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Quality control of herbal drugs and preparations: The methods of analysis, their relevance and applications

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ABSTRACT

Medicinal plants have been used for a very long time to improve human health; they are gaining increasing popularity globally as drugs, complementary and alternative medicines, food supplements, cosmetics and, more surprisingly, as medical devices. The complexity of herbs and extracts, supplied to such a wide range of markets and in different requlatory environments, raises major quality issues, increasing the need for appropriate analytical methods for their identification and standardization, but also for the detection of adulterants and contaminants. Customs laboratories are often confronted with herbal samples which pose a range of challenges, ranging from quality issues to safety and even legal issues. Selecting a relevant analytical method, from the many available (microscopy, spectrometry, spectroscopy, chromatography…), is a crucial point that mainly depends on the set analytical goals. This review aims to detail such analytical goals and their complexity to propose a selection of analytical methods likely fit for each purpose. Major limiting factors, such as herbal product naming, sampling and sample preparation are also discussed.

Introduction

Medicinal plants are a very important source for new natural compounds, which offer a chemical diversity important for the pharmaceutical industry [1–3], and are the cornerstone for many complementary and alternative medicines [4], food supplements, cosmetics and, more surprisingly, medical devices. The complexity of herbs and extracts, supplied to such a wide range of markets and in different requlatory environments, raises major classification and quality issues, increasing the need for appropriate analytical methods for their identification and standardization, but also for the detection of adulterants and contaminants.

Customs laboratories are often confronted with herbal samples (herbal medicines, food supplements, tobacco, cannabis, …), thus facing many different questions that range from identity confirmation to contamination assessment, risk identification, classification problematics or legal issues. Significant advances have been made in recent years in the processing and study of medicinal plants, including modern extraction methods and identification of key components. Many analytical methods, often hyphenated, including chromatography, microscopy, spectrometry, spectroscopy, DNA barcoding etc., have been applied to determining the identification and quality of herbal products, [5–7]. Moreover, modifications to published methods are continuously being added and improved [5], yielding an even wider variety of possible analytical choices. But the selection of a given method should be carefully pondered, according to the set analytical objectives.

A general problem is the authentication of plant material. The application of analysis of the genetic profile of plants is gaining inclusion in state and commercial analytical laboratories. The authentication of medicinal plants through DNA barcoding is a gold standard that has been successfully applied to detect adulteration in several high-value species [107,108]. However, there are limitations within genetic profiling, and also in the accessibility of such technology in basic laboratory settings. In many cases the identification of key chemical biomarkers, active or purely analytical, is often the main approach used; this can be done through direct comparison with chemical reference compounds or by hyphenated methods (GC- and HPLC-MS or MS/MS), referring to public or commercial libraries of compounds. However, many phytochemicals are not commercially available nor described in databases [8,9]; also, the phytochemistry of many herbal medicines can

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be quite complex and still relatively unknown, questioning the choice of such identification or standardization strategies. The concept of analytical profiling is then proposed, aiming to define a reference chromatographic, spectroscopic, or chromato-spectrometric profile or fingerprint, for a given plant or extract, including a documentation of its robustness and acceptable variations. This approach requires a considerable amount of work to establish an authenticated library of acceptable samples and likely adulterants or counterfeits.

The present review describes possible analytical goals that can be set for herbal samples and their complexity, to help in the selection of analytical methods appropriate for each purpose.

The importance of quality monographs compendia

Important analytical approaches are documented in international Pharmacopoeias for the quality control and analysis of recognised herbal medicines. These notably include the European [10], French [11], German [12], British [13], United States [14], Chinese [15], Japanese [16], and Taiwanese [17] Pharmacopoeias. Within these compendia, individual monographs present guidelines for best practice in macro- and microscopical identification, and in determination of quality. Modern methods, outside of the current monographs, have also been developed; these can be needed for particular analytical purposes (application in regions with particular substituents or toxic contaminants) and may complement the methods described therein. An important consideration of Pharmacopoeias is that, depending on where analysis is being performed, some techniques that are simple, such as TLC and HPTLC, can be valuable if more sophisticated hyphenated techniques are not available.

