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Herbal Reference Standards: applications, definitions and regulatory requirements

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Abstract: This review concerns the definitions and appropriate analytical characterisations of herbal reference standards within the framework of regulatory requirements. It describes currently applicable rules and regulations, as well as future issues relating to the European Pharmacopoeia and United States Pharmacopoeia. It provides an update on the use and availability of pharmacopoeial (EP and USP) herbal reference standards since our last review was published in 2009. The continuing challenges facing manufacturers, suppliers and analysts are discussed on the basis of exemplary reference substances for herbal products in medicinal and food products. The article also reviews the special aspects of Brazilian stipulations (Brazilian Pharmacopoeia, Anvisa) by comparison with European regulations.

The term *herbal products* as used throughout this article refers to herbal drugs, herbal preparations and finished herbal medicinal products unless a different meaning is obvious from the context. More specific terms are used where necessary.

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Introduction

Safety assessments must be a major concern for all manufacturers and suppliers of botanicals and botanical preparations intended for use as ingredients in pharmaceuticals and food supplements; not only with respect to their sense of responsibility to the customers but also to ensure compliance with the stringent statutory requirements (Ruparel & Lockwood, 2011). Unlike chemically defined products, the constituents responsible for the therapeutic activity and efficacy of most herbal products are only known to a certain extent, if at all. For this reason, the active substance in an herbal medicinal product or food/dietary supplement is always the herbal preparation in its entirety and complexity, *e.g.* in the form of an extract or plant powder. The selection of constituents, so-called phytochemical markers, that are suitable for control purposes for herbal products is therefore essential (Reif et al., 2004; Helliwell, 2006; Veit & Wissel, 2007).

Herbal medicinal products have to meet many statutory requirements relating to their manufacture, constitution, testing, storage and distribution. This means that the reference substances used to calibrate and validate the testing methods that are applied within the framework of quality control have to comply with regulations as well. Active substances in an HMP must be declared and tested on the one hand, while comprehensive validation must ensure that the applied methods provide precise, reproducible and accurate results on the other. Analytical methods are always affected by numerous variables, such as the matrix, the reagents and material used, as well as light, temperature, equipment and the mode of operation. The requirements relating to safe herbal products, particularly with regard to the low active constituent content, constitute a hurdle that can frequently only be met with a great deal of effort and expense. This article therefore gives a brief summary of official definitions, practical requirements and applications for herbal reference standards within the framework of regulatory stipulations. It also focuses on current and future developments within the EP and USP, as well as special aspects relating to Brazilian legislation (*e.g.* Anvisa regulations). It should be read in conjunction with our previous review on herbal reference standards, which already contained a description of the basic regulatory requirements and analytical definitions (Schwarz et al., 2009).

Definitions of reference standards

Apart from the challenges of marker selection and method development for herbal preparations, appropriate characterisation of reference standards is absolutely essential (EMA, 2008; Sahoo et al., 2010). There are official, often country-specific stipulations, which define the requirements that must be met by a compound in order to qualify as a reference standard. Furthermore, the term "reference standard" is also used in the context of reference preparations and reference spectra within the European

Pharmacopoeia's scope of application (EDQM, 2012).

European Pharmacopoeia Chemical Reference Substances (CRS) offered by the European Directorate on the Quality of Medicines & HealthCare (EDQM), are primary reference standards by definition. A primary standard is defined as being: "*A standard shown to have suitable properties for the intended use, the demonstration of suitability being made without comparison to an existing standard.*" Establishment and qualification take place within the framework of an elaborate process, which frequently includes testing in several laboratories, as well as the performance of inter-laboratory tests. Secondary standards are derived by comparison with primary standards. The term "working standard" is used to describe secondary standards that serve as standards within the framework of routine analysis. They are derived from primary reference standards and are therefore equivalent to secondary reference standards.

United States Pharmacopoeia (USP) Standards have been established in accordance with an extensive protocol and released for use by the USP References Substance Expert Committee. The rulings of the USP are described in detail in Section <11> of the General Chapters (United States Pharmacopeial Convention 2012). A relatively new development is that substances that were previously labelled "Authentic Substances" (AS) by the USP, which are highly characterised but not required for use in a USP-NF monograph, are now distributed as "USP Reference Standards". Although this transition simplifies the official definition, it does not absolve the customer from his obligation to prove suitability. Attention must be also paid to the validity statement for a current lot because it may be used only for as long as it remains listed on the web site (www.usp.org). Similar batch validity statements for reference substances from the EP are available from the EDQM home page (www.edqm.org). The user assumes responsibility for ensuring the validity of a batch and receives a substance with a label providing information concerning the quality but no certificate of analysis. In contrast to these official reference standards, some well-established and acknowledged suppliers offer marker compounds with comprehensive certificates of analysis, which may be used in the same way.

Analytical characterisation of reference standards

The following requirements for the analytical characterisation of primary reference substances are defined by official guidelines, *e.g.* EU/ICH and FDA Guidelines (European Commission 1998; EMA 1995; 2000a,b; FDA, 1999), the GLP Directive (European Parliament and the Council of the European Union 2004) and the International Pharmacopoeia (WHO) (WHO, 1999). Generally speaking, compounds that are to be used as reference standards are characterised by means of identity testing and exactly defined purity testing to obtain a content assignment. (Belz et al., 2010; Schwarz et al., 2009; Thorpe & Wadhwa, 2011)

Identity

The identity of a substance is verified by means of appropriate chemical attributes such as structural formula, empirical formula and molecular weight. Mass spectra can be recorded to obtain information about the molecular weight of the reference substance and information relating to the empirical formula can be obtained by measuring high-resolution spectra. Typical fragmentation patterns enable initial interpretations of the underlying structural units and the presence of certain substituents. Mass spectrometry alone is seldom sufficient complete determination of the structure. UV and IR spectra may be used in the same manner to obtain structural information about typical functional groups and basic structural elements, but these spectra must always be compared with a spectra library or combined with other identity tests. Nuclear magnetic resonance (NMR) spectroscopy therefore performs a central role in identity testing. Measurement and interpretation 1D and 2D NMR spectra offers a means of achieving nearly complete structural clarification without the need for comparison with reference spectra. Alternative X-ray structural analysis is also possible but is subject to limitations regarding substance amounts and crystallisation properties. Apart from the methods mentioned above, other means of verifying the structural identity of a primary reference standard include measurement of the melting point, determination of optical rotation values and/or the recording of CD spectra for compounds with chiral centres, as well as TLC and elemental analysis.

Purity

A combination of various analytical techniques is required to determine the purity of a primary reference standard. Selective separating procedures - usually HPLC or GC, or less frequently electrophoretic CE - are used to determine organic impurities. Attention must be given to the selection of suitable detection method that ensures that certain impurities are not left unaccounted for. UV or diode array detectors combined with HPLC are representative of fairly universal and sensitive detection technologies, and light-scattering detection techniques may also be used to detect natural substances (*e.g.* saponins), which lack a pronounced UV chromophore. Flame-ionisation detectors (FID) are highly recommended for gas chromatography. Other universal techniques, such as refractive index detection (RID), should not be used for HPLC because of the lack of sensitivity required for precise, quantitative purity testing. To the same extent, thermal conductivity detectors (TCD) and more selective detectors, such as

ECD, NPD, FPD or AED, should not be used for GC for the same reason.

Differences in the response factors of the substance and the impurities (which also vary according to the pertinent method of detection) exert a significant influence on the purity testing process. Elimination of this methodical error is unaffordable for natural substances, however, due to the complex composition which prevents the identification and quantification of all constituents. The area normalisation (100%) evaluation method is therefore adopted, which ensures that the purer the reference substance, the smaller the error. Furthermore, the error is reduced if it is assumed that most impurities, that are co-extracted during the isolation procedure, are similar to the substance in terms of structure, *e.g*. as shown by their UV spectra.

It is also necessary to determine the water content and residual solvent content, as well as any inorganic impurities that were used during the synthesis or extraction process, which have not been removed completely or, in some cases, are integral parts of the substance. The water content can be determined by means of Karl Fischer microtitration and the residual solvent content can be determined by means of headspace GC analysis, whereby both methods only consume relatively small amounts of the reference substance. Organic impurities may be determined by means of ICP-MS, AAS or various titration methods, such as argentometric titration, according to the type of impurity and all of these methods are characterised by the fact that they can be carried out on a micro scale.

