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Research Article

Novel Stability Indicating Analytical Method Development and Validation for The Estimation of Canniflavin In Bulk By Uplc

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ABSTRACT
A novel stability indicating RP-UPLC method was developed for the estimation of Canniflavin in bulk. Canniflavin is a chemical compound found in *Cannabis Sativa Linn.* Chemically, this is a prenyl flavanoid and unrelated to THC and other Cannabinoids. Canniflavin comes under the category of anti-inflammatory agent and inhibitor of prostaglandin E2 production, it shows the inflammatory benefits 30 times more effective than aspirin. Canniflavone is employed as internal standard for canniflavin analysis. The analysis was carried out using Hibar C18 column
(100×2.1mm, 2µ) column. The optimized chromatographic column was achieved at 0.3ml/min. Flow rate using with KH_2PO_4 and Acetonitrile as a mobile phase in the ratio of 55:45.
Temperature was maintained at 30℃. Optimized wavelength selected was 275.0nm. The developed and validated method showed the Retention times 1.191min and 1.941min for
Canniflavone, canniflavin respectively. The proposed method was fully validated in terms of Linearity, LOD, LOQ, Precision, Stability and recovery. All calibration curves showed a good linear relationship (r^2 ≥0.999). The precision evaluated by an intra-and inter day study showed % RSD 0.5% for Canniflavin. By using Standard addition method the % recovery was obtained as 100.1% for Canniflavin. LOD, LOQ values obtained from regression equation of Canniflavin were 0.311, 0.943. Regression equation of Canniflavin is y=0.0092 × +0.014. This method demonstrated excellent reliability and sensitivity. Stability indicating capability of the proposed method was verified by forced degradation experiments which includes (Acid, Base, Peroxide, Thermal, Water, Photolytic conditions) which were under the specification limits. Hence the suggested method is simple, Precise, rapid, sustainable and robust. Therefore it can be reliably utilized in regular quality control tests in industries and also in herbal formulations.

Keywords: RP-UPLC, Canniflavin, Canniflavone, Standard addition method and Stability indicating

INTRODUCTION

Phytochemicals are responsible for color, aroma and flavor and utilization of these phytochemicals commonly furnishes several health benefits, such as anti-inflammatory, anti-oxidant, anti-cancer and other chronic diseases [1-3]. Normally, phytochemicals are categorised into six vital divisions depending upon their chemical structures and characteristics. These devisions comprises of carbohydrates, lipids, phenolics, terpeniods, alkaloids and other nitrogen containing compounds. Canniflavin is a prenyl flavonoid which is categorized under the flavoinoids. These flavoinoids comes under phenolic compounds [4]. Cannflavin is obtained from the plant source *Cannabis sativa Linn* belonging to the family *Cannabaceae*. The IUPAC name of canniflavin is 5, 7-Dihydroxy-2-(4-hydroxy-3-methyl but-2-enyl) chromen-4-one. The structure of canniflavin given in (Fig.1) [5]. Canniflavin has been studied for the potential use as anti-inflammatory activity. The anti-inflammatory property of canniflavin is 30 times more powerful than that of aspirin. This canniflavin is an inhibitor of prostaglandin E2 (PGE2).
Canniflavin has a wide variety of pharmacological activities, such as Neuroprotective, Antioxidant, Anti-viral, Neo plastic, antiviral and anticancer activities [6]. Canniflavone is an internal standard, it can be used for the Canniflavin analysis by UPLC. The synonym of canniflavone is Canniflavin-B and 6- prenyl chrysoeriol. The selection of internal standard based on two criterias like pKa and isotopes similar to that of Canniflavin [7]. As per literature survey there are few methods developed for this Canniflavin such as HPLC, UV, and NMR [816]. But there is no UPLC

method developed for the analytical determination of this Canniflavin.
Hence UPLC method selected due to high specificity, selectivity, sensitivity, robustness, economical prospective scenario and low mobile phase consumption when compared to HPLC.

