

Advanced Analytical Approaches for the Standardization and Quantification of Bioactive Flavonoids and Isoflavonoids

Shanmuga Lakshmi Shanmugalakshmikanthan¹, Sedhu Raagavan Sarkgunan¹, Sowmyalakshmi Venkataraman^{1*}

¹ Department of Pharmaceutical Chemistry (DST-FIST Sponsored Department), Sri Ramachandra Faculty of Pharmacy, Sri Ramachandra Institute of Higher Education and Research (DU), Porur, Chennai, Tamil Nadu – 600116, India.

Corresponding Author

Dr. Sowmyalakshmi Venkataraman

Department of Pharmaceutical Chemistry (DST-FIST Sponsored Department), Sri Ramachandra Faculty of Pharmacy, Sri Ramachandra Institute of Higher Education and Research (DU), Porur, Chennai, Tamil Nadu – 600116, India.

Email: sowmyamahesh30@gmail.com

ORCID: 0000-0001-9693-1472

ABSTRACT

Flavonoids and isoflavonoid are complex compounds of polyphenols with high pharmaceutical, nutraceutical, and cosmeceutical significance. Their medicinal applications are restricted by the complexity of the matrix, the isomerization of structures, fluctuations in quantity, and the instability at the stage of the extraction and analysis. This review analyzes the advanced methods of the standardization and quantitation of characteristic bioactive components quercetin, equol, glyceollins, and cladrin in plants, preparations, and biological samples. The optimization of sample preparation protocols, comprising ultrasonic-assisted hydroalcoholic extraction, hydrolysis using acid/enzymes, and solid phase purification is described to improve extraction, avoid degradation, and manage matrix effect. Chromatographic/spectrometric methods, including HPLC-UV/DAD, HPTLC-densitometry, LC-MS/MS, high-resolution mass spectrometry and UHPLC-MS/MS, are reviewed in terms of selectivity, applicability and sensitivity to the quality control and pharmacokinetics. Method validation is considered in accordance with the guidelines ICH Q2(R2) and WHO recommendations with regard to specificity, accuracy, precision, linearity, robustness, and stability. Modern problems co-elution, ion suppression, isomer separation, the lack of certified reference materials, and intra-laboratory variations are discussed, along with modern solutions, including the green chemistry, microextraction, metabolomics. Overall, these methods allow for scientifically sound standardization of flavonoid and isoflavonoid products, thus enabling batch standardization, regulatory compliance, and eventually the development of standardized botanical medicines and nutraceuticals.

Keywords: Flavonoids, Isoflavonoids, LC MS/MS, HPLC DAD, Method validation (ICH Q2(R2), Extraction

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INTRODUCTION

Flavonoids and isoflavonoids are significant groups of plant-derived polyphenolic specialized metabolites characterized by a common C₆-C₃-C₆ carbon framework and extensive structural diversity. Variations in hydroxylation, methoxylation, glycosylation, prenylation, and ring substitution produce multiple subclasses, which includes flavones, flavonols, flavan-3-ols, flavanones, chalcones, isoflavones, isoflavans, and pterocarpanes. These structural features influence their physicochemical properties, chromatographic behavior, metabolism, bioavailability, and biological activity.¹ Although predominantly associated with higher plants, flavonoid and isoflavonoid biosynthesis has also been reported in selected fungi and actinomycetes, including through enzymatic and microbial transformations such as hydroxylation, methylation, glycosylation, reduction, and cyclization.²

The pharmacological importance of these compounds (**figure1**) has stimulated considerable interest in pharmaceutical, nutraceutical, functional-food, and cosmeceutical research. Flavonoids such as quercetin have been investigated for antioxidant, anti-inflammatory, cardioprotective, antiviral, anticancer, and neuroprotective effects. Isoflavonoids, including equol and glyceollins, are of particular interest because of their estrogen-receptor-related, osteoprotective, antioxidant, antimicrobial, immunomodulatory, and chemopreventive activities.³ Equol is a bioactive metabolite of daidzein and is produced only by a proportion of individuals by gut microbiota, making its accurate determination relevant to pharmacokinetic, nutritional, and personalized-health investigations.⁴ Glyceollins are prenylated pterocarpin phytoalexins formed in soybean in response to biotic or abiotic stress and occur as structurally related isomers that may differ in biological potency and analytical response.⁵

*Author for Correspondence: sowmyamahesh30@gmail.com

Cladrin, a methoxylated isoflavone, has also attracted interest because of its reported osteogenic and estrogen-modulating potential.⁶

The quality, safety, efficacy, and reproducibility of flavonoid- and isoflavonoid-containing products are strongly dependent on reliable chemical characterization. The concentration of these metabolites can vary substantially with cultivar, plant species, maturity, geographical origin, post-harvest processing, harvesting conditions, extraction solvent, formulation composition and storage. In addition, many flavonoids occur as aglycones, O-glycosides, C-glycosides, methylated derivatives, conjugates, or isomeric forms.⁷ Consequently, the measurement of a single total-flavonoid value or the use of nonspecific spectrophotometric assays may not adequately reflect the identity, abundance, or biological relevance of individual constituents. Standardization therefore requires the integration of botanical authentication, chromatographic fingerprinting, marker-compound identification, and quantitative estimation of selected bioactive or chemically representative constituents.^{8,9}

The analytical determination of these metabolites is challenging because herbal extracts and biological samples contain complex mixtures of co-extractives, including phenolic acids, tannins, sugars, lipids, pigments, proteins, and other secondary metabolites. These components may cause chromatographic co-elution, ion enhancement or suppression in mass spectrometry, nonspecific ultraviolet absorption, and inaccurate recovery measurements. Further complications arise from the limited abundance of some analytes, instability during extraction and storage, oxidation of phenolic groups, light sensitivity, thermal degradation, and the structural similarity of isomeric compounds.¹⁰ Therefore, sample preparation is a critical component of the analytical workflow. Conventional procedures such as maceration and Soxhlet extraction remain useful for laboratory-scale investigations, whereas ultrasound-assisted, pressurized-liquid, and other miniaturized extraction approaches increase extraction efficiency while reducing processing time and solvent requirements.¹¹