There are special aspects of impurity detection, which may be encountered in the context of this process. Glycosidic compounds usually form hydrates containing at least one extra molecule of water, which can lead to significant water content values between 5 and 10 %. These should not be dried too harshly, however, because of their hygroscopic properties. Examples that demonstrate the problems associated with residual solvents are hypericin (*Hypericum perforatum*), which incorporates pyridine very well, anisatin (*Illicium anisatum*), which exhibits similar properties with respect to MTBE or diethyl ether, as does silybinin (*Silybum marianum*) with respect to isopropyl alcohol. Isolated natural substances are often found in their salt form because a counter-ion is needed for a structure-specific charge at the core molecule. Salts may also be present for stability reasons or to facilitate the handling of a compound. Ammonium salts, metal salts of glycyrrhizin, the chlorides of diverse alkaloids or anthocyanins/anthocyanidins are just a few examples. Exact determination of the inorganic impurities is absolutely essential because the actual content rarely corresponds to the stoichiometric ratio (calculated according to the empirical formula).

It has recently become evident that a certificate of analysis alone is no longer considered to be an adequate means of verifying the quality of a reference substance used in method validation or quality control if it is included as part of a registration dossier for herbal medicinal products/food supplements submitted to the responsible authority with an application for marketing authorisation. The authorities are demanding raw data to support the analytical data on the certificate of analysis, *e.g.* NMR, MS or IR spectra as well as HPLC or GC chromatograms, to an increasing extent. These demands are not fully comprehensible as a demand for spectral raw data alone would imply that the registration dossier would not only need to be judged from a regulatory point of view, but would need to be seen through the eyes of an analytical chemist experienced in structure elucidation as well. These recent requests may possibly be regarded as a first step towards implementation of the requirements laid down by the German Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte, BfArM) as early as 1996. The BfArM expects detailed evidence of the quality of the reference substance used by means of so-called primary standard documentation as part of the quality module of the marketing authorisation dossier. Such documentation comprises a description of the general properties of the reference substance (*e.g.* appearance, solubility, colour), a brief description of the production method (*e.g*. isolation from natural source, synthetic), raw data and a detailed interpretation of the spectroscopic and spectrometric data – 1H-NMR, ^{13}C-NMR, MS, IR, UV spectra (*e.g*. NMR signal assignment, interpretation of MS data including fragmentation patterns, structural reasons for IR absorption bands), TLC chromatogram including method description, results of elemental analysis (CHNO) and the determined water, residual solvent and inorganic impurity content values, as well as descriptions of the applied GC or HPLC procedures with all relevant chromatograms and particularly including the pertinent validation data (precision, linearity, specificity and accuracy). Furthermore, the BfArM is demanding not just one, but also a second, complementary chromatographic separation technique to verify that the selected chromatographic system is capable of determining the purity of the reference substance under investigation. Although not stipulated in any pharmacopoeia or international guideline, these are ideally two completely different techniques (*e.g*. HPLC and GC where possible) or at least methods that differ in terms of the composition of the stationary phase (*e.g*. C8, C18) and the mobile phase (*e.g*. acetonitrile vs. methanol), as well as in the method of detection (*e.g*. different detection wavelengths when using UV detection) (Bundesinstitut für Arzneimittel und Medizinprodukte, 1996).

Content assignment

When all of the purity tests have been completed

and the methods have been validated to an adequate extent (EMA, 1995), the content of the primary reference standard can be calculated according to the following formula:

Assigned content = (100% - (water + residual solvent + inorganic impurities) x chromatographic/electrophoretic purity) (%).

Recent developments and an increasing number of successful applications have transformed what used to be a purely scientific method into an established approach: quantitative nuclear magnetic resonance spectroscopy qNMR (Pauli et al., 2005; 2008; Diehl et al., 2007). Although qNMR is also subject to certain limitations, it has been included in the methods of analysis section (chapter 01/2009:20233) of the European Pharmacopoeia (EDQM, 2012; Veit et al., 2008) and has now become an accepted content assignment method. However, the qNMR technique has not been described in any pharmacopoeial monographs for botanicals or botanical preparations to date.

Reference standards in practice

Unlike primary reference standards, secondary standards (or working standards) must undergo initial testing according to their intended purpose. They must exhibit identical characteristics to the primary standard and traceability must be assured, whereas less pure compounds or extracts are frequently inadequate as secondary standards in practical applications. Furthermore, the terms "primary standard", "secondary standard" and "working standard" are not used uniformly in practice and are unfortunately used misleadingly on some occasions. According to the Section 5.12 of the EP (EDQM, 2012), the user must ensure that he has a substance at his disposal, which has been characterised to an adequate extent and is of a quality that can be assured by means of a re-testing programme: "*A system is established and implemented to ensure the continued fitness-for-use of the reference standards. Normally, a re-test programme is applied, taking account of the known physico-chemical properties and stability data for the reference standard. Reference standards are periodically tested for stability during storage*." This approach can eliminate the need to maintain more than one batch of a primary standard and avoid any misunderstandings.

The EDQM and the USP Convention distribute pharmacopoeial primary reference standards (CRS, USP-RS), which fulfil all the regulatory stipulations regarding EP and USP monographs (EDQM, 2012; United States Pharmacopeial Convention, 2012). In full consequence, this means that the standards can be used for their intended purpose but may not be suitable for any other purposes. "*Where a reference standard is needed, it is an integral part of the pharmacopoeial monograph or the manufacturer's specification. Where a European Pharmacopoeia reference standard is referred to in a monograph or general chapter, it represents the official standard that is alone authoritative in case of doubt or dispute*." (EDQM, 2012 - Section 5.12) This specificity has been even officially recognised in the introduction of ISO Guide 34 (International Organization for Standardization, 2009). Any deviations regarding usage for other purposes than the one for which the standard has been established must therefore be documented and the user must fully demonstrate the suitability. Furthermore the predefinition "*Any value assigned to a reference standard is valid for the intended use and not necessarily for other uses.*" causes the necessity to accomplish own analytical characterizations in this case. To the same extent, the USP states "*The use of these materials is specified in the article's monograph, and these materials generally are necessary for use in the Assay and/or the Identification tests. The suitability of a USP RS for uses outside those specified in a monograph is the responsibility of the user*." (United States Pharmacopeial Convention, 2012, Section <11>) and of course, certified reference substances which are characterised completely in accordance with the regulatory requirements are frequently used for many other applications than those described in the pharmacopoeial monographs.

During the latest 11th International Symposium on Pharmaceutical Reference Standards in Strasbourg in September 2012 some differences between EP und USP with respect to their self-understanding of their mission became obvious. While USP also provides several reference standards not used in any of the USP-NF monographs, the EDQM clearly defines its mission as providing reference standards solely for the purpose that its user can make unequivocal fail or pass decisions for the product analyzed by applying exactly the procedure as described in the corresponding EP monograph. Consequently, the EDQM has decided not to disclose data on the uncertainty values of the assigned content of its reference standards. This strategy is also recognized in ISO 34: "*The measurement uncertainty associated to the content value of Ph. Eur. CRS is not stated since it is considered to be negligible in relation to the defined limits of the method-specific assays for which they are used*" (International Organisation for Standardization, 2009; section 5.17). The reason for that strategy being, that the EDQM does not want the user of its reference standards to use such uncertainty data to make a product pass a test by re-calculating the test result including the uncertainty data, although the test result itself is outside of the specifications set in the respective monograph. Instead, the uncertainty of the reference standard content is already being included in the specifications of each monograph. In contrast to this approach of the EDQM, USP has started to provide uncertainty information for a growing number of reference

standards. This EDQM strategy also has implications for the users who want to establish secondary standards derived from an official EDQM CRS. While this is still possible for secondary standards used in identification tests such as infrared spectroscopy or for peak identification in separation techniques, it is NOT possible any more to prepare secondary standards to be used for quantitative analysis from an official EDQM CRS. This is because there is no uncertainty associated with "identity", but the uncertainty associated with "content" has to be included once a secondary standards for quantitative purposes is to be established. However, as this data is not available from the EDQM, such secondary standard cannot be directly derived by *e.g.* doing a peak area comparison to the CRS. Instead a user who wants to replace an EDQM CRS with an in-house standard would need to perform a complete mass balance analysis for his in-house standard as described previously in this publication (*i.e.* characterizing the in-house standard as a primary reference substance). Then a sample would need to be analyzed using the official Ph. Eur. CRS and the own in-house reference substance, and results would have to be compared. Thus, instead of tracing back a secondary standards directly to an official CRS, the procedure now is a comparison of results obtained with the CRS and the in-house standard. The EDQM also has announced to communicate this procedure to producers of secondary reference standards and eventually also take legal action against producers claiming that their secondary standards are traceable to an EDQM CRS (Egloff, 2012).

