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Review Article

Chromatographic Techniques for Flavonoids

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ABSTRACT

Flavonoids are a diverse group of polyphenolic compounds widely distributed in plants and possess significant pharmacological and nutraceutical value. Accurate identification, quantification, and structural elucidation of flavonoids are essential for quality control, chemotaxonomy, and bioactivity studies. The focus of this review is to provide an overview of various chromatographic techniques used for the analysis of flavonoids, including TLC, HPTLC, HPLC, UPLC, GC, and their hyphenated mass spectrometric methods. Thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) remain powerful, cost-effective tools for preliminary screening, fingerprinting, and semi-quantitative analysis, enhanced by visualization reagents and densitometry. High-performance liquid chromatography (HPLC) serves as a widely applied method for precise qualitative and quantitative profiling, while ultra-performance liquid chromatography (UPLC) improves resolution, sensitivity, and speed, especially for complex flavonoid mixtures. Advanced hyphenated techniques, such as LC-MS/MS, UPLC-QTOF-MS, HPTLC-MS, and GC-MS (after derivatization), enable confirmation of the structures by molecular mass and fragmentation patterns, thus providing support for comprehensive metabolomic characterization. Collectively, modern chromatographic and mass spectrometric platforms offer robust, sensitive, and reproducible analytical workflows that are essential for standardization, authentication, and in-depth investigation of flavonoid-rich botanical materials.

INTRODUCTION

Chromatography is used to identify the components of a mixture, measure the amount of each component present, and, in certain situations, purify the individual components [1]. Chromatography methods, including gas chromatography (GC) and high-performance liquid chromatography (HPLC), have significant environmental and economic costs, mainly due to their reliance on organic solvents, high energy consumption, and expensive instrumentation [2]. Solubility and different Rf values, as well as their ability to fluoresce in ultraviolet light, allow flavonoids to be easily identified using chromatographic techniques. Paper chromatography is one of the oldest methods used for flavonoid analysis [3].

Whatmann, Schull's paper, among others, is extensively utilised for the identification of flavonoids by paper chromatography. Aqueous solutions of polyvalent metals (lead acetate, aluminum chloride, etc.) can be used for detection [4]. For flavones, the borax solution is also used as a spraying reagent [4, 5].

Thin-Layer Chromatography (TLC)

TLC is a qualitative and semi-quantitative method for separating, identifying, and determining the concentration of flavonoids from plant extracts by comparing the retention factor (Rf) values and UV absorbance of analyzed spots to known standards, often utilizing special visualizing agents and densitometry for enhanced analysis [6].

TLC separates compounds based on their differential partitioning between a stationary phase (the TLC plate) and a mobile phase (the solvent). Most flavonoid analyses use polar silica gel plates (normal-phase TLC), which effectively separate the polar flavonoid compounds [7]. Frequently used mobile phases include mixtures of ethyl acetate, formic acid, water, and various organic solvents like methanol, toluene,

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and butanol. Flavonoids absorb UV light, appearing as spots under UV light at 254 nm. Some also fluoresce under UV light at 366 nm. Thin-layer chromatography combined with UV-VIS spectrophotometric measurement allowed the differentiate structurally between 5,6-dihydroxy and 5,8-dihydroxy flavones [4]. Spraying reagents, such as a solution of 1% ethanolic aluminum chloride, can enhance the visibility of flavonoids by causing them to fluoresce or produce specific colors [8]. The NP/PEG reagent or Naturstoff reagent, Neu's reagent, or DPBA is used to enhance fluorescence [9]. Flavonoids have a poor natural fluorescence characterisation that must be improved for separation on chromatography plates. By spraying several complex agents on the TLC plates, the fluorescence of the flavonoids was increased. The most widely utilised complex compound to enhance the fluorescence of flavonoids was diphenyl-boric acid 2-amino ethyl ester (DPBA) [10].