Several types of analysis tools have been applied for flavonoid and isoflavonoid characterization and quantitation. HPLC using UV or diode array detection still remains a method of choice for QC due to its availability, repeatability, and applicability for assay and fingerprinting analyses. HPTLC/densitometric methods represent rather economic option for the concurrent quantification of several markers in herbal materials. Yet, the latter may lack selectivity in case when compounds have the same ultraviolet spectrum or the presence of target analytes is very low. In turn, UHPLC-MS/MS and LC-MS/MS allow achieving high detection capability and selectivity which makes them especially valuable for the quantitation of low-concentration analytes, metabolites, conjugates, and markers in plasma, urine, tissues, and other biological matrices. HRMS, including QTOF and Orbitrap platforms, can additionally be used for accurate mass measurements, metabolomics, dereplication, and structure elucidation.¹¹

Accordingly, advanced analytical approaches are essential for converting chemically variable botanical extracts into

standardized and reproducible reliable standardization cannot be established solely by selecting a sophisticated instrument; it requires a complete, fit-for-purpose analytical procedure. This includes appropriate selection of extraction conditions, internal or reference standards, calibration strategy, chromatographic separation, detector response, matrix-effect evaluation, analyte stability assessment, and system-suitability criteria. Validation should show that the applicability is suitable for its targeted application with respect to selectivity or specificity, response and range, precision, accuracy, detection and quantification limits, robustness, and stability, as applicable. The current ICH Q2(R2) guideline emphasizes the documented demonstration of the validation process establishes the suitability of an analytical procedure for its specified application and extends validation considerations to advanced chromatographic, spectrometric, and spectroscopic procedures.¹² For herbal materials and finished herbal products, WHO recommendations additionally emphasize the use of marker substances, chromatographic procedures, fingerprinting, and validated methods capable of providing specific and selective measurements.¹³

pharmaceutical or nutraceutical preparations. The combined use of optimized sample preparation, HPLC/HPTLC fingerprinting, LC-MS/MS quantification, high-resolution mass spectrometry, and contemporary validation principles promote the reliable identification and measurement of bioactive flavonoids and isoflavonoids across plant, formulation, and biological matrices. In this context, quercetin, equol, glyceollins, and cladrin represent analytically relevant examples because they encompass different structural subclasses, biological roles, matrix requirements, and sensitivity challenges. This review examines advanced analytical strategies for their extraction, separation, identification, quantification, and validation, with emphasis on method performance, matrix effects, analyte stability, applicability to quality control and pharmacokinetic studies, and the role of these approaches in establishing scientifically defensible standards for herbal and natural-product formulation

Chemistry of the target phytochemicals:

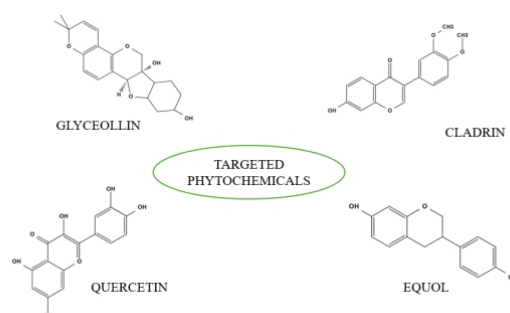


Figure 1: The targeted phytochemicals along with their chemical structures

Sample Preparation

The sample preparation procedure was developed to provide reproducible isolation of bioactive compounds

(flavonoids and isoflavonoids) with minimal losses due to degradation, matrix effects, and fluctuations of water content and particle size of the sample. The collected plants were identified, cleaned up, if necessary, dried in the shadow or in the oven at a stable temperature of up to 40 °C, and ground to form powder. The dried powder was sifted, protected from the light and stored at low temperatures in sealed containers. Around 0.5 g of the powdered sample was weighed precisely into a centrifuge tube and extracted by adding 10 mL of HPLC-grade methanol–water or methanol–aqueous acid solution. For the simultaneous extraction of flavonoid glycosides and isoflavonoids, methanol solution containing 0.1–0.5 mol/L hydrochloric acid may be used if the aim of the analysis is the estimation of total aglycones after hydrolysis. The mixture was sonicated for 30–60 min at a controlled temperature of 25–40 °C and then centrifuged at 4 °C. The supernatant was collected, and the residue was re-extracted with identical conditions of the procedure to increase the analyte recovery. The pooled extracts were evaporated under reduced pressure or under a gentle flow of nitrogen at a temperature up to 40 °C, redissolved in a known amount of methanol or the initial mobile phase composition, vortexed and filtered through a membrane of 0.22 µm or 0.45 µm of regenerated cellulose or PTFE prior to the analysis. For LC-MS/MS analysis, the use of MS-grade solvents is recommended, and the final extract should be diluted with the initial mobile phase to eliminate the effect of the solvent strength mismatching. In case of distinction between the conjugated and free forms, two aliquots should

be prepared: one for the analysis of native glycosides and the second one subjected to validated acid or enzymatic hydrolysis for estimation of the aglycones. Daidzin, genistin, puerarin, rutin, quercetin glycosides, daidzein, genistein, naringenin, quercetin, and kaempferol can be quantified using authenticated reference standards and matrix-matched or solvent-based calibration. The complete procedure should be performed in triplicate, together with procedural blanks, sample-spiked recovery preparations, and quality-control samples. This strategy is consistent with published flavonoid and isoflavonoid methods employing ultrasound-assisted hydroalcoholic extraction, repeated extraction, centrifugation, membrane filtration, and dilution to the initial mobile-phase composition before HPLC–PDA, HPLC–DAD, or HPLC–ESI–MS/MS analysis. For isoflavone aglycones released by hydrolysis, validated RP-HPLC methods have used a C18 column with methanol–0.1% acetic acid and diode-array detection at 254 nm, demonstrating linearity, precision, specificity, robustness, and recoveries close to 98% for daidzein and genistein. The extraction solvent, extraction time, temperature, solid-to-liquid ratio, hydrolysis conditions, filtration step, and sample stability should be optimized experimentally and documented as critical sample-preparation parameters. Method performance should subsequently be established according to ICH Q2(R2), shown in table 1 including specificity, response linearity and accuracy, precision, range, detection limit, quantification limit, recovery, stability and robustness of standard and sample solutions.^{14–16}

