Marker Discovery in Herbal Plants: Recent Advancements in Analytical Techniques coupled with Chemometrics

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Abstract. This review delves into the recent surge of advanced analytical techniques revolutionizing marker discovery in herbal plants. It explores the potential of various chromatography and spectroscopy methods, including hyphenated approaches, for robust identification and quantification of bioactive markers. The power of chemometrics is emphasized as a complementary tool, enabling in-depth analysis of complex herbal matrices and unraveling the chemical fingerprints responsible for therapeutic effects. The discussion acknowledges the analytical challenges associated with marker discovery. Furthermore, it highlights advancements alongside the emergence of novel techniques that are facilitating easier marker discovery from these complex botanical sources.

Keywords: Marker discovery, Chromatography, Spectroscopy, Chemometrics, Advanced analytical techniques

1 Introduction

The World Health Organization (WHO) estimates that 60% of the world's population relies on herbal medicine and about 80% of the population in developing countries depends almost totally on it for their primary healthcare needs (Khan & Ahmad, 2018). The quality of herbal medicine is altered by various physical, chemical, geographical aspects and the time of harvesting, which contribute to the quality of these materials. Apart from that, adulteration is also an increasing concern when it comes to herbal material quality. For the standardization of herbal medicines, various analytical methods and markers were used (Kulkarni et al., 2019).
The markers are typically well-characterized chemical constituents that are unique to a particular plant species or variety. Their selection is predicated upon their chemical and pharmacological characteristics, as well as their ability to exemplify the overall quality of herbal medicine. (Shi et al., 2014).Analytical markers are compounds that are abundant in the plant and can be used to identify and quantify the plant material. Markers are useful for quality control and standardization, useful in identifying herbal materials, and used to detect adulteration (Li et al., 2020).

Analytical techniques are critical in identifying chemical markers for herbal medicines. Through chemical profiling, these analytical methods allow for the comprehensive assessment of the chemical constituents in herbal products. By identifying phytochemical markers

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characteristic of specific botanicals, these methods help verify the presence of authentic ingredients and detect any potential adulteration or substitution (Upton et al., 2020).

Chemometrics is instrumental in analyzing markers for botanical materials, ensuring data quality through meticulous preprocessing to address variations in raw spectral data. It processes and standardizes raw data, enabling targeted analysis which aids in identifying unique metabolites, validating botanical authenticity by comparing compounds against standards, to identify specific markers associated with quality and therapeutic properties. By integrating multidimensional data types, it offers a comprehensive view for marker discovery. The integration of diverse datasets enhances authenticity models, incorporating chemical, bioactivity, and genetic information for comprehensive quality control. Chemometrics' modeling and prediction capabilities differentiate samples based on factors like composition, origin, and adulteration, facilitating authentication and classification. Chemometrics also plays a crucial role in safeguarding the accuracy, reliability, and regulatory compliance of botanical analyses, contributing to the integrity and authenticity of herbal products (Abraham & Kellogg, 2021).

The herbal kingdom remains largely unexplored, with countless potential markers yet to be discovered. Understanding the chemical profiles of medicinal plants through markers can lead to the discovery of novel bioactive compounds for drug development, potentially tackling unmet medical needs. Analytical techniques should be designed to target the principal metabolites, which will streamline marker discovery and accelerate the development of targeted herbal medicines. While LC-MS, GC-MS, and NMR are currently the gold standards for metabolite profiling, there is a need for exploring alternative and complementary techniques. Development of highly sensitive and specific technologies will provide deeper insights into plant metabolomes and identify previously unknown markers. Metabolomics and chemometrics tools will play a key role in extracting meaningful information from large datasets generated by analytical techniques. This review investigates the cutting-edge techniques for discovering and analyzing potential markers within medicinal plants.

2 Results and Discussion

2.1 Marker Discovery

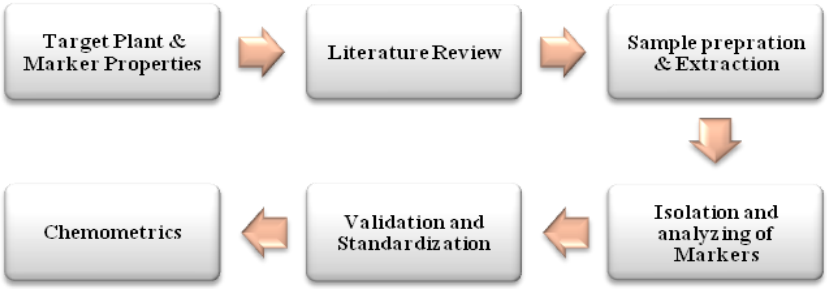

Figure 1. Flow chart on marker discovery. This flow chart illustrates a step-by-step approach for marker discovery(Noviana et al., 2022).