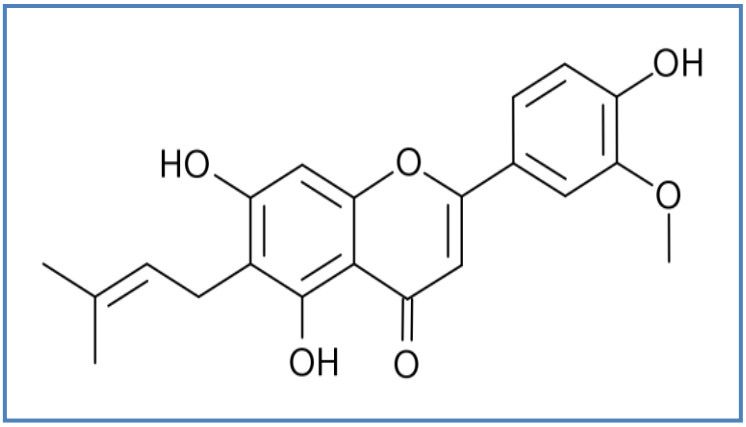


Fig.1.Structure of Canniflavin

MATERIAL AND METHODS

Canniflavin pure drug (API), Canniflavin Natural product, Distilled water, Acetonitrile,
Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-Phosphoric acid. All the above chemicals and solvents are from Rankem. WATERS ACQUITY UPLC SYSTEM equipped with Binary pumps, TUV detector and Auto sampler integrated with
Empower 2 Software.UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Canniflavin solution.

Diluent

Based up on the solubility of the drugs, diluent was selected, Methanol and Water taken in the ratio of 50:50

Preparation of Canniflavin stock solutions

Accurately weighed 25mg of Canniflavin is transferred to 50ml volumetric flask. 3/4 th of diluents was added to the flask and sonicated for 10 minutes. Flask was made up with diluent.
From the above solution, 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent.

Preparation of Internal standard stock solution

Accurately weighed 50 mg of canniflavone is transferred to 50 ml volumetric flask .3/4 th of diluents was added to the flask and sonicated for 10 min. Flask was made up with diluent.
From the above solution, 1 ml from each stock solution was pipette out and taken into a 10 ml volumetric flask and made up with diluent.

Preparation of buffer

0.01N KH_2PO_4 Buffer

Accurately weighed 1.36gm of potassium dihydrogen phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 4.0 with dil. Ortho phosphoric acid.

0.1%OPA Buffer

1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water.

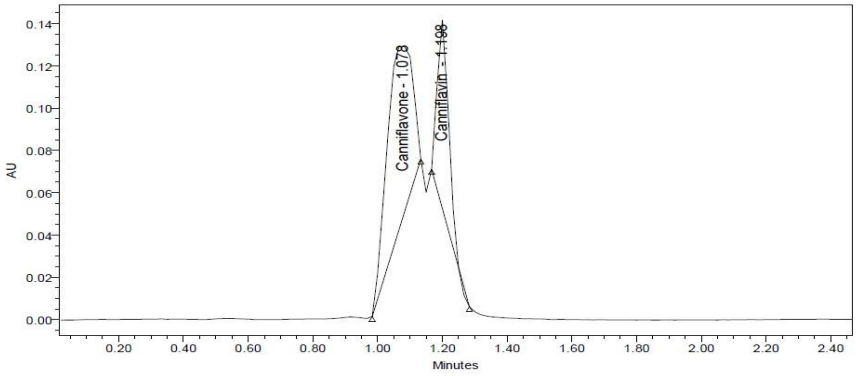
Analytical method development

The first step in the chromatographic approach is to define the objectives of the method. The main objective of the current method was to develop a simple, accurate, precise, robust and specific UPLC method for the estimation of Canniflavin in bulk.
Commencing of the process include the variations in the chromatographic parameters like mobile phase composition and column. Initially the peaks obtained were not that much to the desired level. Finally the mobile phase composition of Acetonitrile: 0.01NKH2PO4 (55:45V/V) with the column Hibar C_{18} (2.1×100mm, 2μm) produced the peaks with good resolution. Peak shape,
USP plate count, USP tailing are found to be within the limits. Therefore this trail is taken as optimized chromatogram. The chromatograms are shown in Fig.2 and Chromatographic
conditions are given in Table 1