Obviously, there are many species for which there are no monographs, and many traditional medicine systems which do not have a written tradition, therefore orally passing down the knowledge of traditional healing plants. In these instances, guidelines need to be firmly established, building a dynamic monographing system:

- Set up criteria to define a *"traditional use"* (e.g. convergence of uses, interdicts, precautions,…) and select species with good chances of activities and probable low risks of toxicity;
- Harvesti botanically identified voucher specimens to define analytical criteria (identification, assay, standardization);
- Collect commercial samples and in-house samples from tradipraticians
- Set up norms and analytical criteria; detect eventual problematic samples
- Develop an iterative quality system by regularly collecting and analyzing samples

Herbal drugs and their preparations

Herbal drugs, that are described as traditional medicines, include aerial parts, flower, fruit, leaves, seed, stem and subterranean parts (such as roots, bulbs, tubers, rhizomes) [31,32]. They are presented in raw form, fresh or dried, extracts, sometimes whole dried plants [18–20] (Fig. 1) and have considerable importance for global international trade. Their clinical, economic, health and pharmaceutical worth is becoming increasingly valued, rightly or wrongly, and their market is steadily growing. Nevertheless, data on the quality, safety and efficacy of many plants, their extracts, preparations, and active compounds are still limited [18]. Controlling their quality is crucial for ensuring their efficacy and safety [32].

Quality of herbal drugs

The quality control of medicinal plants cannot be limited to the botanical level, given the sometimes large variations that occur in chemical composition within the same species [21,22]. Indeed, the

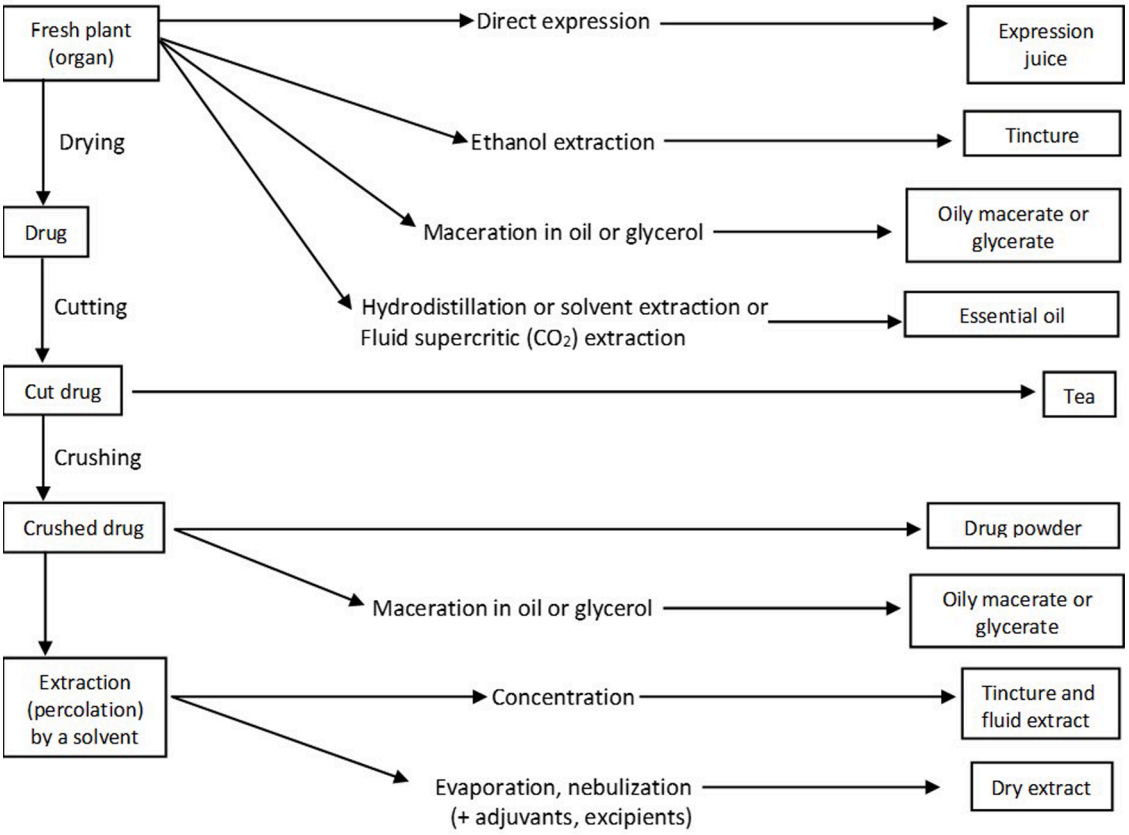

Fig. 1. The major types of herbal preparations.

expression of secondary metabolites in a given plant can be a function of abiotic (climate, geology,…), biotic (predators, parasites interspecific competitions within ecosystems) and genetic factors, the specificity of each taxon (species, variety, cultivar) being related to its genetic constitution [23].

