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REVIEW ARTICLE **OPEN ACCESS**

A Review on Isocratic elution in HPLC methods for Flavonoids

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ABSTRACT
With a wide range of pharmacological and advantageous health effects on people, flavonoids are one of the major subclasses of small molecular secondary metabolites generated in the various plant parts. The analysis of flavonoids by HPLC is one of the most important steps. Isocratic elution is a straight forward, reliable mobile phase with identification for flavonoid analysis in robust chromatographic procedures. Only few of bioactive flavonoids such as querecetine, apigenin, luteolin, catcachin analysis by stable mobile phase, 1-2 min. flow rate in HPLC method. This review will look at the application of flavonoids from the previous five years of research study, discussing the benefits of isocratic elution techniques. Review is done to analyze various factors, such as extraction, isolation, column selection, mobile phase composition, and so on. The objective of this review is to provide evidence of analytical techniques using the isocratic elution method used for different subclasses of flavonoids.
KEYWORDS: *Extraction, Flavonoids, Bioactive, Isocratic elution, HPLC.*

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INTRODUCTION

Flavonoids are more sub classified natural bioactive organic compounds of secondary metabolites with a diversified chemical structure of flavonoid bioactive phytoconstituents. [1-3] They widely exist in plant sources such as roots, stems, leaves, flowers, and fruits, which have numerous categories and chemical structures.[4] Flavonoids are a class of naturally occurring compounds found in various plants known for their antioxidant, anti-inflammatory, anti-cancer, antibacterial, and antiviral properties. Up to 60% to 90% of people in Ethiopian countries are thought to rely on traditional remedies for their medical requirements. [5-7] Flavonoids are associated with a broad spectrum of health-promoting effects and are used in the nutraceuticals, pharmaceutical and medicinal cosmetics industries as pigments and bio preservative applications. This research's capability to precisely identify and quantify certain bioactive flavonoids is essential [8-9]. Phytochemicals may now be simple method, rapidly separated, positively identified, and precisely quantified from plant materials using analytical methods in a matter of minutes. [10-13] The development of low-cost, fast, and simple analytical methods is challenging. Three fundamental stages are needed to conduct a natural product analysis: first, an effective means to extract the desired phytochemicals from the raw plant material; second, a method to separate the different phytochemicals in the extracts; and third, a method to identify and quantify the photochemical [14]. Pure standards are necessary for this. Commercially accessible biomarkers standards are rare and often rather expensive, even with the thousands of flavonoid bioactive photochemical that has been found and reported in the literature [15].

High-performance liquid chromatography (HPLC) is the most preferred analytical approach for quantifying flavonoids due to its high sensitivity, stability, selectivity, and efficiency. Several reported research data guides have been published on gradient elution in HPLC methods on flavonoid bioactive phytoconstituents. [16] Gradient elution is more time-consuming, more organic solvents are used, and gradient elution retention of components is affected.[17] The analysis of flavonoids, which are a collection of compounds with different polarities and structural features, can be quite challenging. However, isocratic elution has proven to be an effective method for separating these compounds in HPLC. [18-20] The isocratic elution method is utilized in herbal industry manufacturing for quality control of bioactive compounds. In this review, we will explore the analysis of flavonoids as bioactive compounds by elution in HPLC methods based on those available in research

articles [21-23]. The primary objective of this review is to present data supporting analytical methods that employ the isocratic elution procedure, particularly is used for separating flavonoids.

Extraction and isolation of flavonoids: Many factors, such as the different solvents used for the extraction of bioactive molecules, the choice of plant parts, and the choice of solvents for the extraction of bioactive compounds, often play vital roles in extracting the bioactive phytoconstituents from plants effectively. [24-25] Sample preparation is a critical step in flavonoid analysis. Techniques like liquid-liquid extraction and solid-phase extraction are commonly used to isolate flavonoids from complex matrices. [26] Different methods for the isolation and purification of bioactive compounds from the extract of the plant are used to carry out analytical techniques such as TLC, column chromatography, HPLC, HPTLC, LC-MS, etc. Novel analytical methods can be developed by using the gradient HPLC method. Medicinal plants play a valuable role in the field of research and development, exploring extraction and chemical isolation.[27-28] Only very few phytoconstituents have been isolated in single form. Very few of the phytoconstituents have been standardized, purified, and studied for their simple analytical methods. [29]

Classification of Flavonoids-

Flavonoids generally refer to the natural product of C6-C3-C6basic structure. The main constituents of flavonoid drugs are 2•phenyl•y-benzopyrones (2-phenyl-chrornone) or structurally related, mostly phenolic, compounds made up of three rings of A/B/C shown in figure No.1.

Depending on the carbon of the C ring, which the B ring is linked to, as well as the degree of unsaturation and oxidation of the C ring, flavonoids can be further classified into various subgroups. [30-32] Isoflavones are flavonoids in which the B ring is bonded to the C ring at position 3. Neoflavonoids are defined as those in which the B ring is joined in position 4, whereas those in which the B ring is linked in position 2 can be further separated into many subgroups according to the structural characteristics of the C ring. [33-35] These subgroups include anthocyanin, chalcones, flavones, flavonols, flavanones, and flavanonols shown in Figure No.2.