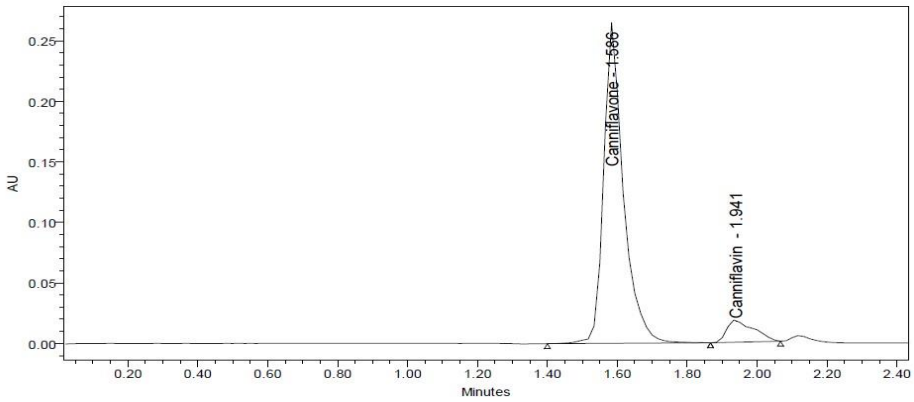
Table 1: Chromatographic conditions

Column	Hibar C_{18}(2.1×100mm,2μm)
Mobile Phase	Acetonitrile:0.01NKH_2PO_4
Flow Rate	0.3ml/min
Detector Wave length	275nm
Injection volume	1μL
Column Temperature	30°C
Run Time	5min
Diluents	Water and methanol (50:50)

