IJPSR (2023), Volume 14, Issue 2 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 09 June 2022; received in revised form, 26 December 2022; accepted, 20 January 2023; published 01 February 2023

SIMULTANEOUS DETERMINATION OF FLAVONOIDS IN POLYHERBAL FORMULATION BY A VALIDATED HPTLC METHOD

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Keywords:

Thin layer chromatography, Method validation, Rutin, Quercetin, Kaempferol, Myricetin, ICH

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ABSTRACT: A rapid, sensitive, selective and robust quantitative densitometric high-performance thin-layer chromatographic method is developed and validated for separation and quantification of flavonoids namely rutin, quercetin, kaempferol and myricetin in herbal formulations. The separation is performed on silica gel $60F_{254}$ high-performance thin-layer chromatography plates using phase cyclohexane: ethyl acetate: formic acid 12:9:0.4 (v/v/v) as the mobile phase. A precise and accurate quantification is performed using linear regression analysis by plotting the peak area *vs* concentration in range of 200–1000 ng/band with regression coefficient, R^2 from 0.997 to 0.998 for selected flavonoids. The developed method is validated in terms of accuracy, recovery and inter- and intraday study as per International Conference on Harmonisation (ICH) guidelines. The method is found sensitive, accurate and reproducible. Therefore, it can be recommended for marker-based standardization and quality assurance. The present research work is an attempt for simultaneous quantification of rutin, quercetin, kaempferol and myricetin in a single mobile phase. The validated HPTLC method can be further applied to evaluate the quality of any plant reported to possess these phytochemicals.

INTRODUCTION: Thin-layer chromatography (TLC) is widely used technique for the primary fingerprint determination of polyherbal formulations which may contain mixtures of various phytochemicals [1, 2]. For the purpose of identification HPTLC is a commonly used technology for identifying and studying the stability of herbal raw ingredients and formulations.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.14(2).876-82

This article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.14(2).876-82

Due to minimal solvent use, cost-effectiveness, and reduced analysis time, the HPTLC method is currently developing as a viable alternative to HPLC [3]. Numerous therapeutic benefits of flavonoids have been linked to the antioxidant activity, which is mostly related to their chemical structure and lowering requirements [4].

Throughout human history, herbal remedies have been used in medical treatments. The recent worldwide upsurge in the usage of herbal medicines has resulted in a boom in the number of herbal medication makers. Because herbal medications are typically polyherbal, determining their chemical stability and purity can be difficult [4]. Herbal medicine manufacturers face numerous challenges, including a lack of readily available,

high-quality raw materials and reference standards, as well as proper standardization methods for single drugs and formulations, quality control parameters and well-established guidelines for drug and formulation standardization. The evaluation of a final product's chemical stability during storage is an essential aspect of quality control. Quality control is the most crucial factor in justifying the approval of crude pharmaceuticals and herbal formulations in modern medical systems [5].

Since their discovery, many groups of flavonoids have demonstrated a variety of important biological effects, including anti-inflammatory, anti-cancer, anti-bacterial, anti-fungal, anti-diabetic and anti-malarial properties [6-9]. These phytoconstituents, along with several other polyphenols have been thoroughly investigated for their medicinal potential during the past few decades [10]. It is carefully determined for their existence, identification and quantification in the various phyto extracts by HPTLC or HPLC. Common flavonoids like rutin, quercetin and kaempferol are also key marker components in this class [11, 12]. There is no report on the simultaneous quantitative measurement of rutin, quercetin, kaempferol and myricetin using HPTLC technique, to the best of our knowledge and literature search. The main goal of this paper is to develop and validate a simple, precise, selective and repeatable HPTLC technique for simultaneous identification and quantification of rutin, quercetin, kaempferol and myricetin in a variety of commercial polyherbal formulations. In terms of accuracy, reproducibility and recovery, the developed method is validated using ICH [13] guidelines.

MATERIALS AND METHODS:

Chemicals: All standard flavonoids were purchased from Sigma Aldrich, USA with 98% purity. Solvents used in this study are Methanol, cyclohexane, ethyl acetate and formic acid (all AR grade) purchased from SRL Chemicals, India. The marketed polyherbal formulations used in this study were in tablet and capsule forms were purchased from local pharmacies of Gujarat and from the online sources.

Preparation of Standard Solutions: 10 mg of rutin, quercetin, kaempferol and myricetin were accurately weighed and transferred to 10 ml standard volumetric flasks, where they were dissolved in methanol to make a stock solution of 1 mg/ml. Starting with the stock solution 1 ml of each solution was pipetted out, transferred to a 10 ml standard flask and the volume was filled up with methanol to achieve 0.1 mg/ml.

