



Advancing Herbal Product Authentication: A Comprehensive Review Of DNA-Based Approach For Quality Control And Safety Assurance

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ABSTRACT

The escalating popularity of herbal products in recent years has given rise to concerns about their quality and safety. The diverse origins of these products, rooted in local and traditional practices, pose challenges in defining and characterizing them, leading to varying regulatory frameworks and an influx of new dietary supplements and herbal medicines. This review explores the significance of DNA authentication methods, including species-specific PCR, DNA barcoding, metabarcoding, and digital PCR, in addressing the complexities of herbal product quality and safety. These techniques offer diverse applications, from rapid species identification to detecting trace contamination and quantifying adulterants. The review emphasizes the need for collaboration between regulatory authorities, industry stakeholders, and scientific communities to standardize and implement DNA-based authentication methods, establishing a new paradigm for ensuring authenticity and safety throughout the herbal product supply chain. The comprehensive examination of these DNA-based methods provides valuable insights into their role in safeguarding against contamination and adulteration while promoting sustainable sourcing practices in the herbal products industry.

Keywords: DNA authentication, species-specific PCR, DNA barcoding, metabarcoding, digital PCR, quality control and safety of herbal products

Introduction:

Herbal preparations have been integral to healthcare systems globally, with an increasing reliance on commercial herbal products in recent years (Raclariu-Manolică et al., 2023). However, the surge in popularity of these products has raised concerns regarding their quality and safety (Ekor, 2014). The diverse origins of herbal products, stemming from local and traditional practices, make defining and characterizing them challenging. This lack of consensus has led to varying regulatory frameworks and an influx of new dietary supplements and herbal medicines (Thakkar et al., 2020).

The terminology used to describe commercial herbal products includes herbal or botanical drugs, traditional or natural medicines, dietary supplements, and natural health products, each posing unique challenges for quality assessment (Heinrich et al., 2022; Shipkowski et al., 2018). As globalization expands the value chains of these products, concerns about responsible sourcing and sustainable supply chains have come to the forefront (Booker et al., 2012). While some herbal products classified as "medicines" undergo strict quality monitoring, "supplements" often face less rigorous regulations, potentially leading to safety issues (Posadzki et al., 2013).

Supply chain weaknesses, driven by high demand and increasing prices for herbal preparations, can result in accidental contamination or intentional adulteration for economic gain (Heinrich et al., 2022). Fraudulent operators may deceive conventional identification assays, substituting lower-cost plant species or adding non-plant materials to mimic the desired herbal product's chemical profile (Booker et al., 2012).

The application of taxonomic and analytical methodologies for the authentication of medicinal plants is widely recognized within the scientific community. The efficacy of analytical techniques in discriminating closely related plant species relies on the specific secondary metabolites present, a profile influenced by environmental variables, as well as variations in the processing and storage conditions of herbal formulations (Pant et al., 2021). However, for quality assurance and consumer safety, precise identification of the bioactive therapeutic components is paramount (Newmaster et al., 2013).

The necessity for universal authentication systems that can offer taxon identification with high confidence and the ability to differentiate among diverse plant species is evident. In the context of DNA-based authentication, DNA serves as a universal marker unaffected by tissue characteristics, age, environmental conditions, and processing/storage variations (Ancuta Cristina Raclariu, Heinrich, et al., 2018a). Major pharmacopeias globally, including the Chinese Pharmacopoeia, United States Pharmacopeia, British Pharmacopoeia, Japanese Pharmacopoeia, and Hong Kong Chinese Materia Medica, advocate the use of DNA-based authentication methods (Wu & Shaw, 2022).

Various techniques such as Species-specific PCR assays (Li et al., 2021; Noh et al., 2021; Sharma & Shrivastava, 2016; Travadi, Sharma, et al., 2022a) and DNA barcoding (Balaji & Parani, 2022; Selvaraj et al., 2012; Vassou et al., 2016; Zahra, 2019) offer cost-effective means of identifying single or targeted plants. Concurrently, high-throughput sequencing-based metabarcoding (Frigerio et al., 2021; Pandit et al., 2021; Seethapathy et al., 2019; Yao et al., 2022) enables the simultaneous identification of multiple taxa within DNA mixtures extracted from herbal products. Consequently, researchers increasingly favor a multi-methodological or integrated analytical approach, combining both traditional analytical methods and advanced DNA-based techniques for enhanced medicinal plant authentication (Frigerio et al., 2021; Intharuksa et al., 2020; Travadi et al., 2023). DNA metabarcoding, while effective for identifying various components in a mixture, has a limitation in providing quantitative information about adulteration (Ancuta Cristina Raclariu, Heinrich, et al., 2018a). In contrast, Droplet Digital PCR (ddPCR) and Digital PCR (dPCR) are robust techniques that enable the detection and quantification of DNA from adulterants. These powerful methods offer the capability to precisely measure the abundance or concentration of specific DNA sequences, providing a quantitative aspect to the assessment of adulteration in a given sample (Travadi, Shah, et al., 2022; Yu et al., 2022a) (Figure 1; Table 1).