The harvest conditions (hygrometry, location, …), the time that has elapsed between the moment of harvest and that of analysis and the phenological stage of the plant (beginning, end of flowering, …) should be points of attention. Storage conditions are very important for microbiological quality, the preservation of secondary metabolites and the conservation of herbal drugs, the easiest being generally to keep them dry, as fresh samples are fragile and tend to deteriorate faster [29]. Poor storage and drying can lead to spoilage, biological contamination, photo-decomposition or atmospheric oxidation [30]. Contamination can also arise during the production stage and this can influence the composition and quality of the final product. Such contamination or degradation can have very important impacts on the chemical composition and, consequently, the therapeutic qualities of a medicinal plant [20,24]. Such sources of variations are usually handled through the development of strict guidelines for good agricultural and collection practices (GACP) and good laboratory/manufacturing practices (GLP/GMP) [33].

Identity of herbal drugs

Adulteration, falsification and contamination of herbal material all present health risks, either by impact on bioactivity or introduction of risks. Effective identification systems and robust methods for the detection of foreign material, both being needed for the purity and quality of medicines, can identify and minimise the risk of these issues [20].

Several taxonomic, chemical, proteomic, and genomic markers allow the authentication and identification of herbal drugs components. These methods comprise morphological identification (macro- and microscopic identification), analysis of molecular markers, i.e. proteins or DNA [20,25], analysis or profiling of secondary metabolites by high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), all methods which can also be hyphenated with mass spectrometry [20,26,27]. These markers can be specific of a taxon (family or species, sometimes variety); they allow determination of the presence of a specific herbal material or can indicate its proportionality in a mixture [20].

Sampling and preparation of samples for analysis

Sampling is quite a problem for unpowdered herbal products; the test samples should be representative even when sampled from a non-homogeneous material. For example, sampling roots of different diameters will yield different bark-to-wood ratios, but biomarkers may be concentrated in only some tissues, resulting in markedly different analytical results; this is for example the case for *Rauvolfia vomitoria* roots [34]. This sampling aspect has been considered by the European Pharmacopoeia, which now proposes a procedure to address sample standardisation, detailing sampling strategy, quartering homogenisation and milling [35]. The main techniques for sample preparation have been refined in recent decades [32–42], indicating that several aspects are to be considered, notably the solubilities and expected concentrations of targeted compounds (that can range from ppm to over 15%), the characteristics of solvent, the solvent-to-sample ratio, the duration, temperature, pressure and type of extraction (static, dynamic or ultrasonic maceration, percolation, soxhlet…). Solvents can be selected based on their physicochemical properties, such as polarity and selectivity, as well as on their toxicity and inertness. Polarity of the solvents plays a crucial role as they will extract the plant components according to the principle of "like dissolves" [28].

Selection of analytical methods according to the questions raised

The questions raised define the complexity of an analysis and influence the choice of analytical methods (Table 1). The following methods are regularly applied for pharmacognostical and phytochemical analyses.

Botanical determination and naming

There are over 400,000 vascular plant species that have been identified on Earth, which represents circa 90% of all vascular plants. Botanical confirmation of the identity of a species is an essential step [38]. Determination of identity is based on the visual and sometimes microscopic observation of key morphological features of the species under investigation [44], which can present some difficulties [47]. Variation in plant nomenclature is also an issue in plant identification. Agriculture and traditional herbal medicine has been practiced for millenia around the world, and frequently plants were named according to the language of each people [39–41]. The diversity of names for the same species and the validity of vernacular names, limited to the geographical region of origin, have shown the need for standardised nomenclature [38]. For this, the binomial nomenclature using Latin names (genus and species, in italics), was adopted as a scientific and international nomenclature [42]. However, a correspondence between vernacular and scientific names can be difficult to apply [43], as a single species can be designated by different vernacular names, and a vernacular name can designate several species, sometimes of different genus or even families [51]; to avoid confusion, it is mandatory to complete the name with the botanical author who described the plant under this name (e.g. *Nicotiana tabacum* L.; *Nicotiana tabaca* St.-Lag.). For many plants there are many different synonyms. For many years known species and their synonyms were registered on 'The plant list' at Kew Gardens (http://www.theplantlist.org/). These lists are now maintained and updated on the 'World Flora Online' website, (http://about.worldfloraonline.org/). Voucher samples stored in official herbaria are the truthful records, linking a scientific name to a botanical species [44], an should be logged for all published studies using plant specimens.