It is worth mentioning that herbal reference extracts are now being included in the European and United States Pharmacopoeias alongside pure reference substances, but this is a very controversial issue. Apart from the advantages – easy availability of larger quantities and the relatively low price compared with the price of an isolated pure substance – major problems are also encountered in the application of reference extracts. Many extracts are hygroscopic, requiring special conditions for packaging, handling and storage processes. Furthermore, the quantification of such complex mixtures, where batches can vary significantly with respect to their matrix components, requires methods with high chromatographic resolution and reproducibility. Finally, a reference extract is always a derived or secondary standard, which means that no other additional standards can be derived from it and the range of applications is very limited, usually restricted to just a single assay in an herbal monograph. One example is "*valerian dry extract HRS*" (corresponding monograph: *valerian root 07/2010:0453*), which is only used for sesquiterpene acid content calculation (sum of valerenic acid and acetoxyvalerenic acid), whereas reagent grade valerenic acids are necessary for identity testing. Apart from this, the EDQM guideline postulates that CRS extracts and drugs are also intended and suitable for single use only, alternative extracts are neither applicable nor allowed.

Regulatory stipulations

Regulatory requirements for herbal medicinal products (HMP) are clearly regulated in the EU (Vlietinck et al., 2009), in the USA and in many other countries. Food supplements have not been defined to the same extent but are covered by food law within the EU (EFSA, 2009; EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2011) and by cGMP for dietary supplements in the USA since June 2007 (United States of America Congress, 1994; FDA, 2012). In contrast to this, Brazil has only just begun to stipulate official regulations for herbal products used in medicinal products, dietary and food supplements. Hence the quality aspect has been brought up more in discussions in recent years and the quality of reference standards and the pertinent documentation will play an important role in this respect.

All globally operating manufacturers of drugs and medicinal products are affected by the guidelines drawn up by WHO, ISO and NIST, as well as by monographs of such pharmacopoeias as the European or United States Pharmacopoeia, which define and provide general and plant-specific requirements for the quality and quality control of plant-based products. Some fundamental requirements for the establishment, documentation and use of reference standards regarding active pharmaceutical ingredients can be found in the *Note for Guidance on Good Manufacturing Practice for Active Pharmaceutical Ingredients* (EMA, 2000b). Although they are not legally binding, ISO Guides 30-35 (International Organisation for Standardization, 1997, 2000a,b, 2006, 2008, 2009) are more essential for reference standard users. ISO Guide 30, for example, which defines a certified reference material (CRM) as follows: "*Reference material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence*."

Particularly important for manufacturers in the pharmaceutical sector, or third parties who establish standards themselves instead of using pharmacopoeial standards, is that each set of rules and standards (ISO Guides and EP) contains cross-references to the other one. Any deviations must be documented with evidence of their equivalence, even if parts of documents, such as Section 5.12 "*Reference standards*" of the EP only have a virtually binding effect within the pharmacopoeia's scope of application. Furthermore, "*Reference standards are shown to be suitable for their intended purpose; they are not necessarily suitable for other purposes*." is an important principle laid down in the EP. The CRS

standards fulfil these specifications in full consequence. Hence reference standards are an important tool to not only ensure analytical quality and method transfer but also to guarantee the safety of herbal products, including the raw material, extracts, pharmaceutical formulations and food or dietary supplements.

Current issues of the EP and USP

The ongoing development of new products, improvements in the field of analytical techniques and the official acknowledgement of such traditional medical concepts as TCM or Ayurvedic medicine leads to a continuous stream of new monographs drawn up by EP and USP committees. This means that the selection of plant-based reference materials will continue to present a major challenge in the foreseeable future. Promising monographs on plants containing caffeine (maté, green tea, guarana), *Crataegi folium cum flore, Echinaceae purpureae succus expressus, Passiflora, Primulae flos, Serratulae coronatae herba, Uncariae tomentosae cortex, Urticae radix, Vitis viniferae folium, Withania somnifera radix* are still being investigated by the EDQM, as well as numerous TCM monographs. State-of-the-art analytical techniques still include qNMR. In addition photometric methods are being replaced by more specific HPLC with different detection modes (UV, ELSD etc.) to an increasing extent.

Overview of the current use of reference extracts, reference substances and reagents

A number of new reference substances and extracts have been introduced since the publication of our last review (Schwarz et al., 2009), which covered European Pharmacopoeia 6.3 and United States Pharmacopoeia 31-NF 26, Second Supplement.

Three new reference extracts have been introduced up to edition 7.5 of the EP: Actaea racemosa dry extract for system suitability in the monograph on Black Cohosh (2069), kudzuvine root dry extract in the monographs on Kudzuvine Root (2434) and Thomson Kudzuvine Root (2483) and saw palmetto extract in the monograph on Saw Palmetto Fruit (1848). An overview of all currently used reference extracts and their application in the European Pharmacopoeia is given in Chart 1. Each of these three newly introduced extracts is used in quite a different way. The 'Actaea racemosa dry extract for system suitability' is a tool for peak identification in the sample to be analysed and also acts as a system suitability test, as its name already suggests, with respect to a minimum peak-to-valley ratio between certain peaks in the chromatogram. Kudzuvine root dry extract is used to both identify various isoflavonoids in the HPLC chromatogram and to quantify puerarin and total isoflavonoids calculated as puerarin in the two monographs mentioned above, but a puerarin R is used as a reference substance in thin-layer chromatography. While use of an extract for peak identification of several isoflavonoids, which are not commercially available as reference substances, seems to be an approach that is not only expedient but also very time and cost efficient, quantitation could also have been easily achieved by means of a reasonably priced puerarin CRS, particularly as reagent grade puerarin material is also needed for TLC identity testing. Furthermore, the puerarin peak in the chromatogram provided with the reference extract is not very well resolved. The saw palmetto extract serves various purposes: it is used to identify peaks within the framework of identity testing and the assay, and to check for a specific peak-to-valley ratio in the system suitability test. Lauric acid CRS and oleic acid CRS are then used for quantitation of saturated and unsaturated fatty acids. This saw palmetto reference extract constitutes a very good example of the way in which an extract can be used to identify a large number of signals in the chromatograms and considerably reduce the requisite number of reference standards at the same time. In previous versions of the saw palmetto fruit monograph, several reagent grade "reference standards" were used in the assay: caprice acid, caprice acid, laurel acid, linoleum acid, linolenic acid, oleic acid, palmitic acid and palmitoleic acid. These reagent grade materials are no longer required for the assay now that the reference extract has been introduced and lauric acid and oleic acid have been changed from reagent grade to CRS.

Apart from the fatty acids mentioned above, berberine chloride and hydrastine hydrochloride have also been changed from reagent grade material into CRS for use in monographs on goldenseal rhizome (1831) and *Hydrastis canadensis* for homeopathic preparations (2500), thus further reducing the number of reagents used for assays in European Pharmacopoeia 7.5. Refer to Chart 2 for a complete overview on remaining reagents used in assays, which hopefully will be replaced by CRS in the future.

In addition to some reagent grade materials being replaced by reference substances, EP has also introduced some new CRS for application in assays of new monographs, *i.e.* astragaloside IV in *Astragalus mongholicus* root (2435), baicalin in Baical Skullcap root (2438), honokiol and magnolol in *Magnolia officinalis* bark (2567; only official from EP 7.6), imperatorin in *Angelica dahurica* root (2556), naringin in *Draynria rhizome* (2563), osthole in *Angelica pubescens* root (2557), rosmarinic acid in Melissa leaf (1447), Melissa leaf dry extract (2524) and Peppermint leaf dry extract (2382), and tetrandrine in Fourstamen *Stephania* root (2478). The already existing rutoside trihydrate is now also used in addition to the previous applications in *Sophora* flower bud (2427) and chlorogenic acid in Artichoke leaf dry extract (2389). A complete overview of the currently available European Pharmacopoeia CRS for herbal monographs is given in Chart 3.