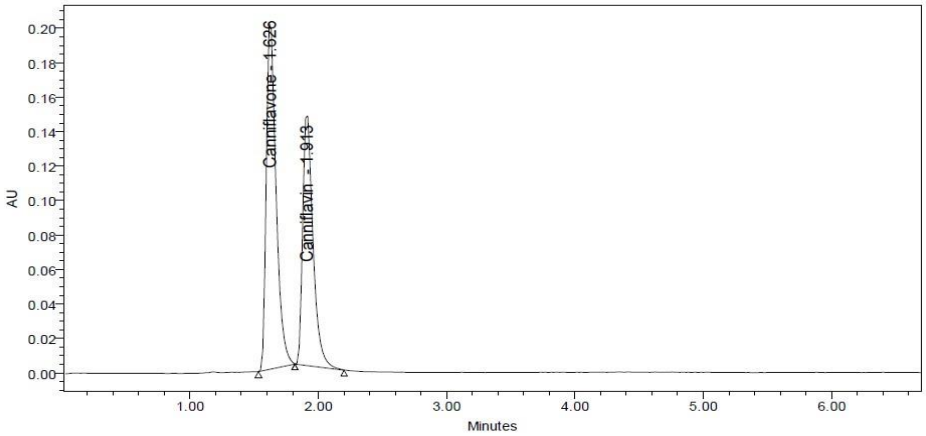
Trail-1



Trail-2



Trail-3



Optimized chromatogram

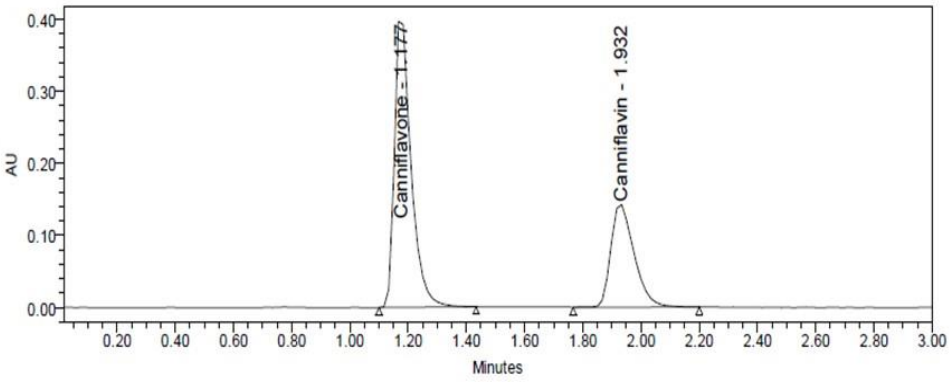


Fig.2 Trails and Optimized chromatogram

Method Validation

System suitability

The performance parameters of the developed UPLC method were determined by standard working solutions (50ppm). Analytical parameters such as number of theoretical plates (N), capacity factor(K), Tailing factor and Resolution were calculated from the obtained from the optimized chromatogram. In addition %RSD of the peak areas of 6 consecutive injections of Canniflavin. The results were given in the Table 2

Table 2: Data for System suitability parameters

S no	Canniflavone(ISD)			Canniflavin			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	RS
1	1.181	2295	1.41	1.915	2765	1.35	5.8
2	1.181	2229	1.42	1.925	2793	1.36	5.9
3	1.185	2377	1.39	1.926	2754	1.37	5.9
4	1.185	2261	1.40	1.936	2692	1.36	6.0
5	1.185	2296	1.42	1.942	2801	1.37	6.1
6	1.186	2295	1.40	1.942	2776	1.37	6.1

Linearity

The peak area response of Canniflavin was linear over the concentration range of 12.5 - 75μg/ml plotting of graph was done by keeping concentration in μg/ml on x-axis and the responses(area) on y-axis from this correlation coefficient, slope, intercept was determined. The results of linear regression was given by the following equation y = 0.0092x + 0.014. The correlation coefficient was obtained as 0.999 for Canniflavin. The calibration curve given in the Fig.4

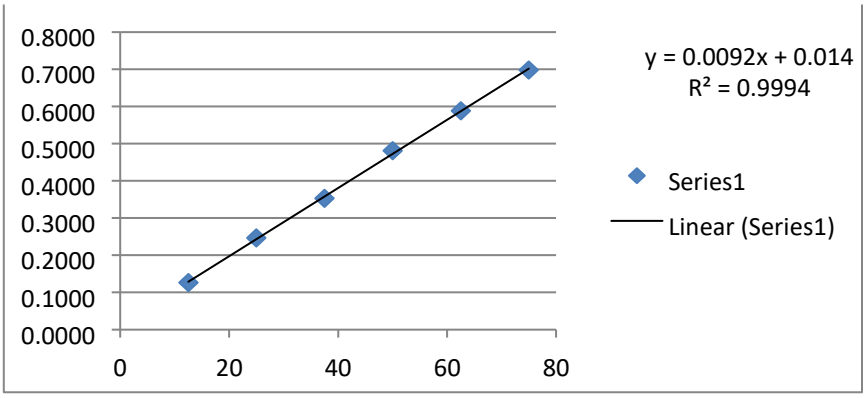
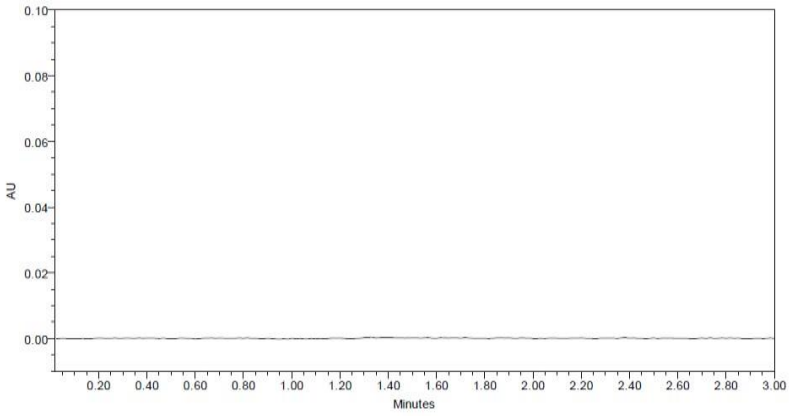