Matrix	Sample Preparation Procedure	Analytical Purpose
Plant Materials	Drying → Grinding → Solvent Extraction → Filtration → Concentration → Reconstitution	Isolation of quercetin, glyceollin, and cladrin from herbal samples.
Soybean Extracts	Methanolic extraction → Resin/Silica gel purification → HPLC analysis	Quantification of glyceollin isomers.
Plasma/Serum	Protein precipitation or SPE → Centrifugation → Filtration	Equol and quercetin bioanalysis.
Urine Samples	Enzymatic hydrolysis → SPE/LLE → LC-MS/MS analysis	Determination of equol metabolites.
Dietary Supplements	Solvent extraction → Filtration → Dilution → HPLC/UPLC analysis	Quality control and standardization.

Table 1. Matrices and the preparation of samples along with their analytical purposes

Extraction Techniques

An efficient extraction method coupled with a quantitative analytical technique is needed for the standardization of

flavonoid- and isoflavonoid-containing herbs, extracts, and medicines. Traditional methods like Soxhlet extraction, maceration, percolation, and reflux still hold value

attributable to their reproducibility and simplicity but often require large volumes of organic solvents, prolonged extraction times and sometimes causes degradation of thermally unstable compounds. Modern extraction approaches like ultrasound-assisted extraction, microwave-assisted extraction, pressurized liquid extraction, enzyme-assisted extraction, and ultrasound-assisted matrix solid-phase dispersion as shown in table 2, improve solvent penetration, mass transfer, yield of extraction, and processing efficiency. Ultrasound-assisted extraction is particularly suitable for flavonoids and isoflavonoids due to the disintegration of plant cells caused by acoustic cavitation leading to the release of intracellular compounds, and pressurized liquid extraction improves the recoveries of analytes by raising the temperature and pressure, using smaller volumes of solvents. Green extraction technologies utilizing aqueous ethanol, ethyl lactate, supercritical CO₂, and natural deep eutectic solvents are gaining popularity since they may help to reduce the disadvantages associated with the use of conventional solvent systems. Deep eutectic solvents may be used together with ultrasound or microwaves to increase the extraction of polyphenols; however, the issues associated with viscosity, water content, hydrogen-bonding network, compatibility of deep eutectic solvents with analytes and their effect on the subsequent chromatographic separation need to be considered. The extraction techniques can be used as methods of post-extraction purification and sample cleanup when determining flavonoids and isoflavonoids in plasma, urine, and other biological matrices. The prepared extracts can be further analyzed using validated RP-HPLC with UV or diode-array detection (DAD), high-performance thin

layer chromatography, or LC-MS/MS. The former two methods are suitable for routine quality control of samples and concurrent analysis of marker compounds like quercetin, rutin, kaempferol, daidzein, genistein, equol, and glyceollins, while the latter method provides higher selectivity and sensitivity useful for trace analysis, metabolomics, and pharmacokinetic studies. Development of an extraction method needs to include optimization of solvent composition, extraction time, temperature, solid-to-liquid ratio, particle size, number of extraction cycles, and sample cleanup by the design of experiments or analytical quality by design. The final analytical procedure should be demonstrated to be fit for its intended purpose through assessment of specificity, selectivity, linearity, working or reportable range, accuracy, precision, recovery, limit of detection (LOD), limit of quantification (LOQ), robustness, solution stability, system suitability, and, where relevant, matrix effects and carry-over. These validation characteristics should be established in accordance with ICH Q2(R2), which provides the current international framework for demonstrating the reliability of analytical procedures used for pharmaceutical quality control and related applications. Collectively, the integration of optimized green extraction, selective sample preparation, chromatographic fingerprinting, quantitative marker analysis, and orthogonal mass-spectrometric confirmation provides a scientifically robust strategy for the standardization, authentication, batch-to-batch consistency, and quality assessment of flavonoid- and isoflavonoid-containing herbal products.¹⁷⁻²⁰ The extracted samples are studied using various analytical techniques, as shown in

Tables 3 to 6.

Extraction Technique	Principle	Typical Solvents/Reagents	Applications to Target Compounds	Advantages	Limitations
Maceration Extraction	Plant material is soaked into the solvent at room temperature to dissolve phytochemicals.	Methanol, ethanol, aqueous ethanol, acetone	Quercetin and cladrin extraction from herbal materials.	Simple, inexpensive, minimal equipment.	Long extraction time and lower efficiency.
Soxhlet Extraction	Continuous hot solvent extraction through repeated washing cycles.	Methanol, ethanol, hexane-methanol mixtures	Isolation of quercetin and isoflavonoids from plant matrices.	High extraction efficiency and reproducibility.	High solvent consumption and possible thermal degradation.
Ultrasound-Assisted Extraction (UAE)	It can destroy the walls of the plant cells, causing greater infiltration of the solvent.	Ethanol-water, methanol-water mixtures	Recovery of quercetin and soybean isoflavonoids.	Reduced extraction time and solvent usage.	Optimization of sonication conditions required.
Liquid-Liquid Extraction (LLE)	A substance is said to undergo partitioning between two immiscible	Ethyl acetate, dichloromethane, hexane	Isolation of equol and glyceollin from biological	Good purification and concentration of analytes.	Labor-intensive and solvent-intensive.

