determining System suitability parameters which includes Tailing factor (Tf), Retention time (Rt), Resolution (Rs) and number of theoretical plates. The proposed method was validated by performing linearity, accuracy, precision and limits of detection and quantitation according to ICH guidelines [13].

Analytical Method Validation

Further, the optimized method was validated according to ICHQ2R2 guidelines. The methodology was examined and found to be linear, repeatable, and accurate both between and within individual days. Using triplicate analysis, precision was determined by determining the variation that occurred within and between days. To establish the linearity of the standard solutions, we utilized all six possible concentration levels [14].

Linearity (Standard solutions)

For linearity study, exact weighed amount of L (10 mg), and C (10 mg) was transferred into the volumetric flask (10 ml) and volume was made up to 10 ml with methanol to provide stock solution contained 1000 µg/ml of L and C each. For further dilution, 1 ml from same solution was transferred to a 10 ml volumetric flask and volume was made up with mobile phase, which gave final stock solution containing 100 ng/ml of L and C each. Different aliquots from this stock solution were transferred to another 10 ml volumetric flask and volume was adjusted up to the mark with mobile phase to have desired solutions contained 100+100, 500+500, 2000+2000, 10,000+10,000 and 20,000+20,000 ng/ml of Luteolin and Curcumin respectively. All above solutions were injected at volume of 20 µl into column by employing optimized chromatographic conditions.

Repeatability

Standard working solutions of compounds containing L (100 to 20,000 ng/ml) and C (100 to 20,000 ng/ml) were injected into a column with a volume of 20 µl under optimized chromatographic conditions. Each standard mixture was injected five times, and the area of each peak was monitored and measured. Repeatability of each concentration was monitored using RSD.

Limit of Detection and Limit of Quantification

LOD and LOQ were determined using two methods: Visual inspection and Statistical method by utilization of repeatability data. Mean of slope and Standard deviation of response were utilized to calculate LOD and LOQ. LOD and LOQ were experimentally confirmed by dilutions of known concentrations of L and C until the average response was approximately LOD and LOQ were determined using a statistical method and repeatability data.

$$LOD = 3.3\ X\ (\frac{\sigma}{S}),\ LOQ = 10\ X\ (\frac{\sigma}{S})$$

Where, σ = Standard deviation of intercept

S = mean of slope

Precision

Precision refers to the degree to which the results of many measurements carried out on the same sample and under the same conditions provide results that are statistically indistinguishable from one another.

Mixture that represents overall range (L and C = 100+100, 2000+2000 and 20000+20000 ng/ml) were analyzed on same day at different time interval for intraday precision. Mixture that represents overall range (L and C = 100+100, 2000+2000 and 20000+20000 ng/ml) were analyzed on different days for inter-day precision.

Robustness

Parameters given below were changed one by one for determination of robustness of the method and the resulting effect was observed by comparing with the standard preparation.

i) Mobile phase flowrate (± 0.1 mL/min), optimized flowrate was 1.0 mL/min.

ii) Mobile phase composition (± 2 mL), in optimized ratio

3 determinations of Q+B = 2000+2000 ng/mL for each alteration were carried out, and RSD was measured [15-20].

Quantitative determination

Sample preparation for Granules

1 gm of prepared granules was crushed and transferred into a 10 ml volumetric flask, and volume makeup was done with methanol. Sonicate the solution under heating conditions and filter it through 0.45µm filter paper. Further, 1 ml of this filtrate was transferred to a 10 ml volumetric flask, and volume make-up was done with the mobile phase. This prepared solution was injected in HPLC under developed chromatographic conditions.

Sample preparation for Tablet

Procedure: 1 prepared tablet was crushed and transferred into 10 ml volumetric flask and volume make up was done with methanol. Sonicate the solution under heating condition and filter it through 0.45µm filter paper. Further, 1 ml from this filtrate was transferred to 10 ml volumetric flask and volume make up was done with mobile phase. This prepared solution was injected in HPLC with well -developed chromatographic conditions [21-25].