Fig. 4 Linearity calibration curve

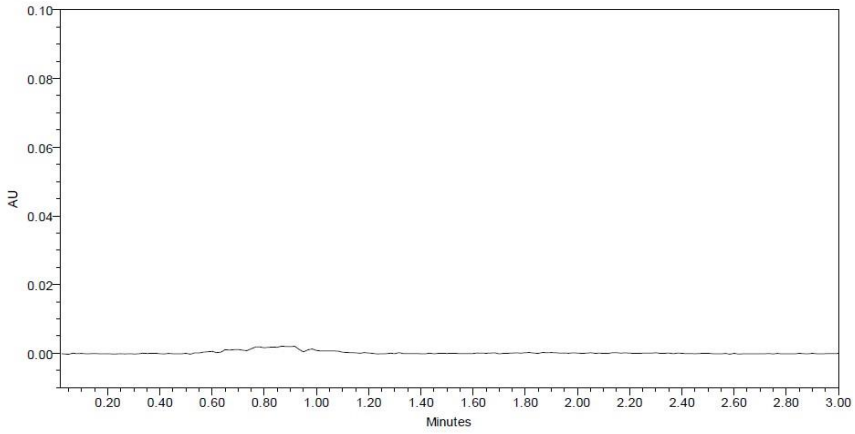
Specificity

It gives information about the interfering compounds present in the sample component. In this, first we inject the blank followed by placebo for checking the interference in the optimized method, the results or specificity were given below in Fig.

Chromatogram of Blank



Chromatogram of Placebo



Optimized chromatogram

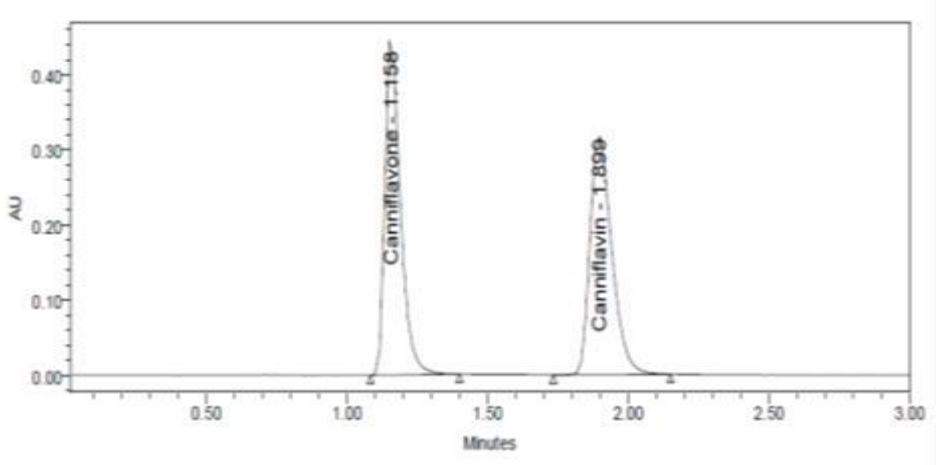


Fig 5: Specificity of Blank, Placebo, Optimized chromatograms

Accuracy

Accuracy studies were carried out to know about recovery level of Canniflavin by spiking method. The recovery study was done for Canniflavin in the range of 50-150 of the initial