Isocratic Elution in HPLC-

A high-performance liquid chromatography technique was widely used for identification, estimation and to check analytical profile of bioactive organic molecule. [36] It will be recognized as a major instrumental technique for phytochemical analysis. With the help of these techniques, we can characterize the chromatogram with no time limit for a large number of parameters. [37] The initial HPLC condition in Table No.1. There are two elution system are present in HPLC first is isocratic and gradients elution in analysis. [38] Isocratic elution involves the use of a single mobile phase composition throughout the entire chromatographic run. Retention of the components is not affected because of the unchanging concentration. [39] Polarity of the mobile phase remains the same throughout analysis. Selectivity or elution order is not dependent on column dimensions. The peaks elute in the same order. [40-42] Unlike gradient elution, where the mobile phase composition changes over time, retention of the components is affected because of the varying concentration because polarity of mobile phase increase or decrease. Isocratic elution offers a stable and constant elution condition this approach can be particularly advantageous for flavonoid analysis, given the variable nature of these compounds. Table No.1: Initial HPLC conditions. [43]

Methodology-

Search Techniques -

A web-based approach for searching study literature was utilized to find publications that discussed the isocratic and gradient elution of bioactive flavonoids using the widely used HPLC technique for investigations. Relevant literature was gathered by searching for published journal articles using international databases, including Google Scholar, Science Direct, and the Web of Science search engine, as a general guideline for supply chain guidelines and standards in Table No.2.

Column Selection: [44]

Several HPLC columns are suitable for flavonoid analysis. C18 and C8 stationary phases are commonly used for isocratic elution due to their good retention of flavonoids. Phenyl and cyano columns may be preferred for specific applications table no. 3.

Mobile Phase Composition:

The mobile phase composition is vital for separating flavonoids efficiently. A typical isocratic mobile phase consists of a mixture of water and organic solvent, often acetonitrile or methanol. The addition of acid, such as formic acid or acetic acid, is often necessary to improve separation and peak shape. Proper adjustment of pH can be crucial in certain cases.

Detection Techniques:
Ultraviolet (UV) detection is the most common technique for flavonoid analysis due to its cost-effectiveness. However, other detection methods such as fluorescence, diode array detection, and mass spectrometry can offer higher selectivity and sensitivity, allowing for the identification and quantification of flavonoids at lower concentrations. [45, 46]

Advantages of Isocratic Elution for Flavonoid Analysis:

Simplicity: Isocratic elution is straightforward and easier to set up than gradient elution methods. This can save time and resources, making it a practical choice for routine flavonoid analysis.

Stability: Flavonoids can be sensitive to changes in mobile phase composition. Isocratic elution provides a stable environment, reducing the risk of compound degradation or changes in retention times during the analysis.

Quantitative Accuracy: Isocratic elution simplifies the process of quantifying flavonoids, as it maintains consistent elution conditions throughout the analysis. This is crucial for accurate concentration determination.

Cost-Effectiveness: Isocratic methods typically require fewer solvents and resources, making them a cost-effective choice for laboratories with budget constraints.

Recent advancement-

In the field of analysis there have been advancements and potential future directions. One noteworthy development is the improvement, in efficiency of HPLC through the use of stationary phase technology superficially porous particles and core shell columns. This innovation has resulted in enhanced selectivity and sensitivity when combined with chromatography and hyphenated techniques, like LC MS. By incorporating these approaches researchers can achieve results in terms of separating and detecting flavonoids.

Table No.1: Initial HPLC conditions.

Particle size	10 or 5 μm
Stationary phase	C8 or C18
Mobile phase	3 for neutral compounds b) 3 and 7.5 for ionic acidic c) 3 and 7.5 for ionic basic
pH of mobile phase	3 for neutral compounds b) 3 and 7.5 for ionic acidic c) 3 and 7.5 for ionic basic
Modifier	10 mM TEA 1% HAS 10 mM TEA
Column length and internal diameter	250 mm x 4.6mm
Column temperature	Ambient to 35°C
Flow rate	1.5 - 2mL/minutes
Injection volume	10 – 25 μL
Buffer concentration	Phosphate 50 mM
% Buffer isocratic	50%
%Buffer gradient	20-80%

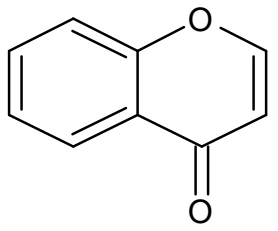
Table No.2: Methods of Isocratic elution in HPLC of selected Flavonoids.