RESULTS

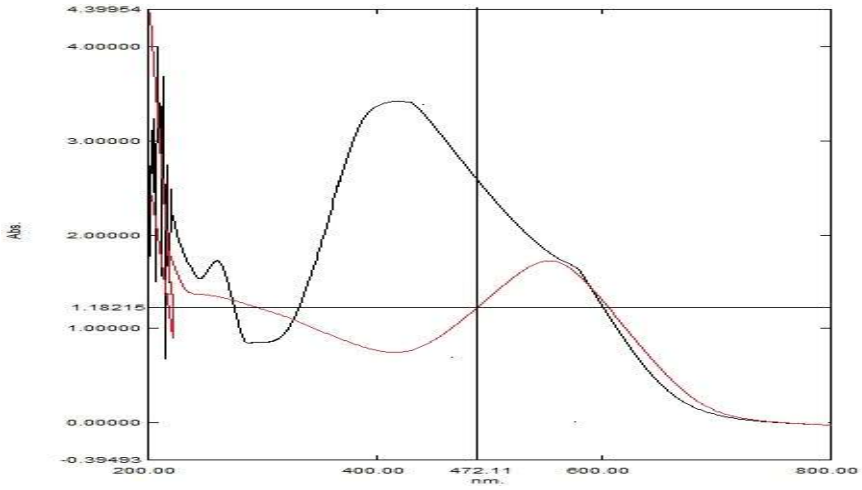


Figure 1: Selection of Analytical wavelength

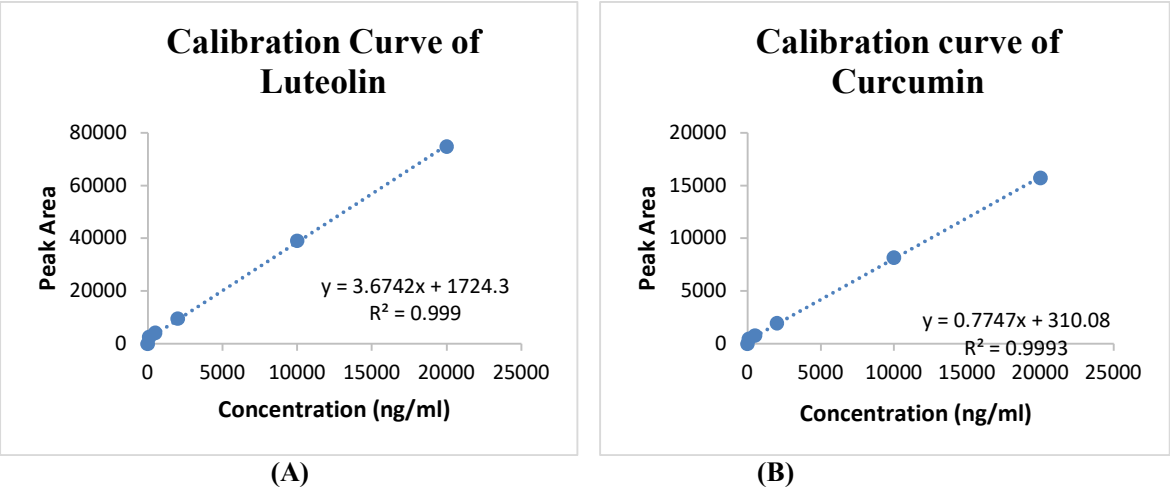
Table 1: Optimized Chromatographic Conditions

Parameters	Optimized condition
Stationary Phase	HYPERSIL ODS C_{18} (250 mm x 4.6 mm)

Mobile Phase (v/v)	Acetonitrile: Methanol: Acetic acid (45:30:25 v/v)
Flow rate (ml/min)	1 ml/min
Detection Wavelength(nm)	472 nm
Temperature	Ambient
Injection Volume (µL)	20 µL
Run time (minute)	20 minutes
Retention Time (minute)	L (2.8 min.) and C (7 min.)

Table 2: System suitability parameter for L+C (100+100 µg/ml)

Parameter	Luteolin			Curcumin		
	Mean	**± SD (n=3)**	**RSD**	**Mean**	**± SD (n=3)**	**RSD**
Retention time (R_t)	2.81	0.01	0.11	7.02	0.01	0.03
Tailing Factor	1.23	0.02	1.25	1.52	0.01	0.66
Number of theoretical plates	6286	95.799	1.52	4621	62.98	1.36
Resolution (R_s) (For both L and C)	7.23		0.08		1.15	

