INTERNATIONAL STANDARD

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Traditional Chinese medicine — Quality and safety of raw materials and finished products made with raw materials —

Part 2: Identity testing of constituents of herbal origin

Médecine traditionnelle chinoise — Qualité et sécurité des matières premières et des produits finis fabriqués à partir de matières premières —

Partie 2: Identification des constituants d'origine végétale

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 249, *Traditional Chinese medicine*.

A list of all parts in the ISO 19609 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

The ISO 19609 series consists of four parts with different content as shown in Figure 1.

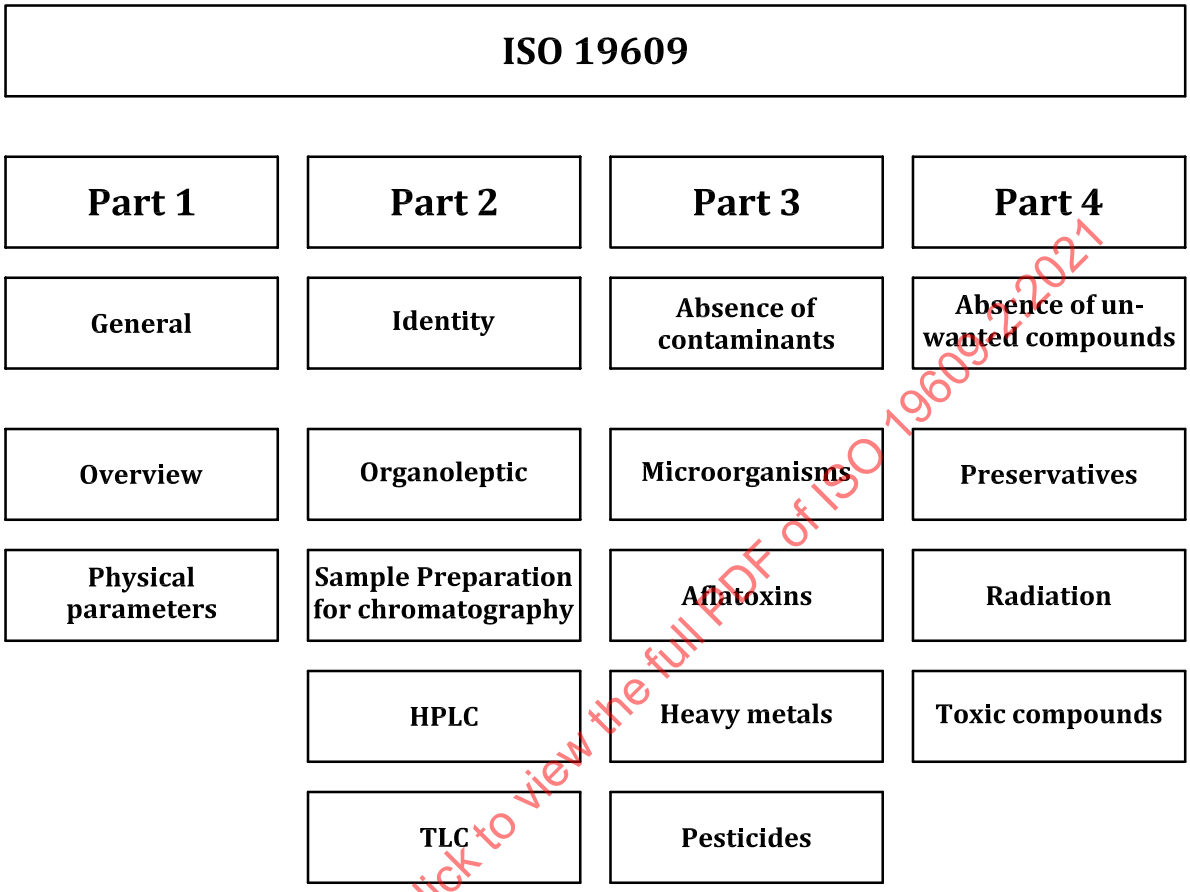


Figure 1 — Overview of the ISO 19609 series

To ensure the safety and efficacy of herbal medicinal products, it is imperative to check the identity of the raw materials and the finished products. One of the aims of the identity check is to prevent the accidental use of falsifications. In order to ensure safety, it is also essential to establish adequate simple testing methods.

As a general basis we can expect in a typical medicinal plant about 1 million constituents. When specific extracts are made from this plant, the number of ingredients is reduced to about 50 000. In classical phytochemical analysis, only one compound is usually analytically identified or quantified as a so-called marker without consideration of the secondary substance matrix from this multicomponent mixture. This results from the test practice for synthetic chemical monopreparations, in which the efficacy is based only on a high-dose active substance. This practice results from the analytical quantification of the relevant effective plasma concentration in the blood of the treated patients.