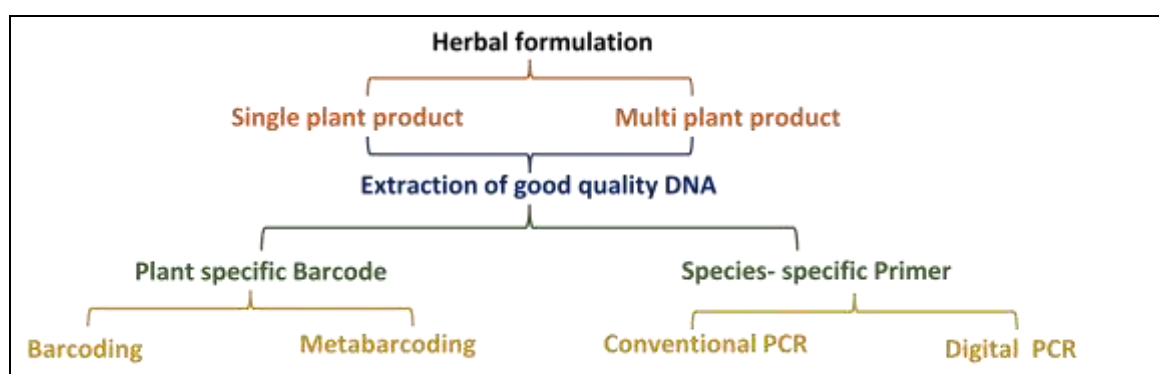


Figure 1. Schematic representation of workflow for DNA-based authentication

Table 1. Key points highlighting advantages and limitations of DNA-based authentication techniques for quality control in herbal products

DNA-based authentication technique	Advantage	Limitation
Species-specific assay	PCR • Rapid detection • Cost-effective • Determination of presence or absence of targeted species	• Inability to detect untargeted adulteration • Potential for non-specific amplification
Barcoding	• Sensitivity and specificity • Robustness • Resolution at Lower Taxonomy	• Inability to determine species in mixtures • Varying resolution power with different barcodes

Metabarcoding	<ul style="list-style-type: none"> • High throughput • Detection of Contamination in Mixtures 	Widespread	<ul style="list-style-type: none"> • Varying resolution power with different barcodes • High sensitivity for trace contamination • Lack of curated reference databases • PCR bias and primer fit compatibility • Not applicable for quantitative determination
Digital PCR	<ul style="list-style-type: none"> • High throughput • Detection of very low quantity of adulteration • Quantitative determination 		<ul style="list-style-type: none"> • Lower dynamic range • Inability to determine untargeted adulteration

This review explores the significance of DNA authentication methods, specifically Species-Specific assay, DNA barcoding, metabarcoding, and dPCR, in addressing the complexities associated with herbal product quality and safety. It highlights their role in identifying and authenticating botanicals, safeguarding against contamination and adulteration, and promoting sustainable sourcing practices within the herbal products industry (Figure 2). Furthermore, the review emphasizes the need for collaboration between regulatory authorities, industry stakeholders, and scientific communities to standardize and implement DNA-based authentication methods. This collaborative effort can establish a new paradigm for ensuring the authenticity and safety of herbal products while fostering transparency and accountability throughout the supply chain (Figure 2; Table 2).