Apart from this scientific binomial nomenclature, herbal medicines are often designated by their pharmaceutical names, as used in official pharmacopoeias; these names, that should not be confused with Latin binomials (which regularly happens in the literature, on labels or on web sites), are built from a Latin word (Table 2) designating the considered organ and the Latin name of the plant (often only the genus is indicated) declined as a Latin genitive (the Latin ending that means *"from"*).

In some cases, the order can be reversed, as Latin name (as genitive) and organ. These pharmaceutical names are never italicized. For example, "Rhizoma Alimatis" or "Alimatis rhizoma" translate as "rhizoma of *Alisma*"; "Radix *Angelicae pubescentis*" or "*Angelicae pubescentis* radix" translate as "root of *Angelica pubescens*". This can yield to confusions and obviously wrong names such as "Rhizoma Alismatidis" or "root of *Angelicae pubescentis*" are regularly seen. Other nomenclature problems are frequently encountered, notably in the field of traditional Chinese medicines where pinyin names can lead to major confusions [45].

Macroscopic and microscopic analysis

Macroscopic observation is often the first step in the identification of plant species or organs [47], completed by organoleptic elements such as color, smell, taste etc. [47] while microscopy brings in complementary information [48]. Macroscopic examination has long been used for the authentication and quality assessment of traditional medicine products. It is important to note that macroscopic analyses concern phenotypic characteristics that can vary according to the conditions under which the plant has evolved, the age at which the plant is harvested, the eventually applied processing or standardisation treatments

Table 1
Selection of analytical methods according to the goals set for an examination of herbal drugs and their preparations.

Set goals of herbal preparation analysis	Appropriate analytical methods[a] Botanical identification	Macroscopic examination [b]	Microscopic examination [b]	Profiling by HPTLC with eventual semi-quantitative determination	Profiling by GC, HPLC, EC hyphenated or not with MS	Determination of defined marker(s) content or contaminants by GC, HPLC, EC, HPTLC, ICP	DNA analysis	Proteomic profiling	FTIR or Raman pattern recognition	Remarks
Control that the labelling of a product is correct										
• Identify an entire plant	X			May be needed			May be needed			
• Identify a single herb in organ form		X	X	X			May be needed			
• Identify a single herb in powder form			X	X	May be needed		May be needed		X	
• Identify a mixture of herbs in organ form		X	X	X	May be needed		May be needed			
• Identify a mixture of herbs in powder form			X	X	X		X		X	Can be difficult
• Identify a liquid or a dry extract of a single herb				X	May be needed	May be needed	May be needed		X	
• Identify a liquid or a dry extract of a mixture of herbs				X	X	May be needed	Rarely possible		Can be difficult	Can be very difficult
Identify an unlabelled (unknown) sample										
• Identify an entire plant	X			X			May be needed			Difficult to reach the exact determination of species without DNA analysis
• Identify a single herb in organ form		X	X	X	May be needed	May be needed	May be needed			Difficult to reach the exact determination of species without DNA analysis
• Identify a single herb in powder form			X	X	May be needed	May be needed	May be needed			May not be possible
• Identify a mixture of herbs in organ form		X	X	X	X	X	May be needed			May not be possible
• Identify a mixture of herbs in powder form			X	X	X	X	X			Frequently not possible
• Identify a liquid or a dry extract of a single herb				X	X	X				Frequently not possible
• Identify a liquid or a dry extract of a mixture				X	X	X				Frequently not possible
Other analytical tasks										
Identify processed samples and/or		X	X	X	X	X		X	X	

(*continued on next page*)

Table 1 (*continued*)

Set goals of herbal preparation analysis	Appropriate analytical methods[a] Botanical identification	Macroscopic examination [b]	Microscopic examination [b]	Profiling by HPTLC with eventual semi-quantitative determination	Profiling by GC, HPLC, EC hyphenated or not with MS	Determination of defined marker(s) content or contaminants by GC, HPLC, EC, HPTLC, ICP	DNA analysis	Proteomic profiling	FTIR or Raman pattern recognition	Remarks
assess the quality of processing										
Identify whether a sample has been extracted or not			X	X		X			X	Determination of total extractive yield by a selected solvent
Assess the content in active compounds				X		X			Semi-quantitation mostly	
Identify an adulteration (substitution by another species)	X	X	X	X			May be needed		X	
Identify contamination by another plant at a given level (higher than an estimated 20%)		X	X	X		X	May be needed		Possible	
Identify contamination by another plant at a given level (lower than an estimated 20%)		X	X		X	X	May be needed			Strongly dependent on the contaminant and its level
Identify contamination by genotoxic compounds, microorganisms, mycotoxins, minerals, heavy metals, pesticides, radioelements			X (microbial and fungal contaminants)			X				Strongly dependent on the contaminant and its level
***Pros* of the method**	"The" basis for botanical identification	Rapid detection of gross errors	Rapid detection of gross errors and dilutions (starch, lactose)	• Rapid and feasible without instruments • Visualization of migrating and non-migrating compounds • Sensitive to variations in chemicals content	• +/- rapid and highly specific • Versatile migration and detection methods • Sensitive to variations in chemicals content	• Highly specific • Allows standardization of herbals and herbal preparations	Highly specific identification down to species, sometimes variety level		Very rapid *"on-spot"* identification	
***Cons* of the method**	Need to examine the taxonomically relevant organs	Organs from different plants may present	• Specialised training	Need for authenticated reference samples	• Cost and equipment	• Cost and equipment • Need for reference compounds	• Need for reference DNA	• Labor-intensive so far	• Needs an extensive training set of	