Table 3: Data for Accuracy Studies

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean %Recovery
50%	25	24.8928218	99.57	100.1%
	25	24.93916	99.76	
	25	25.0137478	100.05	
100%	50	50.0513047	100.10	
	50	50.2274307	100.45	
	50	50.4163032	100.83	
150%	75	75.5205724	100.69	
	75	74.5093109	99.35	
	75	74.9027192	99.87	

concentration. Solutions were injected in triplicate and the method analysis was done. The % recovery, % mean recovery and %RSD were calculated for all the levels. Results were obtain within the limits as shown in Table 3

Precision

Precision studies are done at system precision, Repeatability precision and intermediate precision levels. The %RSD result of all studies were found to be within the specified limits as shown below in Table 4

Table 4: Data for Precision Studies

S. No	System Precision		Repeatability Precision		Intermediate Precision	
	Area of Canniflavin	Area of Caanniflavone	Area of Canniflavin	Area of Caanniflavone	Area of Canniflavin	Area of Caanniflavone
1	780464	1648193	786699	1640407	773915	165587
2	779423	1636644	780692	1673481	764937	1623064
3	776809	1644553	785756	1656160	772683	1646062
4	780888	1638610	778139	1662082	769296	1655046
5	784917	1645650	785427	1672041	762721	1657954
6	774443	1651644	789698	1656013	764134	1626320
Mean	779491	1644216	784402	1660031	767948	1644006
SD	3606.1	5690.8	4223.7	12211.3	4706. 9	155315
%RSD	0.5	0.3	0.5	0.7	0.6	0.9

Sensitivity Studies

Limit of Detection

LOD: Lowest amount of analyte in a sample that can be detected. It can be estimated by Signal to noise ratio and the chromatogarm is shown in Fig 6

LOD = 3.3 × σ/slope, where σ=standard deviation

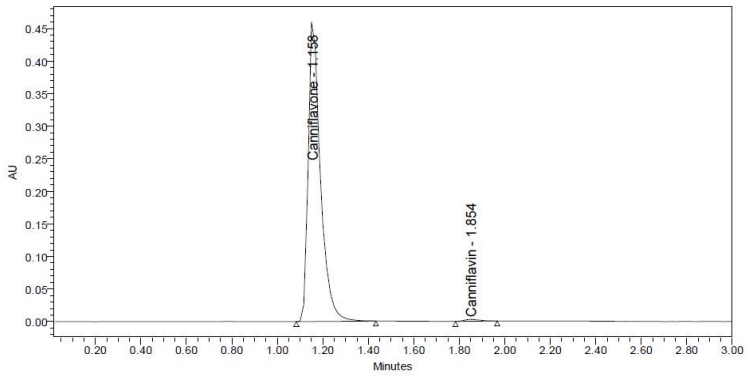


Fig 6: LOD Chromatogram of Standard

Limit of Quantification

Lowest amount of analyte in a sample that can be quantified. It can be estimated by Signal to noise ratio the Chromatogarm is shown in Fig 7

LOD = 10 × σ/slope, where σ=standard deviation
The results of LOD & LOQ were given in Table 5