2.2 Possible hyphenation

Table 1: Various hyphenation techniques used in marker discovery. This table explores the diverse hyphenation strategies employed to identify and isolate marker compounds from plant materials.

Sl no.	Hyphenation	Markers	Plant name	Reference
1.	HPTLC–ESI–MS/MS	cordifolioside A, 20-β-hydroxyecdysone and columbin	*Tinospora cordifolia*	(Patel et al., 2021)
2.	HPLC/ESI-MS	2,3-dihydro flavone,3-hydroxy flavone cinnamic acid and rutin	*Cnicusbenedictus (roots)*	(Rezig et al., 2024)
3.	LC-MS/MS	Quinic acid, isoquercitrin, epicatechin, and routine	*Onobrychisargyrea*	(Yeniçeri et al., 2024)
4.	UPLC-Q-TOF-MS/MS	Succinic acid, quinic acid, Quercetin-O- rhamnoside - hexoside, Laricitrin and Quercetin	*Neolitseapallens*	(Thakur et al., 2024)
5.	LC-UV-SPE/NMR	Valine, tachioside, uridine, adenosine and dimethoxy-1,4-benzoquinone etc.,	*Euterpe oleracea Mart.*	(Thomasi et al., 2024)
6.	GC-MS	β-pinene, 1,8 cineole, nerolidol, bornyl acetate andsabinene	*Achillea millefolium*	(Konarska et al., 2023)

2.3 Chemometrics

Chemometric techniques provide a good opportunity for mining more useful chemical information from the original data. Chemometrics has a natural and essential place in the study of medicinal plants. An increasing number of medicinal plants are being studied using comprehensive approaches and hyphenated techniques associated with chemometrics, which are utilized to extract meaningful information and provide numerous data processing methods. Chemometrics is used to resolve the mixture into linear components, optimize experimental protocols, and extract relevant information from chromatographic results. Chemometrics has been proven to be an effective method for determining the herbal drug's quality (He & Li, 2024).

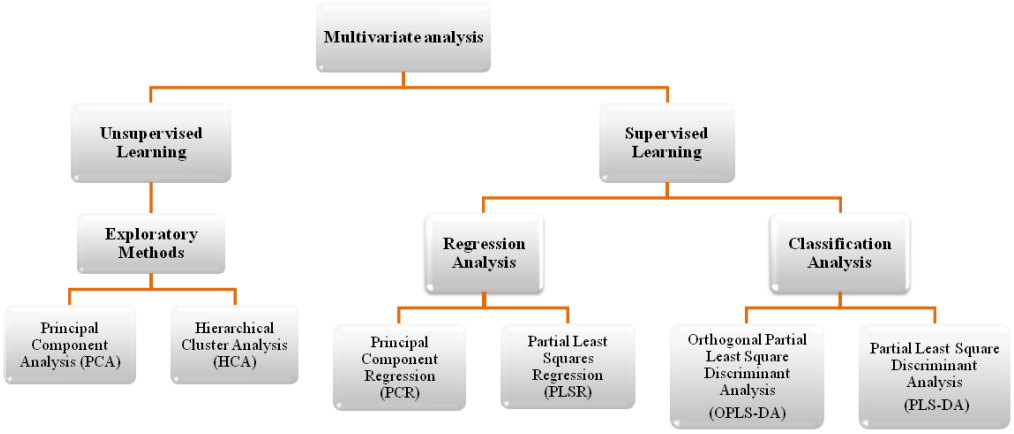

Figure 2: Classification of chemometrics. This diagram illustrates the systematic classification of chemometrics techniques and subfields.

Multivariate analysis

Multivariate analysis refers to statistical techniques used to analyze data sets that contain observations on multiple variables. Multivariate analysis was employed to analyze the

complex chemical composition data obtained from various spectrometry experiments and to discover species-specific markers for the quality and safety control of herbs (Jin et al., 2021).

1) ***Unsupervised Learning*****:**Reduces data dimensionality, detects patterns, and highlights the spectral regions that are connected to quality parameters.