	solvents, for example oil and water, due to differences in the solubility of the substance.		and plant samples.		
Solid-Phase Extraction (SPE)	Sample application: Analyst(s) are adsorbed by a solid sorbent and then sequentially eluted.	C18 cartridges, polymeric cartridges	Widely used for analysis in plasma, serum, and urine before LC-MS/MS.	High recovery, reduced matrix effects, improved sensitivity.	Additional cost of cartridges and optimization.
Protein Precipitation Extraction (PPE)	Proteins are precipitated and removed from biological samples before analysis.	Acetonitrile, methanol	Quercetin and equol determination in plasma samples.	Rapid and simple sample preparation.	Less selective than SPE.
Column Chromatographic Purification	Separation based on differential adsorption on stationary phase.	Silica gel, HP-20 resin, Sephadex	Purification of glyceollin's from soybean extracts.	High purity fractions obtained.	Time-consuming and requires large solvent volumes.
Microextraction Techniques (SPME, DLLME)	Extraction using very small solvent volumes or coated fibres.	Various organic solvents or coated fibres	Emerging approach for trace-level equol and flavonoid analysis.	Environmentally friendly and highly sensitive.	Specialized equipment and method development required.

Table 2. Comparing the extraction techniques performed for the sample preparation, applications, advantages and limitations.

Analytical Methods Performed

Glyceollin

S.NO	METHOD	PARAMETER	RESULTS	REFERENCE
1.	MS-MALDI	Procedure for purification: HP-20 resin column (35cm inner diameter × 50cm): Water→70%methanol→methanol stepwise elution. Silica gel column (35cm inner diameter× 50cm, 20kg silica gel PSQ-100B): Hexane: ethyl acetate = 1:1 elution. HPLC to obtain pure glyceollin II with a UV detector	Procedure: PDA-UPLC-MALDI-MS Identification: ¹ H and ¹³ C NMR (400 MHz, acetone-d ₆ , 25 °C) RETA: Glyceollin II - 11.9min MSions: Daidzein, m/z 253.0 ([M+H]) Glyceollins I-III, m/z 337.1 ([M+H]) MALDI-MS conditions: Negative ion linear mode, resolution 100 m Matrix: 1,5-DAN (10 mg/mL in 70% ACN)	²¹
2.	HPLC	Method: RP-HPLC-UV Instrument: Shimadzu LC-Prominence Column: ODS C18 Sample preparation: concentrated extract redissolved in bi-distilled water, then centrifuged (8000 rpm, 20 min) Injection volume: 5 microliters	All biotic elicitors, like biotic stress, resulted in substantial glyceollin accumulation in comparison with the control. Glyceollin I increased 2–3-fold when adding biotic stress factors alone; a 4–7-fold increase when the cultures were treated with <i>S.</i>	²²

		temperature: 25° C Elution rate: 1.0 ml/min Detection wavelength: 280 nm	<i>cerevisiae</i> , ragi tape in the light, and most notably a 7-fold increase (and the largest quantity of isoflavonoids) when ragi tape + light was used on <i>S.cerevisiae</i> . It concluded that the maximum quantity of isoflavonoid accumulation was seen by use of ragi tape together with light treatment, which led to the largest quantity of isoflavonoids and the strongest antioxidant activity.	
3.	LC-ESI-MS/MS	Method: RP-HPLC+ LC-ESI-MS/MS Column: RP-C18 Mobile phase: ACN:Water Elution: Gradient UV detection: 285nm	Parent glyceollins (MS): Glyceollins I–III: m/z 337 Phase I metabolites: m/z 353: Hydroxylation/ Epoxidation m/z 355: Hydrolysis product m/z 371: Diol addition m/z 381: Diol + oxidation Phase II conjugates: m/z 417: Sulfate conjugate m/z 433: Sulfate + oxygen m/z 451: Sulfate + hydrolysis m/z 513: Glucuronide conjugate m/z 531: Cysteiny glycine conjugate m/z 474: Cysteine conjugate m/z 516: Mercapturic acid conjugate	23

Table 3. Analytical methods performed on Glyceollin**Equol**

S.NO	METHOD	PARAMETER	RESULTS	REFERENC E
1.	HP LC-MS/MS	Technology: HPLC-MS/MS system sample pretreatment methods: SPE(Solid Phase Extraction) elution system: gradient elution eluent: A: 0.1% formic acid; B: 0.1% acetonitrile-formic acid column temperature: 40 C Detector type: ESI - negative ions	Linearity: Serum: 7.8-1000.0 ng/mL; Urine: 39.1-5000.0 ng/mL. Correlation coefficients (r) 0.9948 to 0.9984. Precision, Accuracy & Stability US FDA criteria met. Application: Moderately positive correlation of serum genotype genistein (GEN) to FF level (Spearman's $r=0.44$, $p<0.05$)	24
2.	UP LC-MS/MS	Method: LC-MS Method Column: T3 column Dimensions :2.1 x 100mm Particle size:1.8 um Column temperature: 40 °C Samples storage: Autosampler vial @ 4 °C	Linearity: R 0.996 (Concentration range from 5-1000ng / mL) Precision: Intra-day: 5.3% Inter-day: 8.6% Accuracy: 5.2%	25