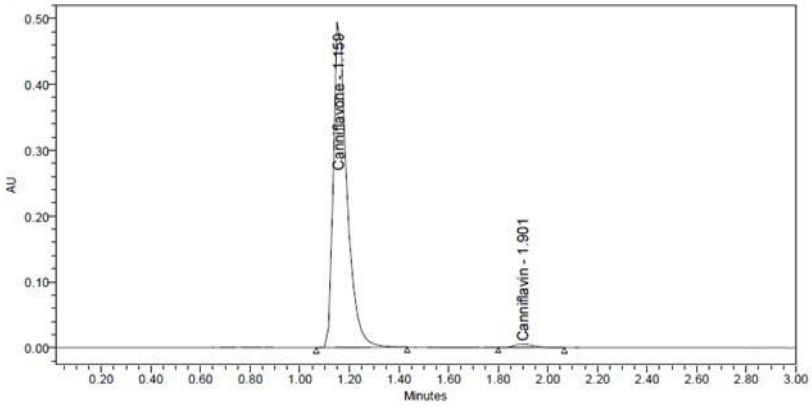


Fig 7: LOQ Chromatogram of Standard

Table 5: Sensitivity data

Molecule	LOD	LOQ
Canniflavin	0.311	0.943

Robustness

Robustness of the optimized test procedure was confirmed by undertaking deliberate changes in optimizes experimental conditions. The robustness parameters include concentration of the mobile phase (±10% v/v), Flow rate (±0.3ml/min) and column temperature (30^0C ± 3). The results for Robustness were mentioned below in table 6

Table 6: Data for Robustness Studies

S.No	Condition	%RSD of Canniflavin	%RSD of Canniflavone (ISD)
1	Flow rate (-) 0.25ml/min	1.1	0.8
2	Flow rate (+) 0.35ml/min	1.1	0.5
3	Mobile phase (-) 65B:35A	0.8	0.9
4	Mobile phase (+) 55B:45A	0.5	0.4
5	Temperature (-) 25°C	0.9	1.0
6	Temperature (+) 35°C	1.4	0.6

Degradation Studies

The stability studies were carried out on Canniflavin to demonstrate the inherent stability. The forced degradation was executed on Canniflavin with current ICH procedures. The acid (2N Hcl, refluxed at 60°C for 30min), Base (2N NaOH, refluxed at 60°C for 30min), Peroxide (20% H_2O_2, refluxed at 60°C for 30min), Thermal (105°C for 6), UV light (exposed to UV light for 7 days), Water (refluxing the drug in water for 6h at 60°C). The results obtained for stability studies were summarized in below Table 7

Table 7: Degradation data of Canniflavin

S.NO	Degradation Condition	% Drug Un Degraded	% Drug Degraded
1	Acid	95.26	4.74
2	Alkali	94.56	5.44
3	Oxidation	95.42	4.58
4	Thermal	96.85	3.15
5	UV	98.19	1.81
6	Water	99.07	0.93

DISCUSSION

An efficient and extensive literature survey reveals that no UPLC and very scanty HPLC methods were developed for the estimation of Canniflavin in bulk. In the HPLC methods, which were reported earlier, Canniflavin was eluted with longer retension times and much lower sensitivity and were not economical as compared with the present method. When compared with HPLC, this UPLC method provides faster elution of analyte as the column is packed with much lower particle size, and provides greater surface area for the analyte to ineract and aids in more separation that is efficient. In this current developed method, a mobile phase combination of Potassium dihydrogen ortho phosphate KH2PO4: Acetonitrile [55:45% v/v] was selected for the analysis of Canniflavin and was eluted at 1.941. Numerous samples can be analysed rapidly by the application of the present method. By the analysis of statistical data,it is confirmed that the developed method has perfect accuracy,ideal specificity and reproducible precision with much high sensitivity.

CONCLUSION

An accurate, simple, rapid highly sensitive novel UPLC technique for the determination of Canniflavin in bulk with good resolution was demonstrated. Moreover, remarkable benefits of UPLC were found in speed and low solvent consumption. Stability studies were also performed for Canniflavin to establish the stability indicating nature of developed UPLC method and therefore method found to be accurate, precise, Specific, robust ,stable. All statistical results were under the satisfactory limits. The proposed method could be used to estimate Canniflavin in bulk as well as marketed herbal formulations.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data and material should be available upon request.

Competing interests

The authors declare that they have no competing interests to declare.

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CRediT authorship contribution statement

Authors Ramya Kuber. B analyzed and interpreted the data of obtained chromatograms and a major contributor in writing the manuscript. Theja.I and Jamuna A, performed the benchwork and experimental work of the stability-indicating liquid chromatographic method development of analytes using UPLC. The authors read and approved the final manuscript.