Table 2: Recent reports on applications of DNA-based authentication in herbal plant material

DNA-based authentication protocol	Application on Plant material	Reference
Species-specific PCR assay	<i>Panax ginseng</i> , <i>P. quinquefolius</i> , and <i>P. notoginseng</i> root materials	(Lu et al., 2022)
Multiplex-SCAR assay	Mistletoe species <i>Taxillus chinensis</i> and <i>Viscum coloratum</i> leaves and branch material	(Noh et al., 2021)
Multiplex-SCAR assay (chloroplast <i>trnH-psbA</i> intergenic spacer)	<i>Isatis indigotica</i> dried leaves powder	(Hsieh et al., 2021)
Duplex and Digital PCR assay	<i>Ocimum basilicum</i> and <i>Ocimum tenuiflorum</i> dried leaves powder	(Travadi, Sharma, et al., 2022)
Duplex and Digital PCR assay	<i>Piper nigrum</i> and <i>Carica papaya</i> dried berries	(Travadi, Shah, et al., 2022)
Species-specific PCR and Metabarcoding	<i>Bacopa monnieri</i> and <i>Centella asiatica</i> dried leaves powder	(Shah et al., 2023)
<i>RbcL</i> minibarcoding	Polyherbal formulation of Indian market	(Pandit et al., 2021)
<i>ITS2</i> and metabarcoding	<i>rbcL</i> Single and Polyherbal formulation of Indian market	(Travadi et al., 2023)
qPCR and dPCR	<i>Actaea racemosa</i> (Black cohosh)	(Shanmughanandhan et al., 2021)
Droplet digital PCR <i>ITS2</i> and metabarcoding	<i>rbcL</i> <i>Panax notoginseng</i> 39 Thai herbal products listed on the Thai National List of Essential Medicines	(Yu et al., 2022) (Urumarudappa et al., 2020)

<i>psbA-trnH</i> and metabarcoding	ITS2	(NLEM) 15 Herbal Teas	(Frigerio et al., 2021)
<i>ITS2</i> metabarcoding		62 products, containing basil, oregano, and paprika collected from different retailers and importers in Norway.	(Ancuța Cristina Raclariu-Manolică et al., 2021)
<i>ITS2</i> metabarcoding		18 Milk thistle botanical formulations (teas, capsules, tablets, emulsion)	(Ancuța Cristina Raclariu-Manolică et al., 2023)
<i>ITS2</i> metabarcoding		71 herbal medicinal products were randomly purchased from Greek markets	(Anthoos et al., 2021)
<i>psbA-trnH</i> metabarcoding		3 Hedyotis herbal products collected from China and Thailand	(Yik et al., 2021)
<i>ITS2</i> , <i>matK</i> , <i>rbcL</i> and <i>trnH-psbA</i> barcoding		DNA barcode library of plants listed in the Thai Herbal Pharmacopoeia (THP) and Monographs of Selected Thai Materia Medica (TMM) for 101 plant species	(Urumarudappa et al., 2022)
<i>ITS</i> and <i>matK</i>		Six <i>Momordica</i> species	(Kumar et al., 2020)
<i>ITS2</i> barcoding and metabarcoding	and	30 raw material samples, 10 food products and 12 herbal products	(Zhang et al., 2020)
<i>ITS2</i> barcoding		52 Licorice products from Chinese market	(Li et al., 2023)
<i>ITS2</i> metabarcoding	and <i>trnL</i>	4 TCM preparations from Chinese market	(Yao et al., 2022)



Figure 2. Utilizing DNA-based methodologies for the quality assessment and monitoring of herbal preparations in the value chain

Rapid and cost-effective Species-specific assay

Species-specific PCR assays play a pontial role in the authentication of herbal products, providing a rapid, accurate, and cost-effective means of identifying specific plant species within complex herbal formulation (Wu & Shaw, 2022). The development of these assays involves the use of species-specific primers derived from various sources such as Random Amplified Polymorphic DNA (RAPD)(Dnyaneshwar et al., 2006), Sequence Characterized Amplified Region (SCAR)(Dhanya et al., 2009; Kim et al., 2019; Shah et al., 2023), Inter-Simple Sequence Repeat (ISSR)(Kumar et al., 2018) or multiple sequence alignments (MSA) utilizing chloroplast genomes(Travadi, Shah, et al., 2022; Travadi, Sharma, et al., 2022b) and various barcode combination(Sharma et al., 2017). Chloroplast genome DNA barcode regions increase the probabilities to detect in compare to single locus barcode region due to sheared DNA in process herbal product, have been