		Injection volume:10 L (full loop injection) Mobile phase eluent A: water:acetonitrile (95:5, v/v) 0.01% formic acid; B: Acetonitrile:water (95:5, v/v) 0.01% formic acid	Recovery during extraction: 60.6 to 82.3% Repeatability <15% Matrix-effect Not detected Carry-over Not detected Stability Stable up to 7 days stored at 21C and 5C	
3.	LC-MS/MS	Technique LC-MS Column Phenomenex C18 Particle size 3.5 µm Mobile phase eluent A 0.1% HOAc/ 0.1% MeCN/ 13 mM N H₄OAc/ (pH = 4); B 10 0% MeCN/ 0.1% HOAc Elution rate 0.2 5 mL/ min Column temperature:40°C Detection: HPLC stream through ESI source	Precision (%RSD): 3.33-9.24% within Day; 8.5-19.1% day to Day. Accuracy: <100% at low and >90% at medium and high concentrations. Sensitivity: LOD: 2ng/ml. LOQ: 6 ng/mL. Application: 36% of subjects analyzed were equol producers (>68.3mol/d); equol excretion was detected during the first 6 and 12 months.	26
4.	HPLC AND GC	Methods: HPLC-UV and GC HPLC Method: Column HP-Inertsil ODS2 C18 (250 4.6mm, 5m) with guard column Mobile phase eluent Acetonitrile: 5% with Acetic acid (23:77, v/v) Elution rate: 1.0mL/min Detection Wave Length: 254nm GC method: Column BP-1capillary column Carrier He Flow Speed: 40cm/s Oven :150~300Cat4C/mins	Sensitivity: LOD = 8 ng/mL (S/N=2) Linearity: By dilution: R = 0.9982 By addition: R=0.9989 Accuracy (Recovery): Recover y: 97.0% Expected/ Observed ratio: 92.0-100% Precision: %CV (RSD): 1.27-4.86%	27
5.	LC-E SI-MS/MS	Column: Atlantis C18 Mobile phase eluent: ACN: 0.1% CH₃COOH Elution: Isocratic Elution rate: 0.6 ml/min	Linearity: 0.5-500 ng/ml. Matrix effect: No obvious matrix effect was found. Application: LC-MS/MS validated assay in rat plasma for formononetin (IFN), daidzein (DZN), and equol. Also used to study permeability, protein binding, and pharmacokinetic properties of IFN with the LC-MS/MS method. High permeability and bioavailability (oral 21.6%) were found.	28
6.	GC-MS	Method: GC-MS Matrix: Human urine Sample preparation: SPE, Enzymatic hydrolysis Internal standards: d-DAI, d-GEN and 13C BPA	Sensitivity (LOD) DAI: 4ng/ml EQ:4ng/ml GEN: 5ng/ml BPA: 3ng/ml Precision (Inter-assay %CV) DAI: 9% EQ: 15% GEN: 18% BPA:10% Application: 8 to 128 ng/ml of EQ can be observed in more than half of the subjects. The	29

			highest urinary concentration following a meal containing 50g soybean protein can reach GEN:820ng/ml, DAI: 960ng/ml, and EQ:1740ng/ml.	

Table 4. Analytical methods performed on Equol**Cladrin**

S.NO	METHOD USED	PARAMETER	RESULTS	REFERENCE
1.	LC -MS/MS	Method: Analysis by RP-HPLC Column: XBridge Shield RP-18 Particle size: 5 m Eluent: ACN: Aq. FA (70:30, v/v)	Linearity: 1-600 ng/mL Sensitivity: LLOQ = 1 ng/mL Pharmacokinetics of test: RL: C _{max} = 4.23 ± 0.61 ng/mL, T _{max} = 5.5 ± 1.0 h CL: C _{max} = 26.97 ± 1.14 ng/mL, T _{max} = 3.5 ± 1.0 h	³⁰
2.	LC -MS/MS	Technique: RP-HPLC-MS/MS analysis, Column XBridge Shield RP-18, size:4.6x50 mm, Mobile phase eluent: Acetonitrile: 0.1% aqueous and formic acid (70:30, v/v). Detection: MS/MS.	Linearity: 1-600 ng/ml Sensitivity: LLOQ = 1 ng/ml Application (Pharmacokinetics): RL: C _{max} =4.23 ± 0.61 ng/mL, T _{max} =5.5 ± 1.0 hr CL: C _{max} =26.97 ± 1.14 ng/mL, T _{max} =3.5 ± 1.0 hr	³¹
3.	UPLC - MS/MS	Column: C18 column Elution: Isocratic Mobile phase eluent: acetonitrile: 0.10% and formic acid (65:35) Elution rate: 0.60 ml/min	Run time: 2.50 min Linearity: R > 0.99 (0.20-200 g/L concentration range). Sensitivity: High sensitivity. Application (Pharmacokinetics): Absolute bioavailability: 16.58%, 19.04%, and 6.76% (5, 10 and 20 mg/kg orally administered). T _{max} : 3.0 h	³²

Table 5. Analytical methods performed on Cladrin**Quercetin**

S.NO	METHOD USED	PARAMETER	RESULTS	REFERENCE
1.	HPLC-DAD	Technique: RP-HPLC-DAD Instrument: JASCO HPLC system Pump: PU-4180 Autosampler: AS-4050 Detector: DAD (MD-4010) Column: LiChroCART C18 (LiChrosorb RP-18) Elution: Isocratic Elution rate: 1.0 to 1.5 ml/min Wavelength: 254nm, 368nm Injection volume: 10 microliters	linearity: >0.995 precision: (repeatability) (%CV) 2.4-6.7 (intermediate precision) 7.2-9.4% accuracy:88.6-110.7% sensitivity: LOD = 0.046g/mL LOQ = 0.14g/mL Stability: Quercetin was more stable at 4C rather than Room temperature and 20C.	³³
2.	HPLC DAD	Mobile phase eluent: Methanol: Methanol/water/CH ₃ COOH (100:150:5) Elution rate: 1.3 ml/min Column temperature: 30°C	Linearity Rutin R=0.995 (60–180g/mL) Quercetin R=0.9907 (2–10g/mL) Sensitivity Rutin LOD=6.36g/mL, LOQ=19.28g/mL Quercetin	³⁴