The USP, on the other hand, has introduced nine new reference extracts and 28 new reference substances up to edition USP35 – NF 30, Second Supplement. Of these 28 USP RS, 26 are pure compounds, and two are mixtures of oligomeric grape procyanidins. The newly introduced reference extracts are Andrographis, Ashwagandha, Bacopa, *Boswellia serrata, Centella asiatica*, Curcuminoids, Forskohlii, *Garcinia* hydroxycitrate, Guggul, Malabar-Nut-Tree leaf, maritime pine and *Phyllanthus amarus* extract. This means that reference extracts are used for all of the monographs that were scheduled for introduction in the near future at the time of last review (Schwarz et

Chart 1: CRS reference extracts in the European Pharmacopoeia (Issue 7.5).

CRS reference extract (EP)	Monograph #	Monograph	Identity	Assay
Senna extract	0206	Senna leaf	x	
	0207	Senna pods Alexandrian	x	
	0208	Senna pods Tinnevelly	x	
	1261	Senna leaf dry extract standardised	x	
Milk thistle standardised dry extract	1860	Milk-thistle fruit		x
	2071	Milk thistle dry extract refined and standardised		x
Valerian standardised dry extract	0453	Valerian root		x
	1898	Valerian dry hydroalcoholic extract		x
	1899	Valerian tincture		x
	2400	Valerian dry aqueous extract		x
	2526	Valerian root cut		x
Agnus castus fruit standardised dry extract	2147	Agnus castus fruit		x
Ginkgo dry extract for peak identification	1827	Ginkgo dry extract refined and quantified		Peak ID only
Ginkgolic acids	1827	Ginkgo dry extract refined and quantified		x
St. John's wort standardised dry extract	1874	St. John' s wort dry extract quantified		x
Bilberry dry extract	2394	Fresh bilberry fruit dry extract refined and standardised		x
Ivy leaf standardised tincture	2148	Ivy leaf		x
Standardised artichoke leaf dry extract	2389	Artichoke leaf dry extract		SST and peak ID only
Kudzuvine root dry extract	2434	Kudzuvine root		x
	2483	Thomson kudzuvine root		x
Actaea racemosa dry extract for system suitability HRS	2069	Black cohosh		SST and peak ID only
Saw palmetto extract	1848	Saw palmetto fruit		SST and peak ID only

Chart 2: Reagents used for assays in the European Pharmacopoeia (Issue 7.5).

Reagent R in EP	Monograph #	Monograph	Method	Required purity	Remarks
11-keto-β-boswellic acid	2310	Indian frankincense	HPLC	90 % (HPLC)	Percentage purity taken into consideration
acetyl-11-keto-β-boswellic acid	2310	Indian frankincense	HPLC	90 % (HPLC)	Percentage purity taken into consideration
apigenin 7-glucoside	0404	*Matricaria* flower	HPLC	95 % (HPLC)	Percentage purity taken into consideration
asiaticoside	1498	*Centella*	HPLC	97 % (HPLC)	HPLC purity taken into consideration
cinchonine	0174	*Cinchona* bark	Photometry	-	
	1818	*Cinchona* liquid extract standardised			
eugenol	2094	Oriental cashew for homoeopathic preparations	Photometry	-	

ferulic acid	1419	*Eleutherococcus*	HPLC	99 % (HPLC)	Reagent used in spite of ferulic acid CRS being available
ginsenoside Rb1	1523 2383	Ginseng Notoginseng root	HPLC	95 % (HPLC)	HPLC purity taken into consideration
ginsenoside Rg1	1523 2383	Ginseng Notoginseng root	HPLC	95 % (HPLC)	HPLC purity taken into consideration
hederacoside C	2092	*Hedera helix* for homoeopathic preparations	HPLC	95 % (HPLC)	Percentage purity taken into consideration
marrubiin	1835	White horehound	HPLC	95 % (GC)	Percentage purity taken into consideration
parthenolide	1516	Feverfew	HPLC	90 % (HPLC)	Purity not taken into consideration
quercetin dihydrate	1828	*Ginkgo* leaf	HPLC	90 % (HPLC)	Content calculation allows for percentage of anhydrous quercetin. Reagent used in spite of quercetin dihydrate CRS being available
quinine	0174 1818	*Cinchona* bark *Cinchona* liquid extract standardised	Photometry	-	
sinensetin	1229	Java tea	HPLC	95 % (HPLC)	Purity not taken into consideration

Chart 3: CRS reference substances in the European Pharmacopoeia (Issue 7.5).

CRS reference substance in EP	Monograph #	Monograph	Identity	Purity/Limit	Assay
arbutin	1054	Bearberry leaf			x
astragaloside IV	2435	*Astragalus mongholicus* root			x
baicalin	2438	Baical skullcap root			x
benzyl alcohol	1827	Ginkgo dry extract refined and quantified			x
berberine chloride	1831	Goldenseal rhizome			x
	2500	*Hydrastis canadensis* for homeopathic preparations			x
boldine	1396	Boldo leaf			x
	1816	Boldo leaf dry extract			x
caffeine	1504	Cola			x
capsaicin	1859	*Capsicum*		x	x
	2336	*Capsicum* oleoresin refined and quantified		x	x
	2337	*Capsicum* tincture standardised		x	x
cephaeline hydrochloride	0094	Ipecacuanha root	x		
	1875	Ipecacuanha liquid extract standardised	x		
	0093	Ipecacuanha prepared	x		
	1530	Ipecacuanha tincture standardised	x		
chlorogenic acid	1866	Artichoke leaf	x		x
	2389	Artichoke leaf dry extract			x
	1821	Narrow-leaved coneflower root	x		x
	1897	Nettle leaf	x		x
	1822	Pale coneflower root	x		x
	1823	Purple coneflower herb	x		x
	1824	Purple coneflower root	x		x
coumarin	2120	Melilot	x		x

cyanidin chloride	2394	Fresh bilberry fruit dry extract refined and standardised		x	
digitoxin	0117	*Digitalis* leaf			x
emetine hydrochloride	0094	Ipecacuanha root	x		
	1875	Ipecacuanha liquid extract standardised	x		
	0093	Ipecacuanha prepared	x		
	1530	Ipecacuanha tincture standardised	x		
ferulic acid	1834	Lemon verbena leaf			x
foeniculin for peak identification	0804	Anise oil		x	
ginkgolic acids	1827	Ginkgo dry extract refined and quantified			x
glycyrrhizate (monoammonium)	2378	Liquorice dry extract for flavouring purposes			x
	1536	Liquorice ethanolic liquid extract standardised			x
	0277	Liquorice root			x
harpagoside	1871	Devil's claw dry extract			x
	1095	Devil's claw root			x
honokiol*	2567	*Magnolia officinalis* bark			x
hydrastine hydrochloride	1831	Goldenseal rhizome			x
	2500	*Hydrastis canadensis* for homeopathic preparations			x
imperatorin	2556	Angelica dahurica root			x
lauric acid	1848	Saw palmetto fruit			x
magnolol*	2567	*Magnolia officinalis* bark			x
naringin	2563	*Drynaria* rhizome			x
oleic acid	1848	Saw palmetto fruit			x
oleuropein	1878	Olive leaf			x
osthole	2557	*Angelica pubescens* root			x
purpureaglycoside A	0117	*Digitalis* leaf	x		
purpureaglycoside B	0117	*Digitalis* leaf	x		
quercetin dihydrate	1827	*Ginkgo* dry extract refined and quantified			x
rosmarinic acid	1447	*Melissa* leaf			x
	2524	*Melissa* leaf dry extract			x
ruscogenins	1847	Butcher's broom	x		x
rutoside trihydrate	2427	Sophora flower-bud			x
	2184	Buckwheat herb			x
	1874	St. John's wort dry extract quantified			x
salicin	1583	Willow bark			x
	2312	Willow bark dry extract			x
tetrandrine	2478	Fourstamen Stephania root			x
verbenalin	1854	Verbena herb			x

*from EP 7.6 only.

al., 2009), *i.e*. turmeric, soy and the monographs from ayurvedic medicine (*Andrographis*, Ashwagandha, *Boswellia, Garcinia* and Guggul). The USP uses these reference extracts exclusively in the chromatographic procedures involved in identity testing, peak identification and/or system suitability testing in line with its previous activities. Maritime pine bark extract is the only one that is also used in a photometric assay. Where one or more new pure reference substance(s) is/are introduced in a new USP monograph, such substances are also used in the quantitative assays. These include the following substances: 3-acetyl-11-keto-β-boswellic acid for *Boswellia serrata,* andrographolide for *Andrographis*, asiaticoside for *Centella asiatica*, bacoside A3 for *Bacopa*, bisdemethoxycurcumin,

curcumin and demethoxycurcumin for turmeric, calcium hydroxycitrate for *Garcinia*, daidzein, daidzin, genistein, genistin, glycitein and glycitin for soy, eleutheroside E for *Eleuthero*, forskolin for Forskohlii, guggulsterone Z for Guggul, phyllanthin for *Phyllanthus amarus*, withanolide A and withanoside IV for Ashwagandha and vasicine for Malabar-Nut-Tree. A complete overview on currently available USP reference extracts and USP reference substances and their applications is given in Charts 4 and 5 respectively.