Exploratory Methods: Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) are two of its components. Its primary applications include locating and eliminating peripheral observations, reducing data dimensions, and verifying the existence of samples in the dataset. The unsupervised pattern recognition method can cluster the samples and understand the data structure, but it lacks the standard between recognition and sample classification (Liu et al., 2023).

a) PCA- Unsupervised visualization of data structure for initial exploration.Use PCA to project data into principal components (PCs) for visualization. Visualize the distribution of different species in the PC space to see how they are separated (Abdelmigid et al., 2023).Suitable for large data sets but ignores category information. Use of PCA to remove noise in the data sets. Reduce data dimensionality by identifying latent variables (principal components) that capture most of the variance in the dataset while retaining statistical information. Finds linear combinations with the greatest variance (principal components)(Ivanović et al., 2023). Overall PCA helps in efficient data visualization, identifying clusters, visualizing relationships between samples/variables, identifying outliers, and also dimension reduction for further analysis (Sharma & Yadav, 2023).

b) HCA- Characterizes samples based on relationships between data points. Groups similar samples together into hierarchical clusters.Can cluster data without labels into homogeneous subsets (hierarchical or K-means). It helps in grouping samples based on similarities in metabolite profiles (Rai et al., 2023).

2) ***Supervised Learning****:* Supervised learning utilizes labeled data (training samples) to train a model for classifying new, unknown samples. It applies the trained model to predict categories for new, unknown samples. It performs consistently across diverse datasets but may require retraining for different classification tasks. This method is crucial for identifying HM quality based on their spectral fingerprints (Boateng et al., 2024).

*Regression Analysis:*It establishes a quantitative relationship between spectral data and continuous (numerical) attributes of HMs. It predicts the content of compounds based on spectral data and it determines levels of key bioactive molecules. The key difference from Classification Analysis is that it focuses on predicting numerical values instead of discrete categories (Kucharska-Ambrożej&Karpinska, 2020). It mainly includes Principal Component Regression (PCR) and Partial Least Squares Regression (PLSR). PCR explains variability in predictors without considering response variables.PLSR considers both predictors and response variables and it reduces overfitting risk (Dai et al., 2022).

*Classification Analysis:*Analyze discrete data for classifying HM samples into predefined categories. It also emphasizes similarities within a single class and describes its characteristics. It is used for species origin verification, impurity detection, and production batch consistency analysis. Classification Analysis mainly includes Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA) and Partial Least Square Discriminant Analysis (PLS-DA)(Klein-Junior et al., 2021).

a) Orthogonal Partial Least Square Discriminant Analysis (OPLS-DA): It integrates partial least squares regression with discriminant analysis. It is specifically designed for discrimination and classification tasks. Discriminates the closely related species and is also involved in identifying potential chemical markers that could distinguish them effectively. This model reveals a good classification and prediction ability, allowing for accurate

prediction of the origin of ungrouped samples. OPLS-DA integrates the systematic variation in the data into predictive and orthogonal components. The predictive component is related to class discrimination, while the orthogonal component captures the fluctuation unrelated to class separation. This method focuses on maximizing the separation between different groups in the data. OPLS-DA improves PLS-DA, overcomes overfitting and data distribution issues, and is appropriate for collinear and linear data (Ikhsanet al., 2021).

b) Partial Least Square Discriminant Analysis (PLS-DA): It is a supervised method that combines partial least squares regression (PLSR) and linear discriminant analysis (LDA).PLSR considers both predictors and response variables and it reduces the overfitting risk. LDA finds a linear combination of features that best separates classes. PLS-DA is good for collinear and linear data and provides insights into discrimination causes. This method is mainly used to identify differential metabolites between groups (Abraham & Kellogg, 2021).

2.4 Recent advancements in analytical techniques

Metabolomics is a field of omics science that focuses on the comprehensive study of tiny molecules, known as metabolites, inside biological systems such as cells, tissues, or organisms. Metabolites are the end products of cellular processes and their levels can provide valuable insights into the biochemical pathways and physiological status of an organism. Metabolomics key tool in the identification of markers in herbal plants by providing valuable insights into the metabolic profile of these plants. There are two types of metabolomics targeted and untargeted metabolomics. Untargeted metabolomics makes it possible to thoroughly analyze every metabolite in a sample without prior knowledge of their identities. Targeted metabolomics aims to quantify particular metabolite classes, such as phenolic compounds, alkaloids, terpenoids, and flavonoids. Advances in metabolite annotation and identification techniques, such as high-resolution mass spectrometry and database matching, facilitate the accurate identification of metabolites in herbal plants. Metabolite imaging techniques, such as mass spectrometry imaging (MSI) and nuclear magnetic resonance imaging (NMRI), allow for the spatial visualization of metabolites within plant tissues. The availability of metabolomics databases and bioinformatics tools facilitates the analysis and interpretation of metabolomics data from herbal plants. Integrating metabolomics data with genomics, transcriptomics, and proteomics data provides a comprehensive view of the molecular mechanisms underlying the biosynthesis and regulation of metabolites in herbal plants(Yang et al., 2022)(Gajula & Nanjappan, 2020)(Oh et al., 2023).