increasingly employed for the development of species-specific primers (You et al., 2021). Zhang et al. (2017) demonstrated the use of chloroplast genome DNA sequences for the identification of *Echinacea* species (Zhang et al., 2017), and He et al. (2017) illustrated their application for barcoding *Lonicera japonica* (He et al., 2017). The development of specific primers for the detection of *Carica papaya* adulteration in Piper nigrum products and for *Ocimum sanctum* and *Ocimum basilicum* in Tulsi products has been instrumental in enhancing the authentication of botanicals (Travadi, Shah, et al., 2022; Travadi, Sharma, et al., 2022b). The criteria considered, such as high copy number of chloroplast DNA, short amplicon length, specificity, and sensitivity, have significantly contributed to the effectiveness of these primers in accurately identifying and differentiating the target botanicals within herbal products. In the context of lower taxonomic level identification, the utilization of Species-specific Primers derived from the Internal Transcribed Spacer (ITS) region has proven to be a more effective alternative for detections (Hsieh et al., 2021; Sharma et al., 2017). British pharmacies have been pioneers in adopting trnH-psbA-derived species-specific primers for the authentication of *Ocimum tenuiflorum* (Cartwright, 2016). Similar studies have been conducted on *Terminalia arjuna* (Sharma et al., 2017), *Senna tora* (Seethapathy et al., 2015), *Portulaca oleracea* (Xu et al., 2023), *Viscum coloratum* (Noh et al., 2021), *Aristolochia species* (Doganay-Knapp et al., 2018), and *Isatidis Folium* (Hsieh et al., 2021). These investigations contribute to the expanding scientific knowledge and emphasize the critical importance of rigorous authentication protocols in maintaining the quality control of herbal products.

Species-specific DNA markers, developed based offer high specificity, sensitivity, and applicability to single or multi-ingredient formulations. They are robust, rapid, and cost-effective, making them suitable for standard techniques in evaluation of raw material in value chain. However, it is important to note that species-specific assays are limited to detecting known target species and cannot identify unknown samples or unexpected contaminants. Sequencing-based identification, such as DNA barcoding, complements these assays by providing a broader scope for detecting known and unknown species. Overall, species-specific PCR assays are valuable tools in the authentication of herbal products, contributing to the overall quality and reliability of the herbal industry.

Advancements in DNA Barcoding for Herbal Products

DNA barcoding is a technique that uses a short, standardized fragment of the genome known as a "DNA barcode" to identify species (Hebert et al., 2003). This brief sequence of nucleotides can be derived from the chloroplast, mitochondrial, or nuclear genome and enables the identification of organisms at the species level. As such, DNA barcoding is considered to be the most effective method for species-level resolution and taxonomic identification (Little, 2014a). The process typically involves DNA extraction, polymerase chain reaction, sequencing, and sequence analysis. A comprehensive review analysed 17 potential barcode regions (*matK*, *rbcL*, *ITS1*, *ITS2*, *psbA-trnH*, *atpF-atpH*, *ycf5*, *p sbKI*, *nad1*, *trnL-F*, *rpoB*, *rpoC1*, *atpF-atpH*, and *rps16*) extensively used in authenticating and identifying medicinal plants (Tehen et al., 2014).

Global-level online data analysis was conducted to detect adulterants in herbal products using DNA barcoding. Adulteration rates were highest in Australia (79%), followed by South America (67%), Europe (47%), North America (33%), Africa (27%). Asia had the lowest percentage with Brazil being the highest distantly followed by Taiwan India USA Malaysia Japan South Korea Thailand China (Ichim, 2019). DNA barcoding has been successfully used in a range of studies to detect adulteration in products derived from *Ginkgo biloba* (Little, 2014b), *Actaea racemosa* (Baker et al., 2012), *Senna*, and (Seethapathy et al., 2015). It has also been applied to identify common adulterants of endangered species such as genus *Panax*, and to recognize species with high toxic or allergenic potential found in herbal products (Wallace et al., 2012). Several studies have demonstrated the effectiveness of DNA barcoding in herbal product authentication and identification (Balaji & Parani, 2022; Vassou et al., 2016). These studies have shown that DNA barcoding can accurately distinguish between different species of plants used in herbal products, allowing for quality control and preventing mislabelling and adulteration.

Based on the extensive studies carried out with 7 barcode regions (*psbA-trnH*, *matK*, *rbcL*, *rpoC1*, *ycf5*, *ITS2* and *ITS*), DNA barcoding has shown promising potential for authenticating and identifying medicinal plants. These regions have been used to study 8557 specimens from 5905 species belonging to 1010 diverse genera of 219 families in 7 phyla (Chen et al., 2010). The results have also led to the recommendation of using *ITS2* as a potential barcode, with the ability to discriminate species up to 92.7% efficiency. Additionally, the creation of an API reference DNA Barcode library for 374 medicinal plants with the *rbcL* barcode has been instrumental. This library covers species from 308 genera, 112 families, and 45 orders (Vassou et al., 2016). Furthermore, the testing of 100 raw herbal products using the *rbcL* barcode revealed that 21% of the products were reported to contain adulterants (Vassou et al., 2016). Notably, DNA barcoding has made its debut in the British Pharmacopoeia for the authentication of *Ocimum tenuiflorum* (Cartwright, 2016). This marks a significant step in the integration of DNA barcoding for the authentication of herbal products.