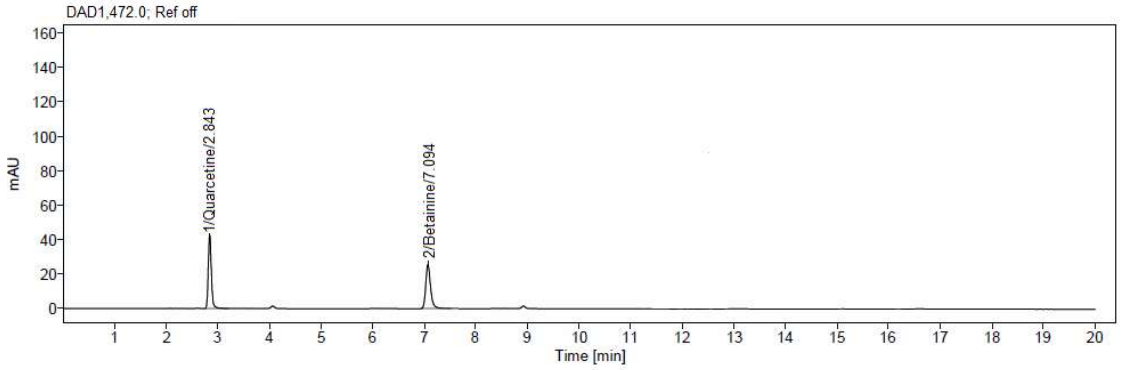


(A) **(B)**

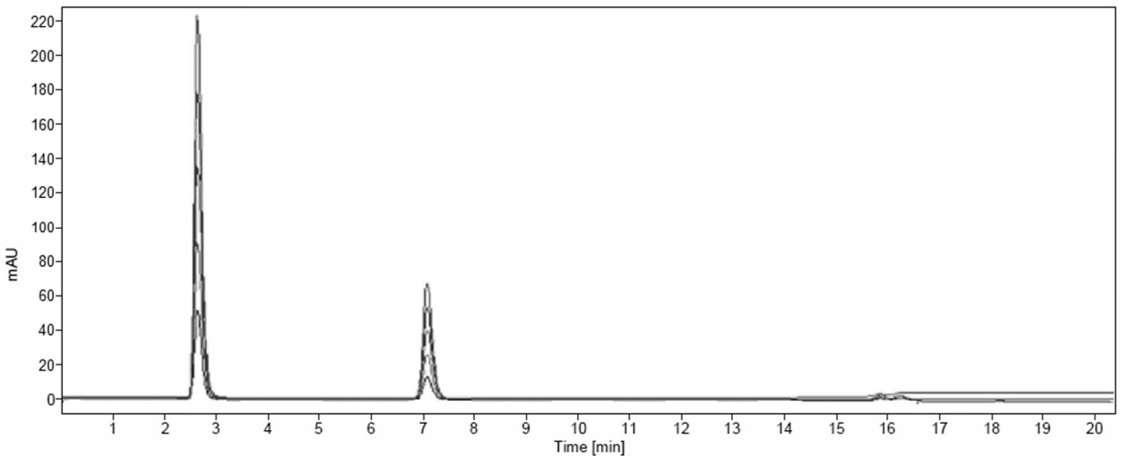
Figure 2 : (A) Regression analysis for Luteolin, (B) Regression analysis for Curcumin

Table 3: Summary of All Validation Parameters

Parameter	Limit	Result		Conclusion
		Luteolin	Curcumin	
Linearity and Range	$R^2 > 0.995$	0.999 (100-20000 ng/ml)	0.9993 (100-20000 ng/ml)	Method was linear
Repeatability	RSD < 2	0.71-1.02	0.72-1.14	Method was repeatable
Intraday Precision	RSD < 2	0.55-0.73	0.96-1.36	Method was precise
Inter-Day Precision	RSD < 2	0.65-1.10	1.11-1.62	Method was precise
Robustness	RSD < 2	0.26-0.63	0.23-0.73	Method was robust



(A)



(B)

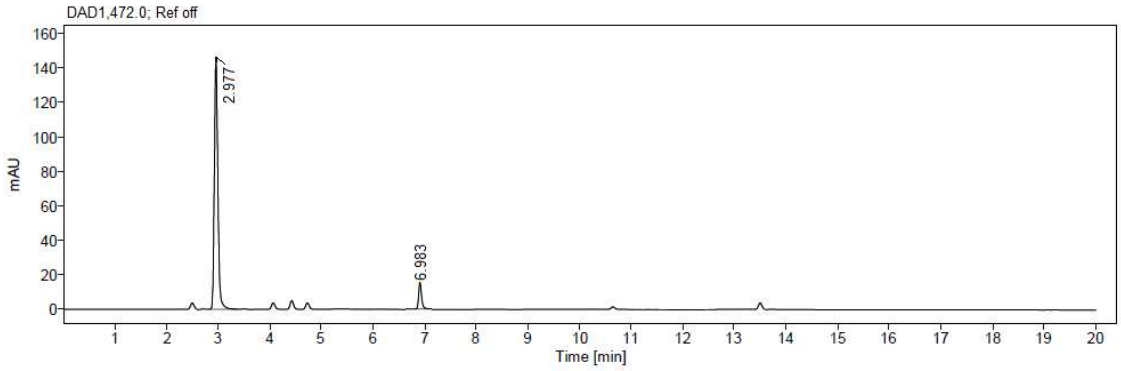
Figure 3: (A) Chromatogram of L+C (100+100 ng/ml), (B) Overlain chromatogram of L and C of Linearity study

Table 4: Limit of detection (LOD) and Limit of quantification (LOQ)