(*continued on next page*)

Table 1 (*continued*)

Set goals of herbal preparation analysis	Appropriate analytical methods[a]									
	Botanical identification	Macroscopic examination [b]	Microscopic examination [b]	Profiling by HPTLC with eventual semi-quantitative determination	Profiling by GC, HPLC, EC hyphenated or not with MS	Determination of defined marker(s) content or contaminants by GC, HPLC, EC, HPTLC, ICP	DNA analysis	Proteomic profiling	FTIR or Raman pattern recognition	Remarks
	that may not be available	+/-similar characters (notably roots and barks)	• Limited to organ type, occasionally to family or genus, rarely to species	or reference profiles	• Non-detection of non-migrating compounds/ impurities • Need for authentic reference samples or reference profiles		• Selection of amplicons difficult • NGS still in development • No information on quality	• Still to be developed	authentic samples • Needs continuous re-training	

[a] HPTLC, high-performance thin-layer chromatography; GC, gas chromatography; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; MS, mass spectrometry; ICP, inductively coupled plasma analyser; FTIR Fourier-transform infrared spectroscopy; NGS, next-generation sequencing.
[b] Macroscopic and microscopic examinations can be completed with chromogenic chemical reactions; although useful, these reactions often lack specificity.

Table 2
Some of the common Latin names applied to plant organs.

Latin word	English word
Radix	Root
Semen	Seed
Caulis	Stem
Cortex	Bark
Fructus	Fruit
Flos	Flower
Folium	Leaves
Herba	Aerial parts
….	…

or the storage conditions of the drug [47]. Whereas, for experienced practitioners [47] and some expert systems [49], these analyses can help discriminate confused species or drugs, it can also be difficult to distinguish related species or varieties of the same species or to highlight adulterations [50].

Faced with the weaknesses of macroscopic analyses [51], microscopy of a cross section or powder of an organ [51] has the advantage of allowing analyses at the cellular level [51] and is more suited to the identification of species or the verification of adulteration [51,52]; it is also the only morphological alternative when drugs are presented as powders, but this identification often needs to be supported by chromatographic evidence. Certain parameters, such as the shape and size of trichomes, crystals, vessels, fibers, pollens or starch granules, the stomata guard cells or the cell walls constitution, are often faithful to a given organ and species or genus [59]. The use of visible, polarisation and fluorescence microscopy as well as scanning electron microscopy has also been reported [53–55]. Unfortunately, microscopic authentication of tissue is not applicable to fresh herbal material. The omission of this parameter is frequently permitted by regulatory authorities when reviewing licensing applications for herbal medicinal tinctures.

As discussed here above, many species are clearly documented macro-, microscopically and chemically in compendia monographs, an important source of reliable information.

Identification of key chemicals by in-tube reactions

Identification of key chemicals by in-tube reactions [56] are still used today in laboratories with limited access to instrumental methods. These reactions are based on the formation of colored, precipitated and/or fluorescent derivatives [57,58] with alkaloids, terpenoids, flavonoids, tannins, anthocyanins, coumarins, quinones, cyanogenic heterosides, etc. present in the plant material [58]. Despite limited sensitivity and selectivity, coupled with a certain subjectivity in assessing the results, these methods can help in a rapid *"possibly yes/no"* answer to an identification question. These methods can be used to distinguish important chemical classes such as tropane alkaloids (Vitali Morin reagent), codeine (Marquis' reagent), opioids (Froehde's reagent), etc. [60]. These methods have now largely been superseded by profiling methods for plant materials, although United Nations Office on Drugs and Crime (UNODC) test kits are recommended and frequently used at border control monitoring for opiate, cocaine and cannabinoids (https://www.unodc.org/unodc/en/scientists/druge-kits-new.html).