Multidimensional chromatography is an advanced analytical technique that involves the use of two or more independent separation mechanisms to enhance the separation capacity and efficiency of complex mixtures. By incorporating orthogonal separation mechanisms in different dimensions. Based on a single set of physicochemical properties, the sample is first separated in one dimension using a particular chromatographic column. The separated fractions or components are then further separated in a second dimension using a different chromatographic column with orthogonal separation mechanisms. It provides higher peak capacity and improved resolution compared to one-dimensional chromatography, allowing for better separation of complex mixtures. By utilizing orthogonal separation mechanisms in different dimensions, multidimensional chromatography can effectively separate compounds that co-elute in one dimension, leading to better peak identification and characterization. It can be applied to various types of chromatographic techniques, including GC, LC, and SFC making it a versatile tool(X. Yang et al., 2024)(Ranjan et al., 2023)(Xu et al., 2023).

UHPLC-MS, a potent and popular method, offers a significant improvement over conventional methods for screening and identifying the active ingredients in natural products.The efficiency increases with decreasing particle size of the column, a reduction in

particle size of less than 2 μm leads to an improvement in efficiency that remains constant at increased flow rates or linear velocities. It offers faster analysis times due to the combination of smaller particle size, and higher pressure which results in quicker separations and higher sample throughput. It offers higher efficiency in terms of peak capacity, peak shape, and resolution. Continued developments in column technology have led to the introduction of novel stationary phases, such as superficially porous particles (SPPs) and core-shell particles. These advancements offer improved efficiency, resolution, and peak shape in UHPLC separations. Miniaturization of UHPLC systems has gained attention, leading to the development of micro and nano UHPLC systems(Ahmed et al., 2023).

Direct Analysis in Real Time Mass Spectrometry (DART-MS) operates by rapidly ionizing compounds directly from the sample surface under atmospheric pressure and at ambient conditions, than ions generated are transformed to MS for analysis. Unlike typical MS procedures, DART-MS enables direct sample analysis without requiring considerable sample preparation, such as extraction, derivatization, or chromatographic separation. DART-MS provides high sensitivity and specificity in detecting markers from herbal plants due to its ability to ionize a wide range of compounds directly from the sample surface. DART-MS offers rapid analysis capabilities, allowing for high-throughput screening of samples. DART-MS can be hyphenated with other chromatography instruments like TLC-DART-MS, LC-DART-MS, GC-DART-MS and CE-DART-MS (Wang & Liu, 2023)(De Angelis et al., 2021)(Wang, 2024).

Ion mobility spectrometry (IMS) is an analytical method that uses an electric field to separate and identify ions according to the degree of mobility in a carrier gas. The ions generated from the sample are introduced into the IMS device, typically a drift tube filled with a buffer gas (e.g., nitrogen or helium). An electric field is applied along the length of the drift tube, causing the ions to move through the buffer gas towards a detector at the end of the tube. The ions in the drift tube experience collisions with the buffer gas molecules. The ions mobility, which is influenced by their size, shape, and charge, determines their speed through the drift tube.At the end of the drift tube, the ions are detected by a detector, typically a Faraday plate or an electron multiplier. IMS is often coupled with mass spectrometry (IMS-MS) to combine the separation capabilities of IMS with the accurate mass measurement of MS, this enhances selectivity and sensitivity for complex samples. IMS is efficient in detecting structurally similar compounds and characterizing complex mixtures of compounds present in herbal plant extracts based on their ion mobility properties (Kaufmann, 2020)(Masike et al., 2021).

3 Conclusion

In conclusion, this paper underscores the critical significance of markers in the quality control and standardization of herbal medicines. Through the utilization of advanced analytical methodologies such as chromatography, and spectroscopy markers play a vital role in ensuring the authenticity, efficacy, and safety of herbal products. The integration of multidimensional chromatography with chemometric strategies and cutting-edge metabolomics technologies enables precise identification and quantification of markers in medicinal plants. By emphasizing the importance of markers in herbal medicine, this review contributes to the advancement of analytical techniques and the application of chemometrics to streamline the process of marker discovery from herbal plants. Looking ahead, future research holds immense promise in exploring the potential of novel analytical platforms for even more efficient and comprehensive marker discovery.

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