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Plant authentication

The Plant used in this work was authenticated by Dr. K. Madhava Chetty, Taxonomist under the Vocher number 0911 for *Cannabis sativa* L., belonging to family Cannabaceae.

Abbreviations

RP-UPLC: Reverse Phase Ultra Performance Liquid Chromatography
ICH: International Conference on Hormonization
ISD: Internal Standard
LOD: Limit of detection
LOQ: Limit of quantification
TUV: Thermal ultraviolet
HPLC: High-performance liquid chromatography
STD: Standard
SB: Stable Bonding
CSH: Charged Surface Hybrid

REFERENCES

1. Yancui Haung, Indica Edirisinghe. (2016). Chemical changes of Bioactive Phytochemicals during Thermal Processing. Elsevier, Reference Module in Food science Pramod Kumar. (2019).Role of Food and Nutrition in Cancer. Elsevier, The Role of Functional Food Security in Global Health. 193 – 203
2. Ipek Suntar, Omer Faruk Yakinci. (2020). Potential risks of phytonutrients associated with high-dose or long-term use. Elsevier, Phytonutrients in Food. 137-155.
3. Monika Thakur, Renu Khedkar. (2020) Phytochemicals: extraction process, safety assessment, toxicological evaluations, and regulatory issues. Elsevier. Functional and Preservative Properties of Phytochemicals. 341-361. https://pubcum.ncbi.nlm.nih.gov
4. Simon Erridge, Nagina Mangal et al. (2020). Cannflavins- from plant to patient: A scoping review. Elsevier. Fitoterapia. https://go.drugbank.com/drugs/DB06605.
5. Wieland Peschel, Matteo Politi. (2015) 1H NMR and HPLC/DAD for *Cannabis sativa L.* chemo type distinction, extract profiling and specification. Talanta (1), 150–165.
6. Wieland Peschel, (2016) Quality Control of Traditional Cannabis Tinctures; Pattern, Markers, and Stability. Sci. Pharm. 84, 567–584.
7. Júlia de, A. Leite, Marcos, V.L. de Oliveira, Raphael Conti, Warley de S. Borges, Thalles R. Rosa. (2018).Extraction and isolation of cannabinoids from marijuana seizures and characterization by 1H NMR allied to chemometric tools. Scijus. Sci Justice, *58*(5):355365. doi: 10.1016/j.scijus.2018.06.005. Epub 2018 Jun 25.
8. Lucia Marchetti, Virginia Brighenti, Maria Cecilia Rossi, Johanna Sperlea, Federica Pellati and Davide Bertelli. (2019). Use of 13C-qNMR Spectroscopy for the Analysis of Non-Psychoactive Cannabinoids in Fibre-Type Cannabis sativa L.
9. Tapan Seal. (2016). Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves, Sonchus arvensis and Oenanthe linearis of North- Eastren region in India, Journal of applied Pharmaceutical Science,*6* (02);157-166.
10. Adam metonnen engida, Novys. Kasim, Yeshitila, Asteraye. Tsigie, Suryadi Ismadji, Lien Huong, Huynh, Yi- Hsuju.(2012) Extraction, identification and quantitative HPLC analysis of flavonoids from sarangsemut (Myrmecodia pendan), Industrialcrops and products, 392-396.
11. Bras H.de oliveira, Tomoe naka shima, Jose D. de souza filho and fabiano L.freshe. (2001). HPLC analysis of Flavonoids in Eupatorium littoral, J. Braz. Chem. Soc, *12*(2), 243- 246.
12. Taleuzzaman M, Ali S, Gilani SJ, Imam SS and Hafeez A. (2015). Ultra Performance Liquid Chromatography (UPLC) - A Review, Austin J Anal Pharm Chem. *2*(6): 1056