			LOD=0.58g/mL, LOQ=1.77g/mL Accuracy (recovery): 96.0% to 100.8%	
3.	RP-HPLC	Method: RP-HPLC Column: C18 Mobile phase eluent: Methanol Elution: Isocratic Elution rate: 1.0 ml/min Wavelength: 370nm	Linearity: 10-70 g/mL (r = 0.9986) Sensitivity: LOD: 1.2 g/mL LOQ: 4.0 g/mL Precision (Reproducibility): Same day: %RSD = 0.05-0.84% Different instruments: %RSD = 0.89-1.55% Accuracy (Recovery): 98.2-101.3% (at 40, 50, and 60 g/mL)	35
4.	RP-HPLC	Instrument: Younglin ACME 9000 Column: C18 (250 4.6 mm i.d.) Mobile phase eluent: Methanol : 0.05% orthophosphoric acid (80:20, v/v) Elution rate: 0.7 mL/min Detection: UV-Visible detector at 254 nm.	Elution time (Rt): 8.78 min Precision (Repeatability): 50.48±0.40 %RSD: 0.78% LOD: 0.0048 g/mL LOQ: 0.039 g/mL Linearity: R >0.99 Method Validation Results: Method meets all validation limits and demonstrated linearity.	36
5.	RP-HPLC	Method: RP-HPLC Column: RP-C18 Elution rate: 1.0 ml/min Wavelength: 392nm Sample preparation: PPE (protein precipitation extraction)	Elution time (Rt): Quercetin (QUE): 5.5 min. Curcumin (CUR): 12.1 min. Linearity: 2-10 g / mL (QUE: R = 0.9998, CUR: R = 0.9993). Accuracy (Recovery): >95%. Precision: %RSD < 2%. Sensitivity (Plasma): (QUE: LOD= 0.18 g/mL, LOQ= 0.54g / mL, CUR: LOD = 0.35 g/mL, LOQ= 1.0g/mL)	37
6.	RP-HPLC	Technique:- RP-HPLC-UV assay Column: RP-C18 Elution: Isocratic Mobile phase eluent: A: Water+0.1% Formic acid; B: Methanol:Acetonitrile(40:15, v/v) Composition: 40:60(v/v) Elution rate: 0.8 ml/min Wavelength of detection: - 254nm Elution time: 7.58 min Validation: - according to ICH Q2B Guidelines.	Linearity: 5.0-17.5 g/ mL (R = 0.996) Sensitivity: LOD:2.28 g/mL LOQ:6.92g/mL Robustness: Changes in composition, wavelengths and elution rate of the mobile phase eluents showed variation % of 4 %. Application: The amount of quercetin in leaves was 0.0055 % w/w.	38
7.	RP-HPLC	Method: HPLC Elution: Gradient Mobile phase eluent: ACN: Glacial Acetic acid Elution rate: 1.0 ml/min Wavelength: 395nm	Linearity: Curcumin: 50-250 ng/mL Quercetin: 800-1600 ng/mL Sensitivity: Curcumin: LOD = 11.76 ng/mL, LOQ = 35.6 ng/mL Quercetin: LOD = 32.94 ng/mL, LOQ = 99.76 ng/mL Accuracy (recovery): 95-105% Precision: %RSD < 2%	39

8.	RP-HPLC	Technique: RP-HPLC-UV analysis Column: Phenomenex C18 column (250 4.6 mm I.D.) Mobile phase eluent: Acetonitrile: 10 mM phosphate buffer (50:50, v/v) Elution rate: 1.0 mL/min Detection: UV-Visible detector (370 nm)	• Elution time (Rt): 3.392 min • Linearity: 5-25 g/mL • Accuracy (Recovery): - For Formulation1: 99.82-99.88% - For Formulation2: 98.18-98.92% • Precision: %RSD = 0.02053-0.05273 • Sensitivity: - LOD: 0.9053 g/mL - LOQ: 2.5435 g/mL • Robustness: By 2/3rd full factorial design (Design Expert software) • Application (Assay): - Market capsule: 98.38 % - In-house cream: 97.40%	40
9.	RP-HPLC	Technique: RP-HPLC-UV analysis. Column: Reversed-phase C18 column Mobile phase eluent: Methanol: Water (70: 30, v/v) + 0.1% trifluoroacetic acid Elution rate: 0.8 mL/min Detector: UV detector Quercetin(Q):368 nm Methyl quercetin (MQ):354 nm	Linearity: 0.15-1.5 g/mL (R > 0.99) Selectivity: There was no significant interference from additives, such as egg-lecithin and octyldodecanol. Precision: %RSD < 2 % (intraday and interday) Accuracy (Recovery): Q: 98.9 to 103.46% MQ: 98.9 to 102%	41
10.	RP-HPLC	Technique: HPLC analysis. Column: HypersilBDS-CPS C18 Particle size: 5µm Mobile phase eluent: Methanol: Water (35:65, v/v) with 2% acetic acid. Elution rate: 1mL/min Injection volume: 20µL Detection wavelength: 254nm	Linearity: 0.05-200 g/mL (R = 0.9997) Selectivity: No interference found in quercetin peak Sensitivity: LOD: 18 ng/mL LOQ: 29 ng/mL Precision: %RSD < 2% Accuracy (Recovery): Non-ionic emulsion: 93% Anionic emulsion: 94%	42
11.	HPLC	Column: LC-18T C18 Mobile phase eluent: 0.3% trichloroacetic acid+water: ACN (50:50) Elution rate: 0.9 ml/min Run time: 13 mins Detection wavelength: 254 nm	Linearity: 0.10-25 g/mL (R > 0.994) Precision: %RSD < 20% Accuracy: deviation from predicted < 20% Conclusion: Method exhibits satisfactory linearity, precision and accuracy over a determined range of analytes tested	43
12.	HPLC	Method Technique: HPLC-UV Column: Thermo Hypersil Gold C18 Particle Size: 5 m Guard column: Zorbax Eclipse Plus C18 cartridge Size: 12.5×4.6 mm Particle Size: 5 m Mobile phase eluent: ACN: 2% acetic acid (40:60) Elution: Isocratic Elution rate: 1.3 mL/min	Linearity: 1.25-200 g/mL (R = 0.99993) Sensitivity: LOD: 0.00488 g/mL LOQ: 0.03906 g/mL Precision (%RSD): Intraday: 0.432-0.806% Interday: 0.323-0.9968% Accuracy: Intraday: 99.589-100.821%	44