TLC Instrumentation and Conditions: Chromatography was carried out on methanol-prewashed aluminium-backed silica gel 60 F_{254} plates on10 cm × 10 cm (E. Merck). Using a CAMAG (Switzerland) Linomat V sample applicator with a 100- µL syringe, samples were applied to the plates in bands 6 mm wide and 5 mm apart (Hamilton, Bonaduz, Switzerland).After saturation of the chamber with mobile phase vapour for 10 minutes, linear ascending development was done in a twin trough glass chamber (CAMAG) using Cyclohexane: Ethyl acetate: Formic acid (12:9:0.4 v/v/v) as the mobile phase. Densitometric scanning was used to collect all measurements with a CAMAG TLC Scanner III at 254 nm. The scanner was ran using Win CATS software.

Preparation of Calibration Curves: For calibration curves, five different concentration solutions were freshly prepared ranging from 200-1000 ng/ spot of all four reference compounds (rutin, quercetin, kaempferol and myricetin). Using the Linomat V sample applicator and a 100-µL syringe, these solutions were applied as bands on TLC plates. To establish the association, the study was repeated three times with the mean peak area responses to the concentrations recorded at 254 nm. **Table 1** shows the values for the regression parameters. For rutin, quercetin, kaempferol and myricetin, calibration plots of peak area versus concentration were established individually.

Analysis of Marketed Polyherbal Formulations: The average weight of ten marketed polyherbal formulation tablets was calculated. A quantity of the powder equal to the average weight of one tablet or capsule was precisely weighed and dissolved in 50 mL methanol. The shell of the capsule was removed after dissolving in methanol. In the sample F-5, a second dilution was performed by taking 1ml of the stock solution (as described before) and dissolving it in 10 ml of the same solvent. The samples were then sonicated for 20

minutes before being filtered through a 0.45 m PVDF filter membrane; the filtrate was utilized as the sample solution. The sample solutions were applied on a TLC plate and developed under the parameters stated. Peak areas were measured after scanning the produced plate at 254 nm. Each component in each formulation was quantified using a multilevel calibration established on the same plate under the same circumstances using a linear regression equation.

RESULTS AND DISCUSSION:

Optimization of Procedures: Pure flavonoids were dissolved in methanol and used as a standard solution. For the simultaneous analysis of rutin, quercetin, kaempferol and myricetin, the prepared solution was scanned in the UV-region between 200 and 400 nm, using 254 nm as the detecting wavelength. By trial and error, several compositions including varied ratios of solvents depending on the polarity and solubility of components were used to select and optimize the mobile phase. The initial step was to use some of the emerging solvent systems that had been used in previous investigations to separate single chemicals and other flavonoids [14-19].

To avoid peak fronting on silica gel plates, mixtures of pure solvents, such as ethyl acetate, chloroform, ethyl methyl ketone, methanol, ethanol, propanol, butanol, cyclohexane, toluene, water and others, were combined with formic acid or acetic acid. To get the simultaneous separation of all the phytochemicals, all of these distinct solvent combinations as well as varied ratios were tested.

Various trials taken to select suitable mobile phase are given below.

Chloroform: Ethyl acetate: Methanol (9:2:1 v/v/v) mixture tried as mobile phase gave separation of only two components. Ethyl Acetate: Toluene: Formic acid: Water (5: 1: 1: 1.5 v/v/v/v) gave separation of all four components, but two components having same peak height.

Toluene: Methanol: Butanol: Ethyl acetate (7.5: 2.5: 1: 1.5 v/v/v/v) mobile phase gave separation of only three components.

Ethyl acetate: Glacial acetic acid: Formic acid: Water (10: 1: 1: 1:2.5 v/v/v/v) showed the separation of three components.

Out of all mobile phases tried, combination of cyclohexane, ethyl acetate and formic acid gave the best separation for rutin, quercetin, kaempferol and myricetin with good peak shape. For simultaneous good separation of all for components different ratios of cyclohexane: ethyl acetate: formic acid (10: 8: 2, 8:11: 1, 15: 5: 1.5, 6: 6: 3, 12: 9: 0.4 v/v/v) were tried. It is found that 12: 9: 0.4 v/v/v ratio gave good separation. The chromatogram for this showing separation of rutin, quercetin, kaempferol and myricetin is shown in **Fig. 1.**

Identification of Peaks: After the separation of four flavonoids with the developed method, the identification of each peak was carried out using the same chromatographic conditions for each standard flavonoid namely rutin, quercetin, kaempferol and myricetin. The results for each of them are given in Supporting **Fig. 1.**

Validation: The developed method was validated in accordance with the recommendations of the International Conference on Harmonization (ICH) [13].