Pyrrolizidine alkaloids and steviol glycosides: two prominent examples

Pyrrolizidine alkaloids and steviol glycosides are two examples that demonstrate the ongoing challenges facing manufacturers and suppliers who produce and distribute herbal products that are subject to the requirements or national and international legislation.

For some time, now, people have been aware that some foodstuffs, particularly including mixed

Chart 4: CRS reference extracts in the US Pharmacopoeia (up to and including USP35-NF 30 through Second Supplement, official on December 1, 2012).

USP reference extract	Dietary Supplements Monograph	Identity	Content
P. *Andrographis* extract	*Andrographis*, P. *Andrographis* and P. *Andrographis* extract	x	x (peak ID)
P. *Ashwagandha* extract	*Ashwagandha* root, P. *Ashwagandha* root, P. *Ashwagandha* root extract	x	x (peak ID)
P. American ginseng extract	American ginseng, capsules, Ttblets, P. american ginseng, P. american ginseng extract	x	x
P. Asian ginseng xxtract	American ginseng, capsules, tablets, Asian ginseng, Tablets, P. American ginseng, P. American ginseng extract, P. Asian ginseng, P. Asian ginseng extract	x	x
P. *Bacopa* extract	*Bacopa*, P. *Bacopa*, P. *Bacopa* extract	x	x (peak ID)
P. Bilberry extract*	P. Bilberry extract	x	x (peak ID)
P. Black cohosh extract	Black cohosh, fluid extract, tablets, P. black chosh, P. black cohosh extract	x	x (peak ID)
Boswellia serrata extract	*Boswellia serrata, Boswellia serrata* extract	x	x (peak ID)
P. Cat's Claw Extract	Cat's claw, capsules, tablets, P. cat's claw, P. cat's claw extract	x	x (peak ID)
P. C*entella asiatica* extract	*Centella asiatic*a, P. *C.asiatica*, P. *C. asiatica* extract, *C. asiatica* triterpenes	x	x (peak ID)
Curcuminoids	Turmeric, P. turmeric, P. turmeric extract, curcuminoids, capsules, tablets	x	x (system suitability)
P. Decaffeinated green tea extract*	P. decaffeinated green tea extract	x	x (peak ID)
P. *Echinacea angustifolia* extract	*Echinacea angustifolia,* P. *E. angustifolia*, P. *E. angustifolia* extract	x	-
P. *Echinacea pallida* extract	*Echinacea pallida*, P. *E. pallida*, P. *E. pallida* extract	x	-
P. *Echinacea purpurea* extract	*Echinacea purpurea* aerial parts, root, P. *E. purpurea*, P. *E purpurea* extract	x	-
P. Eleuthero extract	Eleuthero, P. eleuthero, P. eleuthero extract		x
P. Forskohlii extract	Forskohlii, P. forskohlii, P. forskohlii extract	x	x (peak ID)
P. *Garcinia* hydroxycitrate extract	*Garcinia cambogia*, P. *Garcinia cambogia*, P. *Garcinia hydroxycitrate* extract, *Garcinia indica*, P. *Garcinia indica*	-	x (system suitability)
Purified guggul extract	Guggul, purified guggul extract, native guggul extract, guggul tablets	x	x (peak ID)
P. malabar-nut-tree, leaf extract	Malabar-nut-tree, leaf, P. malabar-nut-tree, leaf extract P. Malabar-nut-Tree, leaf	- x	x (peak ID) x (peak ID)
Maritime pine extract	Maritime pine, maritime pine extract	x	x (photometric)
P. Milk thistle extract	Milk thistle, capsules, tablets, P. milk thistle, P. milk thistle extract	x	x (peak ID)

P. *Phyllanthus amarus* extract	*Phyllanthus amarus*, P. *P. amarus*	x	x (peak ID)
P. Red clover extract	P. Red clover, P. red clover extract, red clover, tablets	x	x (peak ID)
P. St. John's Wort extract	P. St. John's Wort, St. John's Wort	-	x (peak ID)
	P. St. John's Wort extract	x	x (peak ID)
Powered chaste tree extract	Chaste tree, P. chaste tree, P. chaste tree extract	x	-
Pygeum extract	*Pygeum*, capsules, extract	x	-
Tomato extract containing lycopene	Tomato extract containing lycopene	-	x

P.: Powdered

Chart 5: USP reference substances (USP 35-NF30 through second supplement).

USP reference substance	Monograph	Identity	Purity/limit	Content
3-acetyl-11-keto-β-boswellic Acid	DS *Boswellia serrata*, *B. serrata* extract	x	-	x
(S)-allyl-L-cysteine	DS Garlic fluid extract	x	-	x
andrographolide	DS *Andrographis*, P. *Andrographis*, P. *Andrographis* extract	x	-	x
actein	DS Black Cohosh, fluid extract, tablets, P. Black Cohosh, P. Black Cohosh extract	x	-	x (system suitability)
agigenin	DS Garlic, P. Garlic	x	-	-
agnuside	DS Chaste tree, P. Chaste tree	-	-	x
	DS P. Chaste tree extract	x	-	x
alliin	DS Garlic, Garlic delayed-release tablets, P. Garlic, P. Garlic extract	x	-	x
apigenin	DS P. Soy isoflavones extract, capsules, tablets	-	-	x (internal standard)
apigenin-7-glucoside	DS Chamomile	-	-	x
asiaticoside	DS *Centella asiatica*, P. *C. asiatica*, P. *C. asiatica* extract, *C. asiatica* triterpenes	x	-	x
aspartic acid	DS P. Stinging nettle, P. Stinging Nettle extracts, Stinging nettle	-	-	x
atropine sulphate	USP Belladonna extract, Belladonna extract tablets, Belladonna leaf, Belladonna tincture	-	-	x
bacoside A3	DS *Bacopa*, P. *Bacopa*, P. *Bacopa* extract	x	-	x
berberine chloride	DS Goldenseal, P. Goldenseal, P. Goldenseal extract	x	-	x
bisdemethoxycurcumin	DS Turmeric, P. Turmeric, P. Turmeric extract, curcuminoids capsules, tablets, curcuminoids	x (HPLC)	-	x
caffeine	DS P. decaffeinated green tea extract	-	x	-
calcium (-)-hydroxycitrate	DS *Garcinia cambogia*, P. *G. cambogia*, P. *Garcinia hydroxycitrate* extract, *Garcinia indica*, P. *Garcinia indica*	x (HPLC)	-	x
capsaicin	DS Ginger, tincture, P. Ginger	-	x	x
	DS Ginger capsules, USP capsicum oleoresin	-	-	x
casticin	DS Chaste tree, P. Chaste tree, P. Chaste tree extract	x	-	x
(+)-catechin	DS Grape seeds oligomeric proanthocyanidins	x	x	x (system suitability)
chlorogenic acid	DS *Echinacea angustifolia*, *E. pallida, E. pupurea* aerial parts, *E. purpurea* root, P. *E. angustifolia*, P. *E. angustifolia* extract, P. *E. pallid*a, P. *E. pallida* extract, P. *E. purpurea*, P. *E. purpurea* extract,	-	-	x
	DS *Ginkgo*, P. *Ginkgo* Extract	x	-	-