Table 1: Identification of flavonoids in different plant sources by TLC

Source	flavonoids	Mobile phase	Rf	Color	ref
Benincasa hispida	Rutin	Methanol: Glacial Acetic Acid: Formic Acid: Water (3: 0.9: 0.9: 0.5)	0.58		[11]
Benincasa hispida	Quercetin	Toluene: Ethyl Acetate: Formic Acid (5: 4:0.2)	0.82		[11]
Abelmoschus Manihot	Hyperoside	ethyl acetate: formic acid: water 8:3:1	-	Yellow	[12]
Justicia Gendarussa	Vitexin	ethyl acetate: acetic acid: formic acid: water (100:11:11:26)	0.77	Green	[13]
Citrus fruit	Naringin	Methanol, water, and acetic acid (5:4:1)	0.6	Yellow	[14]

HPTLC

Extract flavonoids from the plant material using solvents such as methanol or ethanol. Flavonoids are highly soluble in these polar solvents. A suitable stationary phase, such as silica gel plates (silica gel 60 F254), is used for analysis. Apply the prepared samples and flavonoid standards to the plate using an automated TLC sampler. Saturate the development chamber with the mobile phase to ensure optimal and reproducible separation. Develop the plate in the saturated chamber using a carefully selected mobile phase (e.g., n-hexane–ethyl acetate–formic acid) to achieve the separation of flavonoids. Common solvent systems for flavonoid analysis include mixtures of ethyl acetate, methanol, formic acid, and water in varying ratios. After development, the plate is dried and visualized under UV light at different wavelengths (typically 254 nm and 366 nm) to detect the separated flavonoid bands. To enhance visualization or confirm the presence of flavonoids, the plate may be derivatized by spraying it with a reagent. Aluminium chloride ($AlCl_3$) is a common reagent used for flavonoids, causing them to show a characteristic yellow or green fluorescence under UV light due to chelation. Sodium borate, aluminium chloride-nitric acid- sodium hydroxide [15], Zirconium citric acid, and N- bromosuccinimide are also used for the detection of flavonoid [4]. Quantification is performed using a densitometer, which scans the plate and measures the intensity of each band. The data is processed by software (e.g., WinCATS) to generate densitograms [16]. The amount of a specific flavonoid can be quantified by comparing its peak area or height to a calibration curve generated from the standard solutions. This approach is used to determine the concentration of individual flavonoid compounds, unlike traditional colorimetric methods, which only measure total flavonoid content. For the preparative HPTLC analysis of flavonoids, the primary goal is not just identification or quantification, but the isolation of individual flavonoid compounds in sufficient quantities for further study. Preparative HPTLC involves applying a larger volume of extract as a band to thicker plates to isolate sufficient material for subsequent characterization, often using techniques such as mass spectrometry (MS) [17] or nuclear magnetic resonance (NMR) [17]. The crude plant extract, known to contain flavonoids, is concentrated and then applied as a broad, single band onto a preparative-grade HPTLC plate. These plates have a thicker layer of stationary phase than analytical plates, allowing for a higher sample volume. UV-Visible spectrophotometry to confirm the flavonoid's spectral properties. Coupling with Mass Spectrometry (HPTLC-MS) to precisely determine the molecular weight and structure. Nuclear Magnetic Resonance (NMR) spectroscopy for detailed structural elucidation.

Lower yield: This method is generally limited to isolating milligrams or smaller quantities of a compound and is not suitable for large-scale production [18]. For larger-scale isolation, techniques like preparative HPLC are more suitable. Potential for impurities: Some compounds may still be co-eluted [19], and the final product might contain some silica particles [20]. Further purification may be necessary.

HPTLC-Mass Spectrometry (HPTLC-MS)

HPTLC-MS is the most common hyphenated technique for characterizing flavonoids after separation on an HPTLC plate. It provides the molecular weight and fragmentation pattern of the compounds [21]. MALDI-TOF MS technique, a matrix is sprayed onto the HPTLC plate after separation. The plate is then analyzed directly in the mass spectrometer, saving time by avoiding elution steps [22].

Direct Analysis in Real Time (DART-HRMS) is recent innovation that allows for rapid, high-resolution MS analysis directly from the HPTLC plate without a complex interface [23].

HPTLC-Nuclear Magnetic Resonance (HPTLC-NMR) is a powerful tool for structural elucidation, but coupling it with HPTLC is more complex due to the larger sample size required for traditional NMR compared to the small amounts separated on an HPTLC plate [24]. The band corresponding to the flavonoid is scraped off the plate, and the compound is eluted from the silica using a suitable solvent. The purified compound is then dissolved in a deuterated solvent for analysis by NMR spectroscopy. The resulting NMR spectra provide detailed information on the chemical environment of the atoms in the molecule, allowing for the definitive confirmation or elucidation of the flavonoid's structure.