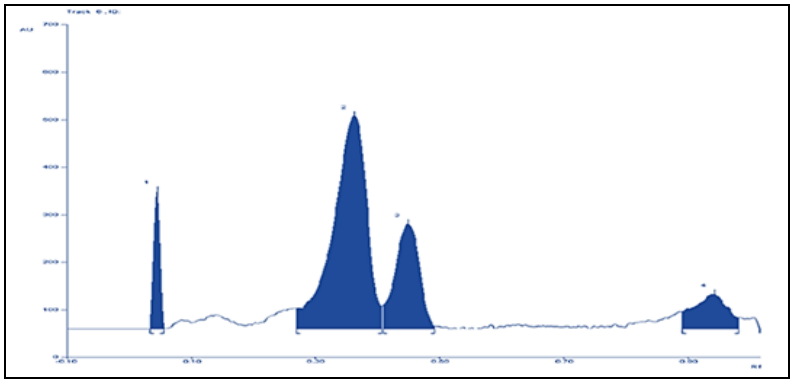


FIG. 1: DENSITOGRAM SHOWING SEPARATION OF STANDARDS PEAK 1 AS RUTIN, PEAK 2 AS QUERCETIN, PEAK 3 AS KAEMPFEROL AND PEAK 4 AS MYRICETIN AT 254 NM

Linearity: Beer's law governs the linearity of all substances at all five concentrations. The results demonstrate a strong relationship between peak area and concentrations, values of correlation coefficients are R^2 = 0.997, 0.998, 0.998 and 0.997 for rutin, quercetin, kaempferol and myricetin respectively. The values of regression parameters are given in **Table 1**. Calibration curves for rutin, quercetin, kaempferol and myricetin are given in Supporting information **Fig. 2.**

TABLE 1: CHARACTERISTIC PARAMETERS FOR THE REGRESSION EQUATIONS OF FOUR FLAVONOIDS

Compound name	Linearity range ng. spot^{-1}	Calibration equation y = a = bx	Correlation coefficient R^2
Rutin	200-1000	y=6.06x-628.7	0.997
Quercetin	200-1000	y=8.25x-566.1	0.998
Kaempferol	200-1000	y=5.05x-533.1	0.998
Myricetin	200-1000	y=1.83-80.07	0.997

LOD and LOQ: The detection limit (LOD) and quantification limit (LOQ) were derived using the equations below.

$$LOD = 3.3 \times \sigma/slope$$

$$LOQ = 10 \times \sigma/slope,$$

Where, σ = Standard Deviation, Slope = Slope of the calibration curve.

LODs for rutin, quercetin, kaempferol and myricetin were 0.236, 0.460, 0.265 and 0.548 ng/spot respectively and LOQs were 0.718, 0.755, 0.802 and 1.659 ng/spot respectively.

Precision:

Method Precision: Instrumental, intraday, and interday accuracy were used to confirm the method's repeatability. Instrumental accuracy was determined by spotting each solution six times. The relative standard deviation (RSD) of peak area for the all four flavonoids was used for measurement of repeatability. For calculating repeatability 600 ng/spot solutions of every standard solution was used. By using the peak area % RSD was calculated and the values of it are shown in Table 2.Intraday relative standard deviation of rutin was between 2.21% to 2.55%, quercetin was 2.25% to 2.82%, kaempferol was 2.28% to 2.72% and for myricetin was 2.32% to 2.49%. While inter day relative standard deviation of rutin was between 3.02% to 3.60%, quercetin was 3.20% to 3.79%, kaempferol was 3.10% to 3.70% and for myricetin was 3.32% to 3.74%.

TABLE 2: REPEATABILITY AND PRECISION DATA OF FLAVONOIDS (RUTIN, QUERCETIN, KAEMPFEROL AND MYRICETIN)

Compound	Repeatability %RSD (n=6)	Precision % RSD (n=5)						
		Intraday				Interday		
		Conc. ng. spot^{-1}	0 hours	3 hours	6 hours	Day 1	Day 3	Day 5
Rutin	1.55	200	2.21	2.25	2.31	3.02	3.08	3.12
		400	2.48	2.55	2.61	3.22	3.26	3.33
		800	2.42	2.50	2.56	3.43	3.51	3.60
Quercetin	1.75	200	2.25	2.29	2.35	3.47	3.54	3.59
		400	2.70	2.76	2.82	3.20	3.26	3.30
		800	2.52	2.58	2.62	3.67	3.73	3.79
Kaempferol	1.69	200	2.28	2.35	2.39	3.10	3.14	3.19
		400	2.64	2.69	2.72	3.57	3.65	3.70
		800	2.50	2.46	2.52	3.32	3.36	3.41
Myricetin	1.27	200	2.37	2.43	2.48	3.68	3.71	3.74
		400	2.35	2.41	2.49	3.65	3.69	3.73
		800	2.29	2.32	2.39	3.32	3.38	3.41