β-chlorogenin	DS Garlic, P. Garlic	x	-	-
citric acid	DS Cranberry liquid formulation	x (HPLC)	-	x
	DS *Garcinia cambogia*, P. *G. cambogia*, P. *Garcinia hydroxycitrate* extract, *G. indica*, P. *G. indica*	-	x	-
curcumin	DS Turmeric, P. Turmeric, P. Turmeric extract, curcuminoids capsules, tablets, curcuminoids	x (HPLC)	-	x
cyanidin 3-*O*-glucoside	DS P. Bilberry extract	-	-	x
cyanidin chloride	DS P. Bilberry extract	-	x	-
daidzein	DS P. Soy isoflavones extract, capsules, tablets	x (HPLC)	-	x
daidzin	DS P. Soy isoflavones extract, capsules, tablets	x (HPLC)	-	x
demethoxycurcumin	DS Turmeric, P. turmeric, P. turmeric extract, curcuminoids capsules, tablets, curcuminoids	x (HPLC)	-	x
23-*epi*-26-deoxyactein	DS Black Cohosh, fluid extract, tablets, P. Black Cohosh, P. Black Cohosh extract	x	-	x
dextrose	DS Cranberry liquid formulation	-	-	x
digitoxin	USP P. *Digitalis*	x	-	-
diosmin*	DS Diosmin	x (HPLC)	x	x
diosmin for system suitability*	DS Diosmin	-	-	x (system suitability)
docosyl ferulate	DS *Pygeum* capsules, *Pygeum* extract	x (HPLC)	-	x
eleutheroside B	DS Eleuthero, P. Eleuthero, P. Euthero extract	x	-	x
eleutheroside E	DS Eleuthero, P. Eleuthero, P. Eleuthero extract	x	-	x
emetine hydrochloride	UPS Ipecac oral solution, Ipecac, P. Ipecac	-	-	x
(-)-epigallocatechin-3-*O*-gallate	DS P. decaffeinated green tea extract	x	-	x
escin	DS Horse chestnut, P. Horse chestnut, P. Horse chestnut extract	x	-	x
formononetin	DS P. Red clover, P. Red clover extract, Red clover, tablets	x (HPLC)	-	x
forskolin	DS Forskohlii, P. Forskohlii, P. Forskohlii extract	x	-	x
fructose	DS Cranberry liquid formulation	-	-	x
genistein	DS P. Soy isoflavones extract, soy isoflavones capsules, tablets	x (HPLC)	-	x
genistin	DS P. Soy isoflavones extract, soy isoflavones capsules, tablets	x (HPLC)	-	x
ginger constituent mix (6-gingerol and 6-shogaol)	DS Ginger, capsules, tincture, P. Ginger	-	-	x (system suitability)
Ginkgo terpene lactones	DS *Ginkgo*, P. *Ginkgo* extract	-	-	x
	DS *Ginkgo* capsules, tablets	x (HPLC)	-	x
ginkgolic acids	DS P. *Ginkgo* extract	-	x	-
gitoxin	USP P. Digitalis	x	-	-
glutamic acid	DS P. Stinging nettle, P. Stinging nettle extract, stinging nettle	-	-	x
gammy-glutamyl-(*S*)-allyl-cysteine	DS Garlic, P. Garlic	-	-	x
glycitein	DS P. Soy isoflavones extract, soy isoflavones capsules, tablets	x (HPLC)	-	x

glycitin	DS P. Soy isoflavones extract, soy isoflavones capsules, tablets	x (HPLC)	-	x
glycyrrhizic acid	DS Licorice, P. Licorice, P. Licorice Extract	x	-	x
grape seed oligmeric proanthocyanidins	DS Grape seeds oligomeric proanthocyanidins	-	x	-
purified grape seed oligmeric proanthocyanidins	DS Grape seeds oligomeric proanthocyanidins	x	-	x
guggulsterone Z	DS Guggul, purified Guggul extract, native Guggul extract, Guggul tablets	x (HPLC)	-	x
hexacosanol	DS Saw palmetto capsules	x	-	-
	DS Saw palmetto extract	-	-	x
2*E*, 4*E*-hexadienoic acid isobutylamide	DS *Echinacea angustifolia*, *E. pupurea* aerial parts, root, P. *E. angustifolia*, P. *E. angustifol*ia extract, P. *E. purpurea*, P. *E. purpurea* extract	-	-	x
hydrastine	DS Goldenseal, P. Goldenseal, P. Goldenseal extract	x	-	x
hyperoside	DS Hawthorn leaf with flowers, P. Hawthorn leaf with flowers, P. St. John's Wort, St. John's Wort	x	-	-
isopteropodine	DS Cat's Claw, capsules, tablets, P. Cat's Claw, P. Cat's Claw extract	-	-	x
isorhamnetin	DS *Ginkgo*	-	-	x
	DS P. *Ginkgo* extract, *Ginkgo* capsules, tablets	x (HPLC)	-	x
kaempferol	DS Ginkgo	-	-	x
	DS P. Ginkgo extract, Ginkgo capsules, tablets	x (HPLC)	-	x
levomenol	DS Chamomile	-	-	x
L-methionine	DS Garlic, Garlic delayed-release tablets, P. Garlic, P. Garlic extract	x	-	-
lutein	DS Lutein preparation	x	-	x
lycopene	DS Lycopene preparation	-	-	x
	DS Tomato extract containing lycopene	x	-	x
malic acid	DS Cranberry liquid preparation	x (HPLC)	-	x
methyl caprate, methyl caproate, methyl caprylate, methyl laurate, methyl linoleate, methyl linolenate, methyl myristate, methyl oleate, methyl palmitate, methyl palmitoleate, methyl stearate	DS P. Saw palmetto, Saw palmetto	-	-	x
	DS Saw palmetto capsules, extract	x	-	x
oxybenzone	DS P. St. John's Wort, P. St. John's Wort extract, St. John's Wort	-	-	x
parthenolide	DS Feverfew, P. Feverfew	x	-	x
phyllanthin	DS *Phyllanthus amarus*, P. *P. amarus*	x	-	x
protocatechuic acid	DS Maritime pine, Maritime pine extract	-	-	-
quercetin	DS *Ginkgo*, Hawthorn leaf with flowers, P. Hawthorn leaf with flowers	-	-	x
	DS *Ginkgo* capsules, tablets, P. Ginkgo extract	x (HPLC)	-	x
quinic acid	DS Cranberry liquid formulation	x (HPLC)	-	x

reserpine	USP P. *Rauwolfia serpentina*, *R. serpentin*a, tablets	-	-	x
rutin	DS Feverfew, *Ginkgo*, P. Feverfew, P. *Ginkgo* extract	x	-	-
scopolamine hydrobromide	USP Belladonna extract, tablets, Belladonna leaf, tincture	-	-	x
scopoletin	DS P. Stinging nettle, P. Stinging nettle extract, Stinging nettle	x	-	x
sennosides	USP Senna leaf, pods	-	-	x
silybin	DS Milk Thistle, capsules, tablets, P. Milk Thistle, P. Milk Thistle extract	-	-	x
silydianin	DS Milk Thistle, capsules, tablets, P. Milk Thistle, P. Milk Thistle extract	x	-	x
β-sitosterol	DS P. Stinging nettle, P. Stinging nettle extract, *Pygeum* capsules, *Pygeum* extract, Stinging nettle	x	-	-
	DS *Pygeum*, Saw palmetto capsules	x	-	-
	DS Saw palmetto extract	-	-	x
sorbitol	DS Cranberry liquid formulation	-	x	-
sucrose	DS Cranberry liquid formulation	-	x	-
withanolide A	DS Ashwagandha root, P. Ashwagandha root, P. Ashwagandha root extract	x	-	x
withanoside IV	DS Ashwagandha root, P. Ashwagandha root, P. Ashwagandha root extract	x	-	x
valerenic acid	DS P. Valerian, P. Valerian extract, Valerian, tablets	x	-	x
vasicine	DS Malabar-Nut-Tree teaf, P. Malabar-Nut-Tree leaf, P. Malabar-Nut-Tree, leaf extract	x	-	x
vitexin	DS Hawthorn leaf with flowers, P. Hawthorn leaf with flowers	x	-	x

*will become official with USP35-NF 30 Second Supplement on December 1, 2012; DS: Dietary Supplements; P.: Powdered.

salads and animal feed may be contaminated with plant material containing pyrrolizidine alkaloids (Röder, 1995). Pyrrolizidine alkaloids and their *N*-oxides have been predominantly found in Asteraceae (*e.g. Senecio*, familiarly known as ragwort), Boraginaceae and Fabaceae. They are produced by medicinal plants including comfrey (*Symphytum officinale*), butterbur (*Petasites*) and coltsfoot (*Tussilago farfara*), but may also be found in honey (main contributors are *Echium, Senecio* and *Borago*). Some of these compounds are hepatotoxic, mutagenic and/or carcinogenic *e.g.* echimidine, senecionine and senkirkine. The metabolised Pyrrolo-Pyrrolidine derivatives have an almost equivalent mode of action to genotoxic carcinogens, such as aflatoxins, but not all substances of this compound class exhibit the same toxicity. The "*Scientific Opinion on Pyrrolizidine alkaloids in food and feed*", which was published by the European Food Safety Authority, summarises the level of scientific knowledge regarding PA toxicity and possible resources (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2011). Appropriate analytical methods should therefore be used to detect and quantify every single pyrrolizidine alkaloid.