Chemical profiling by separative methods

Chromatographic methods allow to fractionate a complex mixture of compounds (crude extract), to yield profiles that can be extremely characteristic.

In thin-layer chromatography (TLC), the compounds, separated by planar chromatography on silica, cellulose, polyamide or chemically-modified plates, can be detected, either directly or after reacting with a specified reagent, by their color, under visible light, by their quenching and/or fluorescence properties, under ultraviolet lights, or by their mass

spectrum [61]. High-performance thin-layer chromatography (HPTLC) relies on the use of silica plates with a greater number of theoretical plates than TLC, coupled with instrumental application, migration, spraying and detection systems to yield profiles with considerably higher resolution and reproducibility. HPTLC has become a reference method in modern pharmacopoeias for the identification of herbal drugs, in conjunction with macroscopic and microscopic examinations.

High performance liquid chromatography (LC) [62], gas chromatography (GC) [63] and capillary electrophoresis (CE) are also increasingly used in phytochemical analyses, for both profiling and quantitative determinations. The choice of technique is determined by the nature of the extract to be examined. These fully automated techniques generally provide a high level of selectivity and sensitivity and can be hyphenated with various types of detectors, allowing to highlight contaminants, sometimes at trace level. In particular, the coupling of these highly separative techniques with various mass spectrometry detectors allows identification of compounds [65], but also multidimensional profiling, based on retention times and fragmentation patterns (MS^n) [64]. For example, fingerprints of cigarettes cut tobacco could be obtained by pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS), the profiles being classified by cluster and principal component analysis. From 4 different types of cigarettes, the method yielded 29 common peaks and allowed, through pattern recognition, to distinguish the different cigarette brands [66].

Chemical profiling by spectroscopic methods

In MS^n, fragmentation patterns can be so characteristic that the profiling method can bypass a chromatographic separation, relying on direct infusion in the mass spectrometer; this has been applied for example to the profiling of *Nicotiana tabacum* L. nonvolatile organic acids and polyphenols, allowing a rapid identification of counterfeit cigarettes and discrimination between tobacco blends [67], to *Bocageopsis pleiosperma* Maas alkaloids [68] or to *Acanthopanax senticosus* Harms saponins [69].

Many applications of metabolic fingerprinting, based on nuclear magnetic resonance (NMR) spectrometry [70,71], Fourier transform infrared spectrometry (FT-IR) [70] spectroscopy, coupled with multivariate analysis, have been developed to differentiate closely related plant species, with applications in the quality control of herbal drugs, but also to discriminate between their different geographic origins [62, 71]. For example, a combination of FT-IR, ^{1}H NMR and chemometric analysis allowed the authentication of *Phyllanthus* species [72]. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra allowed, through cluster analysis, to discriminate authentic from counterfeit tobacco batches with high accuracy and precision [73].

Atomic absorption and atomic emission spectroscopies are used for the qualitative and quantitative analyses of mineral elements in herbal drugs, often in the search for contaminants [74,75]. Inductively coupled plasma atomic emission spectroscopy has the advantages of being very sensitive and able to analyse more than 30 elements at a time [62,76]. Trace element analysis has been applied to distinguish between legal and counterfeit cigarettes. The analysis of two genuine-brands and three counterfeit packs by inductively coupled plasma mass spectrometry and cold vapor atomic absorption spectrometry indicated that the mean amounts of Be, As, Mo, Cd, Sb, Tl, Pb, and Hg were higher in counterfeit cigarettes, while the amounts of V, Cr, Mn, Co, Cu, Zn, Se, and Ba were comparable among legal and counterfeit cigarettes; the amount of Ni was higher in the legal cigarettes [77]. An ICP-MS method to evaluate lead isotope ratios could discriminate tobacco samples from genuine and counterfeit cigarettes obtained in the USA; all the genuine cigarettes, grown in different regions, were statistically distinct, based on the measured ratios [78]. Another study indicated that both Pb and Cd concentration in counterfeit cigarettes were markedly higher than those in their genuine equivalents, and exhibited greater sample-to-sample variability [79].

DNA profiling

The species identification of some samples, that may be very similar at macro- and micro-morphological aspects, may require analysis at the molecular level [80]. As there is so far no universal DNA profiling method for plant identification, this is largely a case-by-case problem and several molecular techniques have been proposed to authenticate medicinal plants, based on species-specific variations in the sequences of various chloroplast and nuclear DNA regions [81].