		Detection Wavelength: 370 nm (UV)	Interday: 99.665-103.060%	
13.	HPLC	Column: C18 Mobile phase eluent: Methanol: Water (65:35) + CH ₃ COOH Elution rate: 1.0 ml/min Detection wavelength: 254 nm	linearity: 5-100g/ml (R=0.9995) coefficient of determination: R > 0.999 (proved for good linearity) intra-assay: %RSD < 2% inter-assay: %RSD < 2%	45
14.	HPTLC	Method: HPTLC-Densitometry Stationary phase: F TLC plates Mobile phase eluent: Toluene: Ethyl acetate: Chloroform: Formic acid (6:4:4:1) Detector: densitometric scanner at 240 nm	Linearity: 100-1000 ng/band Quercetin: R = 0.9764 Kaempferol: R = 0.9823 Accuracy (Recovery): Quercetin: 98.60% Kaempferol: 90.08%	46
15.	HPTLC	Method: HPTLC Stationary phase: Silica gel 60F Mobile phase eluent: Toluene: Ethyl acetate: Formic acid (5:4:1) Detection: UV(270 nm)	Linearity: Gallic acid (GA): 100-1000 ng/band (R = 0.9991) Quercetin (QE): 150-900 ng/band (R = 0.9956) Sensitivity: GA: LOD = 23 ng/band, LOQ = 69 ng/band QE: LOD = 41 ng/band, LOQ = 123 ng/band Accuracy (Recovery): GA: 99.4-99.9% QE: 98.7-99.4% Precision: GA: 1.98% QE: 1.97% Application (content test estimation): GA: 0.69% +/- 0.01% w/w QE: 0.57% +/- 0.01% w/w	47
16.	UV	Preparation of the stock solution was done, and then scanning was performed to find out the λ_{max} of the solution. The λ_{max} of quercetin was found to be 376 nm.	Linearity: R = 0.9997. Validation: Was checked by intra- and inter-daily precision studies. Sensitivity: LOD: 0.1805 g/mL LOQ: 0.5470 g/mL.	48
17.	UV	Bahan a. Tumbuhan : Daun Tamarind (Tamarindus indica L.) diambil dari Mantrijeron, Yogyakarta. B. Alat: Genesys 10S UV-Vis Spectrophotometer c. Reagen a. Aquadest b. Etanol pro analysis (Merck) c. Etanol 70% d. Standar kuersetin (Sigma-Aldrich) e. Kertas filter dan alufoil	Maximum wavelength (max): 361.8 nm Calibration Curve Regression equation: $y = 0.0616x - 0.018$ Correlation coefficient (R): 0.9999 Linearity: Vxo value 5% (linear performance was good) Sensitivity: LOD: 0.1515 ppm LOQ: 0.4592 ppm	49
18.	UFLC	The phytochemicals were separated through fractionation using a Phenomenex C18 column and mobile phase eluent: 80% ACN and 20% Ammonium acetate (pH 3) Elution rate: 1 mL/min Wavelength: 380 nm.	Linearity: R = 0.994-0.999 Quantification: - Curcumin: 3.16% w/w Piperine: 13.92% w/w Rutin: 15.19% w/w Quercetin: 0.36% w/w	50
19.	QbD-based HPLC	Column: C18	Linearity: 2-50 g/mL Accuracy (Recovery): 93.6-96.2%	51

		Mobile phase eluent: ACN: Ammonium acetate (35:65) + CH ₃ COOH Elution rate: 0.7 ml/min	Precision (Recovery): 93.7-96.5% Sensitivity: LOD: 12.1 ng/mL LOQ: 36.6 ng/mL Conclusion: The method exhibited linearity, accuracy, precision and sensitivity in good measure.	
20.	RP- and NP-HPTLC	Method: RP/NP-HPTLC Stationary phase: Silica gel 60 RP-HPTLC Mobile phase eluent: ACN:Water (4:6) NP-HPTLC: Toluene:Ethyl acetate: formic acid (6:4:0.8)	Regression Equation: $Y = 10.08x + 216.85$ Linearity: RP-HPTLC: 200-1400 ng/spot; NP-HPTLC: 100-1200 ng/spot correlation coefficient: $R = 0.9982 \pm 0.0001$ Sensitivity (Quercetin): LOD: 9.67 ng/band LOQ: 29.31 ng/band Biomarker content in extract: Gallic acid: 7.01 g/mg, Rutin: 2.42 g/mg, Quercetin: 1.53 g/mg, Naringenin: 0.14 g/mg.	⁵²

Table 6. Analytical methods performed on Quercetin**Challenges and limitations**

Despite significant advances in chromatographic and spectrometric techniques, the accurate determination of glyceollins, equol, quercetin and cladrin remains challenging because these compounds are frequently present in complex plant extracts and biological matrices. Co-extracted proteins, lipids, carbohydrates, pigments and other phenolic constituents may interfere with analyte recovery, chromatographic separation and detector response, resulting in matrix effects and reduced method accuracy.^{53,54} Therefore, efficient sample clean-up using techniques such as LLE, PPE or SPE is often required. The low abundance of equol and glyceollins in plasma, serum and urine further necessitates highly sensitive techniques such as LC-MS/MS and UHPLC-MS/MS, which require expensive instrumentation, regular maintenance and technically skilled personnel.⁵⁵ Glyceollins also occur as structurally related isomers with similar physicochemical properties, making their chromatographic resolution and individual quantification difficult. In addition, flavonoids may undergo degradation during extraction, storage and analysis because of exposure to light, oxygen, temperature variations and changes in pH. Differences in plant species, geographical origin, harvest period, processing conditions, extraction solvents and sample-preparation procedures can produce substantial variation in analyte recovery and reproducibility.⁵⁶ The absence of universally accepted extraction protocols, certified reference materials and harmonized validation procedures for several phytochemicals' limits comparison among laboratories. Although advanced hyphenated techniques improve sensitivity and selectivity, their high operational cost restricts their routine use in resource-limited quality-control laboratories.⁵⁷ These limitations emphasize the need for improved stability control, standardized sample preparation, matrix-matched calibration, isotope-labelled internal standards and inter-laboratory method verification.