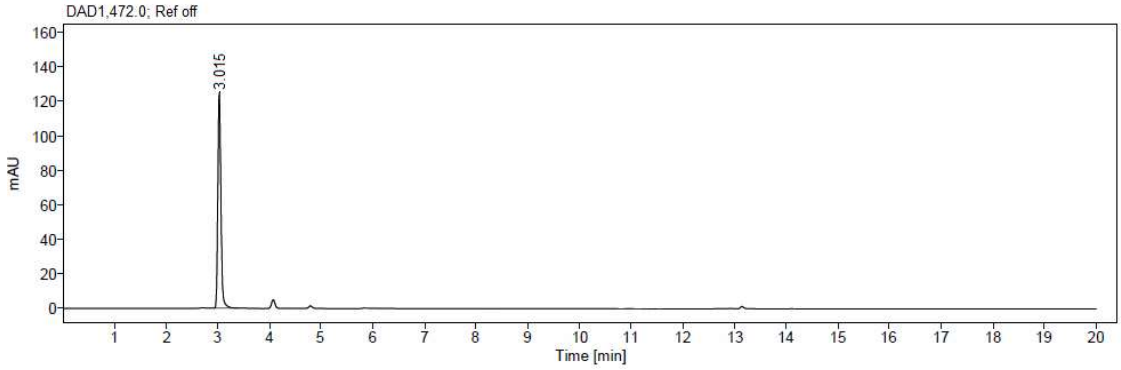
	Luteolin (µg/mL)	Curcumin (ng/mL)
LOD	32.35719	29.91388
LOQ	98.05209	90.64812

Estimation of L and C from herbal formulations

Quantity of L and C were determined from herbal formulations by using AUC and kept the value in equation derived from SCC. Total quantity of L and C found was 800 mg/mL and



(A)



(B)

Figure 5: (A) HPLC Chromatogram of Granules, (B) Chromatogram of Tablet Formulation

DISCUSSION

Selection of Analytical wavelength

After overlapping both the scanned spectra of L and C iso-absorptive point was found. The two iso-absorptive points are 400 nm and 550 nm. At 472 nm both the analytes showed significant absorbance, hence 472 nm was selected as detection wavelength for determination of L and C respectively **(Fig. 1)**.

Optimized Chromatographic conditions

Initial trial was performed using Methanol and Acetonitrile as mobile phase in proportion of 50:50 v/v but separation of each drug was not observed. So, acetic acid was introduced into the system with acetonitrile in 45 parts, methanol 30 parts and acetic acid 25 parts (v/v). Flow rate of system was 1ml/min and detection wavelength was selected 472 nm. Proper separation of L and C was observed using above chromatographic system so this system was kept as optimized chromatographic condition. Observed

Retention time for L and C was 2.8 min and 7 min respectively **(Table 1).**

System Suitability Parameters

After injecting selected solution of mixture 5 times, system suitability parameters showed high column efficiency with great no. of theoretical plates (>2000), Tailing factor for Luteolin was 1.23 ± 0.02 and for Curcumin was 1.52 ± 0.01 observed. Retention time for Luteolin was 2.81 ± 0.01 and for Curcumin was 7.02 ± 0.03 found. Relative Standard Deviation was calculated for each parameter. Calculated RSD was found less than one so we can say that the system is suitable **(Table 2)**.

Validation of Developed Method

The developed Reverse Phase HPLC method has been validated according to ICH-Q2R2 guidelines. The developed method was found to be linear in the concentration range of 100-20000 ng/mL for both Luteolin and Curcumin **(Fig. 3)**. After plotting calibration curve of peak area vs concentration for both drugs, the value of linear regression coefficient was 0.999 and 0.9993 for Luteolin and Curcumin respectively **(Fig. 3)**. Method was found to be repeatable over the range of 100-20000 ng/mL for both Luteolin and Curcumin with %RSD 0.71-1.02 and 0.72-1.14 respectively. When all the mixtures were analysed for all concentration, RSD for each concentration was calculated and it was found to be less than 2. Observed %RSD for intraday precision was 0.55-0.73 and 0.96-1.36 for L and C respectively. Observed %RSD for inter-day precision was 0.65-1.10 for Q and 1.11-1.62 for B. The method was found to be precise because the %RSD value was less than 2. The developed method was found to be robust because there were no major changes observed in the result when deliberate changes were employed into optimized mobile phase system. The % assay was not performed. Summary of all the validation parameters is highlighted in the Table 4.

Conclusion

The combination of Luteolin and Curcumin is innovative, unique and no analytical method available for the determination of same from single or combination dosage forms, therefor proposed method for determination of Luteolin and Curcumin from herbal dosage forms was developed and the developed method was validated as per ICH-Q2R2 guidelines. From the results we can say that developed method is Precise and follow all the ICH regulations. So proposed methodology can be employed to determine Luteolin and Curcumin from herbal formulation.

****Abbreviations**

L – Luteolin
C- Curcumin
LOD- Limit of Detection
LOQ- Limit of Quantification
RSD- Relative Standard Deviation

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