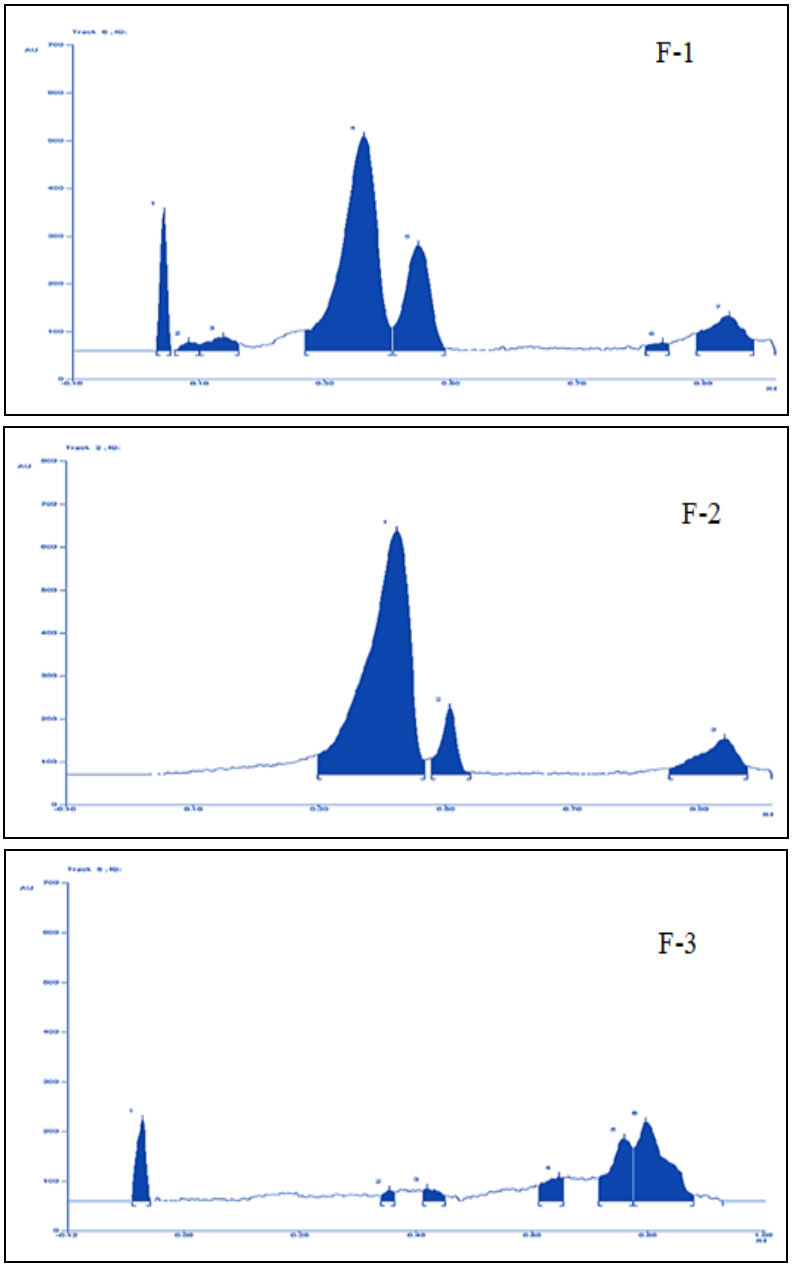
Accuracy and Recovery: Recovery studies were performed at three distinct levels (law, medium, and high) using the standard addition method to determine the accuracy. Known quantities of rutin, quercetin, kaempferol and myricetin standard (160, 200 and 240 ng/spot) were added to the solution of all formulations by spiking on the same plate in triplicate. Results for recovery of the flavonoids are showed in Supporting information **Table 1.**

Application to Marketed Formulations: The developed HPTLC procedure was applied for the

assay of four flavonoids in commercial poly herbal formulations in different tablets and capsules. Five different formulations were spotted in triplicate on TLC plate and developed using the chromatographic conditions as given above. There were no interference peaks in any of the inactive components or the dosage form matrix. HPTLC profile of all five formulations is given in **Fig. 2**. The peaks in the densitogram corresponding to rutin, quercetin, kaempferol and myricetin were identified by comparing their Rf and UV spectra to those of reference substances, as well as spiking studies. The calibration graphs were used to determine the quantities of rutin, quercetin, kaempferol and myricetin and the findings are listed in **Table 3.**