Pharmaceutical applications

Glyceollins, equol, quercetin and cladrin possess considerable pharmaceutical and nutraceutical potential because of their antioxidant, anti-inflammatory, antimicrobial, immunomodulatory, estrogenic, cardioprotective, anticancer and osteoprotective activities. Glyceollins may be useful as bioactive ingredients in products targeting oxidative stress, inflammation, microbial disorders and hormone-dependent cancers.^{58,59} Equol, a microbial metabolite of daidzein, has potential capability in managing menopausal symptoms and in the formulations intended to support bone health and cardiovascular function. However, its therapeutic response may vary considerably between individuals because equol production depends on the composition and metabolic activity of the intestinal microbiota.^{60,61} Quercetin is one of the most extensively investigated flavonoids and has been incorporated or explored in pharmaceutical, nutraceutical and functional-food formulations for antioxidant, anti-inflammatory, antiviral, cardioprotective, anticancer and neuroprotective applications. Validated HPLC and HPLC-DAD methods have also supported the assay and quality control of quercetin in nanoparticles, topical emulsions, creams, capsules, extracts and self-nanoemulsifying drug-delivery system.⁶² Cladrin has demonstrated osteogenic and antioxidant properties and may be investigated as a potential candidate for bone-related disorders, including osteoporosis.⁶³ In pharmaceutical development, these compounds may additionally serve as marker compounds for the standardization of herbal extracts, raw materials and finished formulations. Nevertheless, pharmaceutical translation requires systematic evaluation of their solubility, permeability, stability, bioavailability, metabolism, pharmacokinetics, toxicity and drug-herb interactions.

Future applications

Future research should focus on developing sensitive, selective, rapid and environmentally sustainable analytical

methods capable of simultaneously determining multiple flavonoids, isoflavonoids and their metabolites in pharmaceutical, nutraceutical and biological matrices. UHPLC–MS/MS is expected to support high-throughput trace-level quantification, while high-resolution platforms such as QTOF-MS and Orbitrap-MS may improve accurate-mass measurement, metabolite identification, isomer differentiation and structural characterization. The integration of chromatographic separation with metabolomics, molecular networking and advanced chemometric tools could enable comprehensive fingerprinting and the discovery of previously unrecognized metabolites or degradation products. Automated sample preparation, miniaturized extraction, microextraction, online solid-phase extraction and greener solvent systems may reduce analysis time, solvent consumption and operator-dependent variation. Analytical Quality by Design principles should be increasingly applied to identify critical method parameters, establish method operable design regions and improve method robustness during routine quality control. Future pharmaceutical applications may include standardized botanical drug products, phytoestrogen-based therapies, bone-health formulations, antioxidant and anti-inflammatory products, topical preparations, nano-formulations and targeted delivery systems. Improving the aqueous solubility, chemical stability, intestinal absorption and systemic exposure of these compounds through nanoparticles, liposomes, phytosomes, nano-emulsions, solid dispersions and controlled-release systems may enhance their therapeutic usefulness. In parallel, pharmacokinetic, pharmacodynamic and clinical studies should connect validated analytical measurements with exposure–response relationships and biomarkers of efficacy. Large-scale studies, including investigations of microbiome-dependent equol production, are also needed to support personalized use of phytoestrogen-containing products. Ultimately, harmonized reference standards, validated multi-analyte methods, stability-indicating assays, regulatory-grade quality specifications and well-designed clinical trials will be essential for translating these phytochemicals from promising bioactive markers into reliable pharmaceutical products.

Conclusion

More sophisticated analytical methodologies are necessary for proper standardization and quantitation of bioactive flavonoids and isoflavonoids in herbs, pharmaceutical formulations, nutraceuticals, and biological samples. The reviewed literature clearly shows that there is no one method that can be regarded as sufficient for all possible purposes. HPLC, HPLC-DAD, and HPTLC are still useful for routine quality control, fingerprinting, and marker compound assays, while LC-MS/MS, UHPLC-MS/MS, and high-resolution MS allow higher selectivity and sensitivity to perform trace level quantitation, metabolite profiling, isomeric separation, and pharmacokinetics studies. Optimized sample preparation using ultrasound-assisted extraction, hydrolysis, protein precipitation, liquid-liquid extraction, and solid phase extraction is of equal

importance in order to avoid any losses of analytes and matrix interferences. Analysis of quercetin, equol, glyceollins, and cladrin proves the necessity of developing methods that would be suitable for specific compounds and matrices. However, differences in the origin of plants, extraction procedure, stability of analyzed compounds, matrix effects, lack of reference substances, and expensive instrumentation are still the problems of analytical methods development. In the end, there will be more consistent batches of drugs, which will be used in studies of pharmacology and pharmacokinetics, thus making it possible to translate useful phytochemicals into effective drugs.

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Authors' Contribution

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

Availability of Data and Materials

All data generated or analyzed during this study are included in this article. Further, the data used and/or analyzed during the current study is available from the corresponding author on reasonable request.

Consent to Participate

Not applicable.

Consent for Publication

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Note

Use of Artificial Intelligence-assisted Tools:

The authors declare that they have not used artificial intelligence (AI)-tools for writing and

editing of the manuscript, and no images were manipulated using AI.

List of Abbreviations:

Solid Phase Extraction (SPE); Liquid-Liquid Extraction (LLE); High-Performance Liquid Chromatography (HPLC); Ultra High-Performance Liquid Chromatography (UHPLC); Acetonitrile (ACN); Quality by Design (QbD)

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