The DNA barcode aims at identifying unknown plants based on a short DNA sequence from a standard and agreed position in the genome [82]; DNA barcode sequences are very short compared to the entire genome and can be obtained quickly and inexpensively [83]. Success in species-level assignment of plants using Basic Local Alignment Search Tool (BLAST) [84] with individual barcodes was obtained with matK (99%), followed by trnH-psbA (95%) then rbcL (75%). The matK sequence and nuclear markers such as At103 have been proposed by Bruni et al. [46] to distinguish congeneric edible and poisonous plants.

In a study of simple sequence repeats (SSR) markers in tobacco, 91 clear and polymorphic amplification bands were obtained with polymorphism information content and excellent information index values; clustering analysis showed that the 33 studied varieties, which are standard varieties for flue-cured tobacco, could all be distinguished from one another. A minimum of 25 markers were required to identify the genetic diversity of these varieties [85]. Random Amplification of Polymorphic DNA (RAPD) markers was shown to be a simple and quick method for early selection of *Nicotiana* somatic and traditionally selected hybrids [86]. The polymorphism, similarities, and relationships among *Nicotiana tabacum* L. cultivars were assessed with RAPD analyses. One hundred and forty-nine bands were detected, of which 94 were polymorphic (63.1%). A primer distinguishing all tested cultivars was found. High similarity between cultivars was revealed, and cultivar relationships were estimated through cluster analysis (UPGMA) based on RAPD data [87]. Sequence Characterised Amplified Regions (SCARs) are DNA fragments amplified by PCR using specific 15–30 bp primers, designed from nucleotide sequences established from cloned RAPD fragments linked to a trait of interest. By using longer PCR primers, SCARs do not face the problem of low reproducibility generally encountered with RAPDs [88]. A total of 53 tobacco cultivars could be effectively distinguished by using only 2 SCAR and 7 RAPD markers. A two-step fingerprinting strategy was proposed as a convenient and cost-effective tool to discriminate large numbers of tobacco cultivars [89].

RAPD has also been applied to identify whether a herb sample (*Atractylodes lancea* (Thunb.) DC. rhizome) has been processed or not and data were found to correlate with DNA degradation, depending on processing conditions [90].

Recent improvements in metabarcoding and next-generation sequencing methods further reduced sequencing costs and helped improve the technique for identifying complex multi-ingredient herbal products [91], as recently exemplified for Thailand traditional herbal medicines [92]. Next generation sequencing methods, based on herbarium samples, are certainly the most appropriate methods that will rapidly develop for large scale studies with broad taxonomic sampling [93].

Genetic analyses are considered a very powerful tool for the reliable identification of a given species, even in a mixture, for example to detect toxic contaminating *Senecio* and *Aristolochia* species. The high sensitivity of DNA analyses was shown suitable to even detect and identify traces of DNA in liquid and dry extracts in which the minor amounts of intact plant cells were purified by a repeated dilution/concentration/separation process before extracting the DNA from the plant cells [94].

It should be emphasised that, by themselves, DNA methods are not sufficient for the quality control of a medicinal drug; the DNA authentication of a plant has no relationship with its content in active compounds, which can qualitatively and quantitatively vary, depending on

chemotype and growth conditions.

Protein profiling

Although MALDI-TOF protein profiling is successful and now routinely used for the identification of microorganisms at species level [95], this approach has rarely been employed in plants [96] and there is still a need to develop suitable methods, *M/Z* ranges and markers. Proteomics have been applied to resolve the taxonomic and phylogenetic complexity of some plant taxa, by combining 2-dimensional electrophoresis and MS [97]; in that way, *(i)* the leaves of *Clematis chinensis* Osbeck could be discriminated from those of the very similar taxa, *C. finetiana* H.Lév. & Vaniot and *C. armandii* Franch [98]; and the processing of Hirudo, the dried body of *Whitmania pigra* Whitman (stir--frying with wine) could be identified [99]. Although such studies indicate the potential of proteomics, the large volume of work involved makes it impractical for routine control and progress in MS is still needed. On the other hand, a very simple electrophoresis of proteins may be useful to detect whether a sample has been processed by a treatment involving heat [100].