TABLE 3: ASSAY OF FORMULATIONS

Sample	Amount of flavonoids found in formulations (mg)			
	Rutin	**Quercetin**	**Kaempferol**	**Myricetin**
F-1	0.029	0.036	0.025	0.022
F-2	---	0.041	0.024	0.024
F-3	0.046	0.016	0.021	0.044
F-4	0.056	---	---	0.038
F-5	0.020	0.038	0.029	0.031



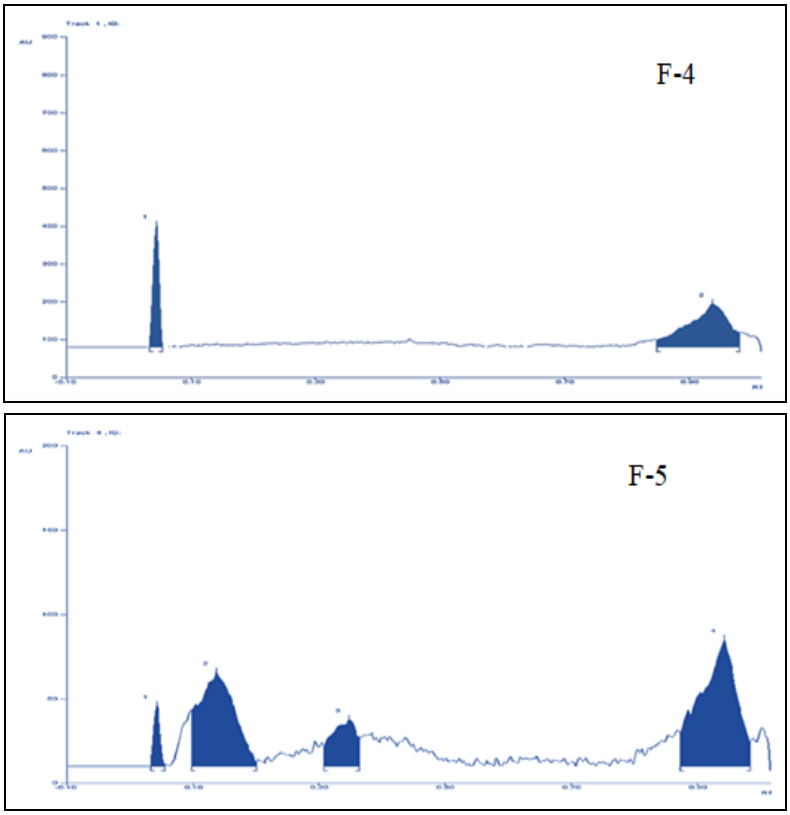


FIG. 2: DENSITOGRAM OF F-1, F-2, F-3, F-4 AND F-5 USING DEVELOPED METHOD

The quantity contained in the marketed formulations is in varying amounts, according to the estimation of four flavonoids using the developed HPTLC technique. The amount of rutin varies from 0.020 to 0.056 mg per tablet/capsule, amount of quercetin ranges from 0.016 to 0.041 mg per tablet/capsule, amount of kaempferol ranges from 0.021 to 0.029 mg per tablet/capsule and the amount of myricetin ranges from 0.022 to 0.044 mg per tablet/capsule.

CONCLUSION: From the **Table 4** and HPTLC profile of all marketed herbal formulations F-1, F-2, F-3, F-4 and F-5, it can be concluded that the amount of every component is different in the formulations. In our study some of components are absent in some formulations as well some were present in very less amount. In the formulations F-1, F-3 and F-5 all four flavonoids are present but, in formulation F-2 rutin is absent and in the formulation F-4 quercetin and kaempferol are absent. The resulting component fingerprint pattern may be utilized to determine not only the presence or absence of pharmacologically active substances of interest, but also the ratio of all detected analytes. It's also been utilized in the quantitative and qualitative examination of adulterants in herbal medications, if any are present. The HPTLC method that has been developed is accurate, specific, and precise. The technique is simple and quick and can be used in routine quality control analysis while also consuming less solvent.

ACKNOWLEDGEMENT: Nil

CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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How to cite this article:
Thakkar M and Sharma S: Simultaneous determination of flavonoids in polyherbal formulation by a validated HPTLC method. Int J Pharm Sci & Res 2023; 14(2): 876-82. doi: 10.13040/IJPSR.0975-8232.14(2).876-82.