Apart from these toxicological studies, the EDQM has not included a corresponding section to the EP chapter or updated a monograph, in spite of the fact that HMP are concerned. At the same time, the safety of herbal products can only be assured by the use of suitable reference substances as suggested by various guidance documents issued by the EMEA Herbal Medicinal Product Committee (HMPC) and the FDA (EMA, 2006a,b; FDA, 2004). The so-called markers are defined as follows: "*Markers are chemically defined constituents of an herbal substance which are of interest for control purposes…*". The pertinent monographs of the European or other Pharmacopoeias, *e.g.* USP etc. define markers for many herbal drugs and drug preparations but do not discharge the manufacturers from their liability to inform themselves about up-to-the-minute news regarding product safety. The framework for selection, characterisation and use of marker substances in other areas, particularly including plant-based food products, such as functional food and food/dietary supplements, has not yet been defined to the same extent as for herbal medicinal products.

Plants with sweet and aromatic flavours have been used as ingredients in food and beverages for hundreds of years. Aztec Sweet Herb (*Stevia rebaudiana*) started its success in South America, the fruit of Luo Han Gou (*Siraitia, Momordica grosvenori*) is well-known in Asia,

not only as a sweetener but also as a TCM drug, and the Chinese Blackberry (*Rubus chingii* var. *suavissimus*) is an essential component for a variety of herbal teas in Europe. A warranty for the quality of appropriate preparations and food requires efficient analytical methods and a broad spectrum of reference substances (Hansen & De Olivieria, 1993; Kedik et al., 2003). Steviol glycosides, such as stevioside and the rebaudiosides, are 300 times sweeter than saccharose, free of calories and have enormous potential for new product developments in the food and luxury food industries, but regulatory stipulations require efficient analytical methods and suitable reference substances here as well. Stevia leaves were not approved as a foodstuff within the European Union until recently. A WHO recommendation (WHO, 2009) regarding *Stevia rebaudiana* with its steviol glycosides and several toxicological studies were required in order to initiate the procedure for EU approval. A positive safety assessment of steviol glycosides from EFSA took place at the end of March 2010 and constituted the basis for the approval procedures in all European countries (EFSA Panel on Food Additives and Nutrient Sources (ANS), 2010). The regulation now permits the sale and use of this natural sweetener and was published on November 12, 2011. The legislation became effective in December 2011 (European Commission, 2011). An efficient analytical HPLC method is suggested and this should be used for content determination in order to comply with the new legal requirements (JECFA 2010). The single components (rebaudioside D, rebaudioside A, stevioside, rebaudioside F, rebaudioside C, dulcoside A, rubusoside, rebaudioside B and steviol bioside) can be determined easily using reference substances. Europe is now becoming an open market for stevia suppliers who offer this much anticipated ingredient.

Brazilian regulatory requirements

In spite of Brazil being one of the ten largest pharmaceutical markets in the world and having the most diverse flora, few efforts have been made to enforce legal rules to ensure the efficacy, safety and consistent quality of phytotherapeutical products. In spite of their importance, around 70% of the phytopharmaceuticals have not been studied to a sufficient extent and the products have been marketed for many years without clear legal registration rules being imposed by the Brazilian government.

Historical background

In the 1930s, phytopharmaceuticals were declared to be pharmacopoeial preparations and, as such, exempted from legal registration. More astonishing was the misconception that "natural products are harmless to the health because they come from nature" resulting from drug registration and evaluation laws in the 1970s that not only confused pharmacopoeial substances with other substances, but also forgot to mention phytopharmaceuticals at all. Only one exception was made in 1991 as a consequence of its prohibition in Germany, which led to the product being banned from the Brazilian market as well (Petrovick et al., 1999): the plant *Symphytum officinale*, widely used for oral administration, contains the toxic pyrrolizidine alkaloids mentioned above. This unacceptable situation was improved by the Ministry of Health Ruling MS/GM, which established guidelines for the National Health Surveillance System (SNVS) in 1994 on the basis of WHO recommendations (Piovesan & Labra, 2007; WHO, 1991; 1995; World Health Organization Research Office for the Western Pacific 1993). A revised proposal was incorporated into directive n. 6, issued by SVS in 1995 to regulate the registration procedure for phytopharmaceutical products in Brazil (Petrovick et al., 1999). There are still many practical problems, however, that not only result from antiquated manufacturing processes, the limited number of research institutions for chemical, botanical, agronomic, pharmacological and toxicological studies and the frequent lack of motivation and financial opportunities on the part of the industry, but also from the ineffectiveness of government inspection agencies (Petrovick et al., 1999). The Brazilian Health Surveillance Agency (Anvisa) was established by Law 9782 in 1999 (Anvisa, 2012). This government regulatory agency is characterised by its administrative independence, financial autonomy and it is governed by the five members of its Collegiate Board of Directors. Anvisa's official mission is: "*To protect and promote public health and to intervene in the risks caused by the production and use of products regulated by health surveillance. This mission must be carried out in coordination with states, municipalities and the Federal District, according to the Brazilian Unified Health System principles, in order to improve the quality of life of the population.*" Anvisa is not only responsible for registering drugs and issuing licenses to pharmaceutical laboratories; the agency also coordinates and controls activities relating to food registration, risk control and inspection. Furthermore (according to Law 9782/1999), Anvisa is responsible for coordinating health surveillance activities of laboratories that participate in the official health quality control laboratory network, which conducts analyses on products including herbal drugs, herbal medicinal products and foodstuffs.

Current rules and regulations

The following (Anvisa) recommendations apply to analytical validation and in cases where there are no standards for herbal medicines: "*The best option to qualify an extract is the usage of isolated markers and/or typical chromatographic profiles.*" Qualified extracts can be used as working standards. Although primary standards in

accordance with the European Pharmacopoeia reference standard (CRS) or USP standard are accepted (RDC 37/2001), RE899/2003 stipulates that laboratory standards with proven identity and purity may also be used where marker compounds are not laid down in the Brazilian Pharmacopoeia or any other legal stipulations. The Brazilian Pharmacopoeia defines a reference substance as a product, which is uniform in its properties, has an appropriate level of purity and in intended for use in assays where one or more of its attributes can be used for comparison with the examined sample. In general, the terms primary and secondary standard are used in the same way as defined for European Pharmacopoeia Chemical Reference Substances. The Brazilian law RDC 249/2005 (GMP) for intermediates and pharmaceutical raw materials, which will also apply to plant-based raw materials in the future, contains the following definitions: "*Primary reference standard: A substance which high purity and authenticity was demonstrated by analytical testing. Secondary reference standard: A substance of established quality and purity as compared to a primary reference standard.*" RDC17/2010 is another important law (GMP), particularly with respect to finished products including herbal drugs. Section X describes best practice for the identification of plant drugs in the absence of pharmacopoeial monographs, as well as the quality control procedures to be adopted for raw materials and herbal medicines:

ART. 602. The reference standard may be a substance chemically defined (for example, a known active constituent or a marker substance or a class of chemical compounds present in herbal raw material) or a standard extract.

§1 reference standards officially approved by the Brazilian Pharmacopoeia or other codes authorized by the legislation in force, or else duly characterized reference standards.

§2 the Reference standard shall have the quality fit for this purpose.

§3 All reference standards should be stored in appropriate conditions to avoid degradation.