Despite its promising potential for authenticating and identifying herbal products, DNA barcoding encounters several limitations that require careful consideration. The extraction of DNA from processed herbal products proves challenging due to their composition, including bioactive secondary compounds, salts, polyphenols and polysaccharide, which readily interact with DNA, hindering the isolation process (Ancuta

Cristina Raclariu, Heinrich, et al., 2018a). To overcome these challenges, substances like bioactive charcoal, PVP, PVPP, and compounds resembling ascorbic acid are employed to remove secondary metabolites (Schenk et al., 2023). The physical processing of herbal products, such as boiling and grinding, further complicates the isolation process, resulting in sheared DNA, making it difficult to amplify barcodes like MatK (Parveen et al., 2016).

The extensive inter- and intra-species divergence in plants, coupled with consensus regions in DNA sequences, poses a challenge in identifying a universal barcode for diverse taxonomic studies. Unlike animals, selecting a single applicable barcode across various studies of plant diversity and taxonomic identification is challenging, given that the resolution and discrimination ability of a DNA barcode are directly proportional to taxonomic and environmental differences (Hollingsworth et al., 2011; Mishra et al., 2016a).

Moreover, DNA is omnipresent in every plant cell, regardless of its function. Consequently, DNA barcoding may not effectively detect specific bioactive compounds present in herbal products. For instance, in *Withania somnifera*, where the bioactive compound withanoid is primarily found in the roots, reported adulterants may involve other plant parts like stems of the same plant (Mundkinajeddu et al., 2014).

Despite these challenges, DNA barcoding remains a trending method for identifying medicinal plants and raw materials due to its resolving power. However, the analysis of DNA barcoding results in herbal products faces multiple challenges, including limited reference databases, inconsistency in the DNA barcode regions used for identification, and the potential presence of contaminants or fillers that can interfere with result accuracy (Mishra et al., 2016b; Parveen et al., 2016; Ancuta Cristina Raclariu, Heinrich, et al., 2018b). These challenges underscore the need for standardized protocols and comprehensive reference databases to enhance the reliability of DNA barcoding in herbal product authentication. In conclusion, while DNA barcoding holds great promise for authenticating and identifying herbal products, addressing these limitations is crucial for advancing the field. Continued research and innovative strategies are essential to overcome these challenges and improve the accuracy and reliability of DNA barcoding in the authentication of herbal products.

High-throughput Metabarcoding application for herbal products

Metabarcoding, a fusion of Next Generation Sequencing (NGS) and DNA barcoding, emerges as a powerful tool for identifying multiple taxa within a given sample, particularly in the realm of herbal products. The workflow involves DNA isolation, PCR amplification, adaptor ligation, library preparation, emulsion PCR, and NGS for bench work, accompanied by a bioinformatics pipeline encompassing sequence trimming, clustering, and BLAST analysis (Coghlan et al., 2012). This methodology combines the strengths of NGS and barcoding, overcoming the challenges of using a single plant barcode for species-level identification due to the plant kingdom's diversity, slow molecular evolution, and frequent cross-pollinations and hybridizations (Fazekas et al., 2009).

Several studies have demonstrated the efficacy of metabarcoding for the authentication of herbal products. Multi-barcode approaches, such as *ITS2* and *trnL*, have been employed to precisely identify plant species in various Chinese medicine and herbal teas (Frigerio et al., 2021; Xin et al., 2018). However, these studies also underscored limitations, including variability in barcode universality and resolution power, the absence of curated databases, and challenges in bioinformatics pipelines (Frigerio et al., 2021; Xin et al., 2018). Metabarcoding has shown excellent outcomes in various studies for the authentication of herbal products. In these studies, results revealed that, labeled species in single drugs of *Echinacea* species (Ancuta Cristina Raclariu, Tebrencu, et al., 2018), *Hypericum perforatum* (Ancuta Cristina Raclariu et al., 2017), and *Veronica officinalis* (Ancuta C Raclariu et al., 2017). Ivanova et al. (2016) identified key ingredient DNA in 8 out of 15 herbal products using the *ITS2* barcode via NGS (Ivanova et al., 2016). Cheng et al. (2014) demonstrated high quality in 27 tested herbal products with a high degree of non-filler and non-listed moieties (Cheng et al., 2014).