Table 2: HPTLC applications for flavonoid analysis

Flavonoid class	Compound	Source	HPTLC Application	Ref
Flavonols	Kaempferol	Heteropogon contortus,	Quantification, fingerprinting, Simultaneous quantification	[25]
Flavonols	Rutin	Morus alba	Quantitative analysis	[26]
Flavones	Luteolin	Cardiospermum halicacabum, Premna mucronata, Vitex negundo,	Quantification, quality control. Simultaneous quantification-analysis with apigenin.	[27]
Flavanones	Hesperidin	Citrus fruits Citrus sinensis, C. paradisi, C. limetta	Quantification	[28]

High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is a precise, sensitive, and versatile analytical technique for qualitative and quantitative flavonoid analysis. It separates compounds using a stationary phase, typically a non-polar C18 column, and a mobile phase, often a mixture of aqueous solvents (like water with acid modifiers) and organic solvents (methanol or acetonitrile). Common detection methods include Diode Array Detection (DAD), which uses UV spectra for identification and purity assessment, and Mass Spectrometry (MS), which provides molecular weight and fragmentation patterns for definitive identification. Quantitative HPLC methods require validation according to regulatory guidelines [29].

Ultra-Performance Liquid Chromatography (UPLC)

Ultra-Performance Liquid Chromatography (UPLC) offers advantages over conventional HPLC for flavonoid analysis, primarily due to its use of less than 2 μm particle size columns as the stationary phase. This enables faster analysis times, increased resolution and sensitivity for detecting low concentrations, and reduced solvent consumption, making it more cost-effective and environmentally friendly. UPLC is particularly suited for complex samples containing numerous flavonoid subclasses. The technique utilizes a UPLC system with a high-pressure pump, autosampler, and a detector, typically a Diode Array Detector (DAD), which monitors absorbance across a UV-Vis spectrum during gradient elution separation on specialized columns with an aqueous and organic mobile. Ultra-Performance Liquid Chromatography (UPLC) coupled with Mass Spectrometry (MS), often using a triple-quadrupole mass

spectrometer (QqQ-MS) or quadrupole-time-of-flight (Q-TOF), enables definitive identification and quantification of compounds. MS provides precise molecular mass and fragmentation patterns for structural information. This UPLC-MS combination offers high-speed separation, capable of distinguishing closely related compounds like flavonoid glycosides, and accurate identification that surpasses UV detection. It serves as a crucial tool for authenticating food products by profiling their characteristic flavonoid content, thereby aiding in the quality control phase [35].

Table 3: HPLC chromatographic conditions for flavonoid analysis in different plant sources

Plant source	Flavonoid	Technique	Mobile phase	Detection	Ref
Morus alba (mulberry) leaves	Quercetin & Rutin	C18 (250 mm x 4.6 mm, 5 µm)	Isocratic: 50:50 (v/v) Acetonitrile:0.1% acetic acid in water	UV detector at 259 nm	[30]
Houttuynia cordata leaves	Rutin, Hyperin	C18 (150 mm x 4.6 mm, 5 µm)	Gradient: 0.1% formic acid in water and acetonitrile	PDA detector at 355 nm	[31]
Bupleurum species	Flavones & Flavonols	ODS C18	Gradient: 0.1% formic acid in water and acetonitrile	UV detector at 254 nm	[32]
Ocimum sanctum, Tinospora cordifolia leaf and stem extracts	Quercetin	C18 (250 mm x 4.6 mm, 5 µm)	Isocratic: Acetonitrile: Methanol (50:50, v/v)	UV 256 nm	[33]
Citrus juice	Hesperidin, Naringin	Zorbax Eclipse XDBC18	Specified Isocratic acetonitrile:water: formic acid (21:78.8:0.2) solution at pH 2.5	280 nm	[34]

Table 4: Advanced Chromatographic–Mass Spectrometric Methods for Flavonoid Identification