Determination of key constituents or analytical markers

The complexity of the chemical composition of medicinal plants is the basis of the underlying challenges in quality control analyses of medicinal plant products [7], where robust, accessible and reproducible methods are neededto guarantee the safety and effectiveness of products in circulation [101]. The markers of quality and biological activity are the frequently the main active constituents, whenever they are known [101]; but, when they are unknown, quality is generally insured by controlling surrogate analytical markers. In all cases, these markers should be preserved and transferred into the product during all the transformation processes that the herbal drug may undergo [101]. Most of the analytical work used to ensure quality and therefore safety and efficacy of plant materials, combines chromatographic (HPLC, GC, CE) methods, hyphenated with spectroscopic (DAD, fluorescence, MS), specific (electrochemical, ion capture, N/P …) or unspecific (FID, charged aerosol, ELSD,…) detectors.

The absolute determination of components relies on reference standards, compound libraries and use of literature. There are of course limitations when we use these methodologies. We now have an increasing awareness of the importance of minor metabolites and their synergistic relationships in the bioactivity of complex plant extracts. It is accepted broadly that key chemical markers, may not be the (only) active metabolites in an extract. Benssousan et al. [102] have proposed an interesting Herbal Chemical Marker Ranking System (Herb MaRS) as a guide for QC and QA of herbal mixtures, to inform laboratory scientists on the selection of the most appropriate chemical markers for quality control of finished complex herbal mixtures, whilst taking into account their bioactivity. This approach, has limitations, and still needs refinement before it can have broad application.

Determination of adulterants, impurities and trace contaminants

The expansion of the medicinal plant market entails a risk of circulation of products with impurities that can alter the effectiveness of the product or lead to its toxicity [103]. In other cases, the difficulty in sustainable supply leads to plant cultivation or storage conditions that can be a source of adulteration or of contamination of medicinal drugs [103]; also the substitution of a cheaper species for an expensive one (Cassia bark for Cinnamon} is common, the reverse being exceptional. Adulteration of the *Illicium verum* Hook. f. fruit with the fruit of *Illicium anisatum* L. is a well-known example. The first species is recognised for interesting therapeutic properties, especially in the treatment of infantile colic, while the second is neurotoxic. Once known possible, the adulteration could be detected by macroscopic examination of the entire fruit and by microscopy of the powder; fluorescence microscopy, gas chromatography [109]), thin-layer chromatography and DNA barcoding [110].

Contaminants or impurities likely to be found in plants can be traces of other plant species, pesticides used for cultivation, heavy metals, radioelements and microorganisms or the products of their metabolism [104]. Thus, the application of well-structured collecting and agricultural practices [105], combined with a control of impurities or contaminants is essential to ensure both the effectiveness of herbal products and the safety of consumers [81]. Since contaminants and impurities are generally present in very low amounts, their analyses also requires the use of highly efficient methods. These include microscopy (i.e. for molds, insect parts or particles), chromatography, sometimes combined with immunoaffinity extractions [106], spectroscopy (MS, NMR, FTIR, ICP-AE, ICP-MS, ESR) as well as DNA-based methods [81,84,95].

Conclusion

When dealing with herbal products, intricate mixtures of compounds embedded in complex matrices, customs laboratories are faced with many different questions, that may involve multifarious and sometimes cumbersome analytical work. This paper aims at summarising the different challenges that can be encountered and the various analytical methods appropriate to answer a given question. It must be stated that, very often, no single analysis will solve a challenge so that a reasoned combination of methods and skills is required, and not all methods are necessarily applicable to all possible components of a mixture or type of matrix. In practice, the selection of an appropriate analysis method should also be based on the nature of the sample and the number of components to be analysed.

In an ideal world, a plant or an herbal product should always be unambiguously identified, with all possible adulterants, contaminants and impurities controlled for. This is hardly possible, technically and economically. And so, a trade-off is often necessary, challenging the whole concept of "proof of quality"; in fact, the intended use of a given material (combined with an accepted level of risk) will condition its more or less enacting quality requirements:

- Registration as a drug
- Marketing as a food, a cosmetic or a medical device
- Use as a raw material for traditional medicine in a developed country
- Use as a raw material for traditional medicine in a developing country

This means a delicate analytical balance between what is needed for safety and efficacy, what is desirable and what is locally feasible, the all depending on available technology, local regulations and acceptable costs. This balance can be determined in part from traditional knowledge (plausibility of safety) or by identification of known toxic markers (e.g. pyrrolizidine alkaloids,…), but also by serendipity, in deciphering causes of accidents (e.g. *Illicium, Aristolochia,*…). And the "proof of quality" certainly evolves with such knowledges.

CRediT authorship contribution statement

N.W. Muyumba: Writing – original draft. **S.C. Mutombo:** Writing – review & editing. **H Sheridan:** Writing – review & editing. **A. Nachtergael:** Writing – review & editing. **P. Duez:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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