Sr. No.	Natural Plant Source	Compound	Column used	Mobile Phase	UV (nm)	References
1	Biomarkar	Apigenin , Luteolin	Hibarlichrospher C18	Methanol: 0.5% Trifluoroacetic acid80:20v/v Flow rate 1ml/min.	269	[47]
2	Orange Peel Powder	Taneretin	RP-C18	Methanol: Water 60:40 v/v Flow Rate 1ml/min	327	[48]
3	*Eugenia Dysenterica Syzygium Cumilini* Extract	Catachin	RP-C18	Methanol: 0.01M Phosphoric Acid15:85 v/v Flow Rate 0.8ml/min	230	[49]
4	Biomarker	Silibinin	Protonsil C18	Acetonitrile :0.2M Sodium dihydrogen Phosphate Buffer with 1.5 ml Triethylamine pH 6.5 , 40:60 v/v Flow rate 1ml/min.	208	[50]
5	Biomarker	Qurecetin	RP-C18	Acetonitrile : Water (Acidify to pH 3.0)30:70 v/v Flow Rate 1.1ml/min	257	[51]
6	Irani Scutellaria	Baicalein, Wogonin, chrysin	C8	Water: Acetinitrile: Methanol Orthophosphoric Acid 60:38:30:1v/v/v/v Flow rate 1ml/min	262	[52]

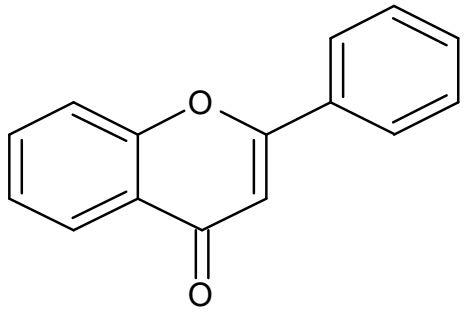
Table No. 3: Various types of columns and their applications.

Columns	Phase	Solvents	Applications
Amine	Amino propyl	ACN, MeOH, THF, $CHCl_3$,CH_2Cl_2	Sugar , Anions
Cyano	Cyan propyl	ACN, MeOH, H_2O, THF	Ketones, Aldehydes
C8	Octyl	ACN, MeOH, H_2O	General, Nonpolar
C18	Octadecyl	ACN, MeOH, H_2O	General, NonPolar
SAX	Aromatic quaternary amine	Salt Buffers, ACN, MeOH, H_2O	Anions

Figure. No.1 Basic Structure of Flavonoids.

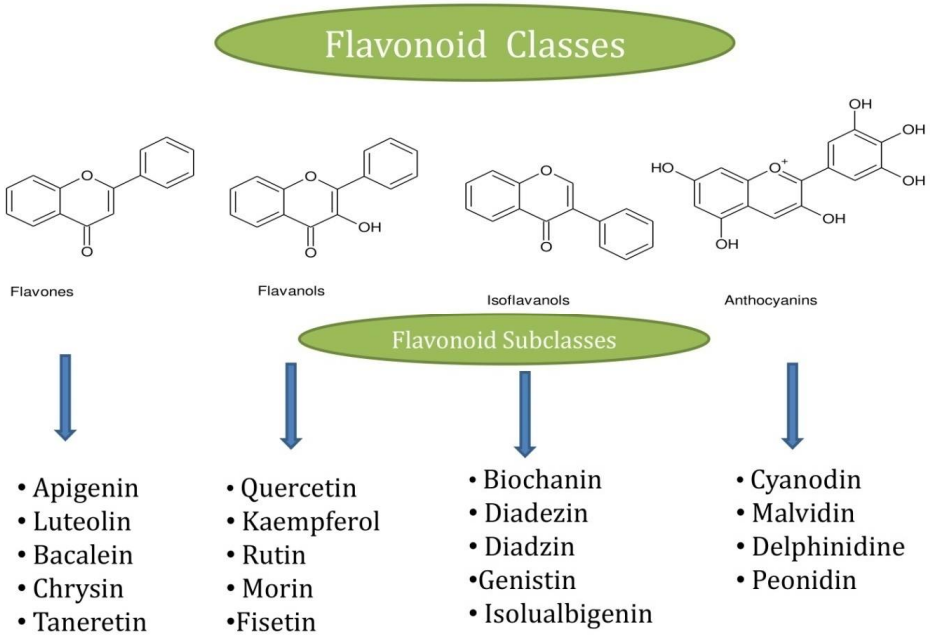


4*H*-chromen-4-one



2-phenyl-4*H*-chromen-4-one

Figure No.2: Flavonoid classes and subclasses.



CONCLUSION

Isocratic elution is a valuable method in HPLC analysis of flovonoid subclasses analysis. Its stable mobile phase, accurate retention time, simplicity, fast, less time consuming make it an attractive choice of for routine analysis, especially in research, quality control and herbal pharmaceutical applications. These days, it's critical to create a procedure in analytical chemistry that minimizes mistakes and overcomes incorrect outcomes. Isocratic elution method which improves the accuracy, precision, specificity and linearity validation method. The isocratic elution method offers a significant challenge in the development of simple analytical methods for naturally present bioactive compound derived from plant constituents.

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AUTHORS CONTRIBUTION

All the authors have contributed equally.

CONFLICT OF INTEREST

All the authors confirm that there is no conflict interest.

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