§4 for characterized reference standards, the full technical report of assessment must be submitted, including nuclear magnetic resonance, mass spectrometry (high resolution), infrared, melting point and/or HPLC (pureness based in the peak-related area).

§5 the standard abstract shall be mentioned in relation to a primary standard, in order to evidence its identity and the marker's content .

This shows that the Brazilian regulatory requirements for herbal products are specified to a much better extent today than they were as recently as twelve years ago. As a result, there is a growing need for appropriate characterised reference standards and validated analytical assays. The manufacturers and suppliers of herbal products are therefore being asked to not only improve their laboratory equipment but also expand their skills to include modern analytical techniques and reference substance establishment to meet all of the pertinent requirements. Helpful information can be found in recent publications, as well as statutory European recommendations, which often contain more stringent and more specific instructions.

The Brazilian Pharmacopoeia

The improvement of Brazilian laws and current activities of Anvisa regarding requirements for herbal products is reflected in the 5th edition of the Brazilian Pharmacopoeia (Anvisa, 2010). Like other Pharmacopoeias this Pharmaceutical Code official for Brazil establishes the minimum quality requirements for drugs, supplies, plants, medicines and health products. The first edition was published in 1929 and since then updated several times. For the first time, the 5th edition launched in December 2010 will make previous editions invalid, which is already common practice for e.g. the European Pharmacopoeia. The current edition of the Brazilian Pharmacopoeia contains 592 monographs, 367 of which are part of the National List of Essential Medicines (Relação Nacional de Medicamentos Essenciais = RENAME). That list also currently contains a total of twelve herbal drugs (Ministério da Saúde, 2012). Regarding HMP the currently official Brazilian Pharmacopoeia contains around 60 monographs dealing with herbs, herbal tinctures, dry extracts and oils. In comparison to the 3rd and 4th edition the number of herbal monographs greatly increased from around 23 to 45 and now to approximately 60. Beginning with the 4th edition more and more medicinal plant monographs (*e.g.* for Brazilian herbs as guaraná, barbatimao, macela and espinheira-santa) were added or updated, respectively (Veiga-Jr & Mello, 2008). Furthermore the 4th edition adopted modern analytical methods and a chapter for pharmacognostical methods was included. In the 5th edition these parts were expanded. Also a significant number of titration assays were replaced by spectrophotometric and HPLC methods. Noteworthy is the newly introduced topic which deals with methods for preparation and analysis of herbal extracts considering also the nature of these extracts (*e.g.* fluid, soft or dry extracst). Six general methods regarding preparation and analysis of herbal extracts were added in the current edition (Pianetti, 2012). An overview on the current herbal monographs in the Brazilian Pharmacopoeia is given in Chart 6.

Conclusions

Herbal medicinal products, as well as dietary and food supplements, have to comply with many statutory requirements relating to their manufacture, constitution, testing, storage and distribution. Within the framework of quality control, the use of appropriate reference standards or so-called phytochemical markers is essential to ensure

Chart 6: Herbal Monographs in the 5th edition of the Brazilian Pharmacopoeia.

Portuguese name	Latin name	Part used
Abacateiro	*Persea americana*	leaves
Alecrim	*Rosmarinus officinalis*	essential oil
Aloe	*Aloe vera*	leaves
Aloe	*Aloe ferox, A. africana, .A. spicata*	dry extract of the leaves
Alteia	*Althaea officinalis*	roots
Anis-doce	*Pimpinella anisum*	fruits
Anis-estrelado	*Illicium verum*	fruits
Arnica	*Arnica montana*	flowers
Bálsamo-do-peru	*Myroxylon balsamum*	balsam
Bálsamo-de-tolu	*Myroxylon balsamum*	balsam
Barbatimão	*Strypnodendron adstringens*	bark
Baunilha	*Vanilla planifolia*	fruits
Beladona	*Atropa belladonna*	leaves
Benjoim	*Styrax benzoin, St. paralleloneuron*	resin
Boldo	*Peumus boldus*	leaves; tincture of the leaves
Calêndula	*Calendula officinalis*	flowers
Canela-da-china	*Cinnamomum cassia*	bark
Canela-do-ceilão	*Cinnamomum verum*	bark
Capim-limão	*Cymbopogon citratus*	leaves
Cardamomo	*Elettaria cardamomum*	seeds
Carqueja	*Baccharis trimera*	herb
Castanha-da-india	*Aesculus hippocastanum*	seeds
Centela	*Centella asiatica*	leaves
Chapéu-de-couro	*Echinodorus grandiflorus*	leaves
Cratego	*Crataegus spp.*	leaves and flowers
Cúrcuma	*Curcuma longa*	rhizome
Endro	*Anethum graveolens*	fruits
Espinheira-santa	*Maytenus ilicifolia*	leaves
Estévia	*Stevia rebaudiana*	leaves
Estramônio	*Datura stramonium*	leaves
Genciana	*Gentiana lutea*	roots and rhizome
Guaraná	*Paullinia cupana*	seeds
Hamamelis	*Hamamelis virginiana*	tincture of the leaves
Hidraste	*Hydrastis canadensis*	roots
Hortelã-pimenta	*Mentha × piperita*	leaves; essential oil
Jaborandi tintura	*Pilocarpus microphyllus tincture*	leaves
Laranja-amarga	*Citrus aurantium*	exocarp
Maracujá-azedo	*Passiflora edulis*	leaves
Maracujá-doce	*Passiflora alata*	leaves
Meimendro	*Hyoscyamus niger*	leaves
Melissa	*Melissa officinalis*	leaves
Noz-de-cola	*Cola nitida*	seeds
Óleo de Amendoim	*Arachis hypogaea*	oil
Óleo de Gergelim	*Sesamum indicum*	oil

Pitangueira	*Eugenia uniflora*	leaves
Polígala	*Polygala senega*	roots
Quebra-pedra	Phylantus niruri; Ph. tenellus	aerial parts
Quilaia	Quillaja saponaria	bark
Quina-amarela	Cinchona calisaya	bark
Ratânia	Krameria triandra	roots; tincture of the roots
Rauvolfia	Rauvolfia serpentina	roots
Ruibarbo	Rheum palmatum; Rh. officinale	roots and rhizome
Sabugueiro	Sambucus nigra	flowers
Sabugueiro-do-brasil	Sambucus australis	flowers
Salgueiro-branco	Salix alba	bark
Sene	Senna alexandrina	leaves

that products are safe and efficacious. A great deal of effort and expense is needed to fulfil all of the official regulations relating to herbal products because of their complex matrices and often low active constituent content. It goes without saying that manufacturers and suppliers are under obligation to ensure that their products are safe, even in the absence of pharmacopoeial monographs. EU/ICH and FDA Guidelines, the GMP Directive as well as International Pharmacopoeias (WHO) define the analytical characterisation for compounds that are to be used as reference standards, especially for herbal medicinal products. The testing parameters must clearly prove identity and purity according to the purpose of the particular reference standard. Apart from CRS and USP primary reference standards, which fulfil the official specifications in full consequence, secondary standards and reference extracts are applied in appropriate manner. A continuous stream of new monographs included in the EP and USP demonstrates the need to lay down guidelines for a growing number of herbal products, including traditional medicines and TCM drugs, along with the need to update analytical methods. Current toxicological research (e.g. on pyrrolizidine alkaloids), which gives rise to new EMEA issues, must be taken into account to an adequate extent. Stevia products have also become the focus of attention as a result of new EU regulations last year. Although Brazil is one of the world's ten largest pharmaceutical markets and has the most diverse flora, regulatory stipulations were neglected to an alarming extent just a few years ago. A growing number of official rules and recommendations (Brazilian Pharmacopoeia, Anvisa regulations and Brazilian law) are now being drawn up for phytotherapeutical products to ensure that they are effective, safe and of a consistent quality standard. Provision of reference standards that are suitable for characterisation of herbal preparations with complex mixtures of active substances therefore continues to be an ongoing global challenge.

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Abbreviations

Anvisa: Brazilian National Health Surveillance Agency
EDQM: European Directorate for the Quality of Medicines & HealthCare
EMA: European Medicines Agency
EFSA: European Food Safety Authority
EP: European Pharmacopoeia
CRS: Chemical Reference Substance
HMP: Herbal Medicinal Product
USP: United States Pharmacopoeia
USP RS: United States Pharmacopoeia Reference Substance

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