In India, where the market for herbal products is rapidly growing, the application of metabarcoding to detect raw plant materials in herbal medicine is not yet well-established. However, the sensitivity of DNA metabarcoding makes it a valuable method for detecting trace amounts of contamination, including pollen and other plant species introduced during cultivation, transport, and production (Pandit et al., 2021; Seethapathy et al., 2019; Shah et al., 2023; Travadi et al., 2023). Despite its sensitivity, DNA metabarcoding is not without challenges. Variables such as DNA quality and quantity, PCR amplification bias, library preparation, sequencing platform, and data analysis parameters can influence the results. Non-curated databases like NCBI GenBank may lead to incorrect identifications at lower taxonomic levels, emphasizing the need for further refinement in reference databases (Arulandhu et al., 2017; Pawluczyk et al., 2015; Staats et al., 2016).

In conclusion, DNA metabarcoding presents a robust approach for herbal product authentication, offering sensitivity to trace contamination. However, addressing challenges related to barcode universality, database curation, and bioinformatics pipelines is crucial for advancing the reliability and accuracy of metabarcoding in the herbal product authentication landscape. Ongoing research in screening new barcodes and variable regions within existing barcodes is essential to enhance the authentication of herbal products.

Exploring dynamics of digital PCR in herbal products

Digital PCR (dPCR) has emerged as a powerful technology for detecting and quantifying DNA or RNA molecules in various applications, including herbal product authentication. The technique involves partitioning a sample into thousands of nanoliter-sized droplets or partitions, enabling the accurate detection and quantification of low-level target DNA, even in complex samples with inhibitors (Hindson et al., 2011). Compared to quantitative PCR (qPCR), dPCR offers higher sensitivity, precision, and the advantage of providing absolute measures of nucleic acid concentration without the need for standard curve (Dingle et al., 2013). This capability makes dPCR a robust tool for authenticating herbal products where DNA quality and quantity can be compromised due to secondary metabolites present in plant material (Morcia et al., 2020).

In the study conducted, a dPCR assay efficiently detected 1.0% (w/w) papaya adulteration in Piper products, showcasing its sensitivity (Travadi, Shah, et al., 2022). Similar studies by Yu et al. (2021a) and You et al. (2021) demonstrated the ability of droplet dPCR to detect 1% adulteration in *P. notoginseng* and *canola honey*, respectively (You et al., 2021; Yu et al., 2022b).

In conclusion, dPCR stands out as a valuable tool for herbal product authentication, offering high sensitivity, specificity, and absolute quantitation capabilities. Its application in detecting adulteration and quantifying target DNA in complex mixtures contributes to the overall quality control of herbal products. Continued research and utilization of dPCR in conjunction with other molecular techniques are essential for advancing the authentication protocols in the herbal industry.

Conclusion

DNA authentication methods have proven to be highly effective in overcoming the intricate challenges associated with ensuring quality control and safety in the realm of herbal products. The holistic approach to quality control within this domain spans the entire spectrum of production, encompassing the procurement of raw materials through to the final product. In this complex landscape, DNA-based methodologies such as species-specific PCR, barcoding, metabarcoding, and Digital PCR have emerged as promising tools with distinct applicabilities. It is imperative to acknowledge that each of these techniques is characterized by specific technological prerequisites, cost considerations, and levels of expertise essential for the accurate assessment of herbal product quality or quantity. Each approach has defined applicability and inherent limitations, emphasizing the importance of judicious selection based on the specific context of analysis.

The diversity of methodologies highlights the growing consensus on the significance of adopting multiple or complementary authentication approaches. This strategic alignment with the "fit-for-purpose" principle is paramount in raising benchmarks for quality control and safety in herbal products. The integration of various complementary techniques allows practitioners to enhance the robustness and reliability of their assessments, effectively addressing dynamic challenges in the herbal product industry. This comprehensive approach is essential for meeting contemporary demands, ensuring the integrity and safety of herbal products, and seamlessly incorporating the latest scientific knowledge and technological advancements in this evolving field.

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Conflict of Interest

The authors have no conflict of interest

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