Plant source	Flavonoid	Method	Mobile phase	Ref
Buckwheat leaves	Rutin and quercetin	UHPLC-MS/MS	Water and acetonitrile UPLC BEH C18 (2.1 x 100 mm, 1.7 µm)	[36]
Grapes	anthocyanins, including peonidin 3-O-glucoside, malvidin 3-O-glucoside, and cyanidin 3-O-glucoside.	UPLC-Q-TOF-MS	1% formic acid aqueous solution and acetonitrile	[37]
Eleutherococcus senticosus	Anthocyanins: Cyanidin-3-O-galactoside, delphinidin. Flavonoids: Quercetin-3-β-D-glucoside, kaempferol-3-O-glucoside, rutin.	UPLC-MS/MS		[38]
Mulberry leaves (*Morus alba L.)*	Quercetin 3-O-rutinoside (rutin), quercetin 3-O-glucoside (isoquercitrin), and various other kaempferol and quercetin glycosides.	UPLC-DAD-QTOF/MS		[39]

Gas Chromatography

Gas Chromatography (GC) is rarely used for flavonoid analysis compared to Liquid Chromatography (LC) because flavonoids are non-volatile and heat-sensitive. To enable GC analysis, a derivatization step is required, most commonly silylation with agents such as BSTFA, which converts hydroxyl groups into trimethylsilyl (TMS) derivatives, increasing volatility and thermal stability. The most common derivatization method involves silylation, where a silylating agent like N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) is used to replace the active hydrogen atoms on the hydroxyl (-OH) groups of the flavonoid molecule with a trimethylsilyl (TMS) group(40). GC–MS, after proper derivatization, allows sensitive profiling of flavonoid aglycones—the non-sugar forms of flavonoids that are often biologically active. For example, quercetin, kaempferol, myricetin, luteolin, and apigenin are commonly detected in a wide range of natural sources. In plant extracts, these aglycones provide chemotaxonomic markers and help evaluate phytochemical diversity. In foods and beverages (such as cranberry juice, tea, citrus fruits, and wine), their quantification is important for nutritional assessment and quality control, since they contribute to antioxidant, anti-inflammatory, and antimicrobial activities. In biological samples (like human plasma or urine), GC–MS enables pharmacokinetic and bioavailability studies by tracking how dietary flavonoids are absorbed, metabolized, and excreted. The fragmentation patterns obtained from MS spectra not only confirm their presence

but also help distinguish structurally similar compounds [40, 41].

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

LC-MS/MS is the preferred method for analyzing flavonoids, offering high precision and efficiency by combining chromatographic resolution with mass spectrometry for structural data. Cheminformatics and LC-MS/MS enable high-throughput surveys of flavonoid diversity, allowing for the separation of various flavonoid forms like glycosylated, acylated, and prenylated types, along with their aglycones. Molecules are ionized and fragmented, and their fragments are analyzed after LC separation to generate unique mass spectra for each compound. ESI, APCI, and APPI are the primary ionization sources used in LC-MS for flavonoid analysis. API sources include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure chemical ionization, which are presently the most pertinent LC-MS ionization sources [42].

LC–MS/MS has become a gold standard for flavonoid profiling across diverse plant and biological matrices. For example, a stable-isotope dilution LC–MS/MS assay was recently developed for the accurate quantification of morusin and mulberrin in mulberry, ensuring high sensitivity and reproducibility [43]. In strawberries, UPLC–MS/MS metabolomic approaches using C18 columns and acidified methanol extractions have enabled detailed characterization of flavonoid subclasses, including quercetin derivatives and anthocyanins. Similarly, flavonoid constituents such as hesperidin, neohesperidin, and quercetin in Zanthoxylum zanthoxyloides were successfully identified and quantified through LC–MS/MS with multiple reaction monitoring (MRM) [44, 45]. Moreover, UPLC–ESI–MS/MS profiling of Apocynum venetum employed HSS-T3 columns to resolve hyperoside, isoquercitrin, and malonylated glycosides with high precision [45]. Together, these examples highlight the versatility and robustness of LC–MS/MS platforms in flavonoid analysis.

CONCLUSION

Chromatographic techniques such as HPLC, UPLC, LC-MS/MS, and GC-MS provide highly sensitive, selective, and reliable approaches for the qualitative and quantitative analysis of flavonoids in plant extracts, foods, and biological samples. These methods enable accurate profiling of diverse flavonoid classes, even in complex matrices, by offering superior resolution, reproducibility, and detection limits. Overall, chromatography-based analysis plays a crucial role in standardization and quality control of flavonoid-rich products, supporting both research and industrial applications.

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ETHICS DECLARATION

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