Phytopharmaceuticals are not based on only one active substance, but on a combination of synergistic compounds (many to thousands). Classical phytotherapy worldwide does not usually use only individual herbs but combinations of several herbs in preparations containing a correspondingly higher number of individual compounds (multitarget theory).

The analytical methods of the pharmacopoeias use one or more marker compounds without significance for the respective effectiveness. This cannot reflect the difficulty of synergistic substances as mentioned previously.

For preparations based on several raw herbal materials or extracts there are currently no test methods available. In the Chinese Pharmacopoeia[7], only one or two markers are often used for such finished products, although many more different raw materials are integrated.

Legally, the quality of these products as remedies must be estimated in an appropriate way.

For analytical quality assessment of such combinations, there are thus two basic approaches:

1) Marker-based identification test

   For each herbal raw material or extract used, a test methodology that can be implemented in the entire product must be individually redeveloped and validated for each of the associated markers. For a combination product with six herbals, herbal parts or exudates, as well as animals and minerals, six independent test methods for each single material based on these six marker compounds are required for release. This approach is currently mandatory for phytopharmaceuticals in countries which apply the European Pharmacopoeia[11].

2) Three-dimensional ingredient overview chromatography (fingerprint)

   With this novel valid method, it is possible to record the entire visible and ultraviolet (UV-VIS) spectroscopic detectable ingredient spectrum of a combination product (finished product) with only one liquid chromatographic separation method [one high-performance liquid chromatography (HPLC) run instead of *x* different ones]. In comparison with corresponding reference extracts of the associated individual materials, this is a clear assignment without compulsory use of the various expensive marker substances possible. This makes a cost-effective, reliable and fast product release possible, without sacrificing product quality.

NOTE Experts agree that the presence of a marker compound as the only criterion for the identity of the used material is not sufficient. Experience of recent years has shown that synthetic active principles or only defined marker constituents were used instead of the real herb material (ephedra problem). Over a long time, high risks resulted from the addition of a racemic mixture of ephedrine to optimize the ephedra material (with a mixture of natural and non-natural isomers), which led in the end to a total prohibition of this material worldwide.

As a method for determining adequate identity, a non-specific HPLC fingerprint method is suitable. This method makes it possible to ensure the identity of the material, both in terms of the retention times of various ingredient patterns and in terms of the UV-VIS-spectra of the individual signals.

Here the question arises as to whether an individualized testing method for each herb (as mostly required in pharmacopoeias) or a general fingerprint method over the whole range of ingredients is to be used. The disadvantage of a method which is not optimally matched to each single herb, however, is easily outweighed by the advantage that complex mixtures of different raw materials in the resulting product can also be identified only with one single method. In addition, the found distribution pattern can give further conclusions on the used extraction procedure.

A universal method must be established over the entire hydrophilic to lipophilic region to realize efficiently the plurality of components in one analytical method, in a sufficiently secure way, with a photodiode array detection (PDA) as well as a diode array detection (DAD). Now the achieved spectra can become assigned to the underlying components of the individual raw materials. In exceptional cases it might be necessary to make certain improvements for individual products which have to be analysed (modification of one of the three-dimensional specifications: time, intensity and spectrum).

Traditional Chinese medicine — Quality and safety of raw materials and finished products made with raw materials —

Part 2:
Identity testing of constituents of herbal origin

1 Scope

This document specifies requirements for identity testing within a quality control framework for raw materials and finished products used in and as traditional Chinese medicine (TCM) and derivative forms. It is applicable to natural products used in TCM, including starting materials and finished products of herbal origin.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 19609-1, *Traditional Chinese medicine — Quality and safety of raw materials and finished products made with raw materials — Part 1: General requirements*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
active substance
substance of physiological or pharmacological action

3.2
decoction piece
prescription medicinal processed from Chinese Materia Medica under the direction of TCM and processing methods for Chinese medicines and derivative forms, which can be directly used in clinical practice or the production of prepared medicines

[SOURCE: ISO 18668-1:2016, 3.3, modified — Note 1 to entry amalgamated with definition.]

3.3
finished product
commercial product intended for sale and use, including *decoction pieces* (3.2)

3.4
manufacturing
process that creates a *finished product* (3.2) from a *starting material* (3.8) in a form suitable for its intended purpose, including packaging

3.5
monograph
detailed written study of a single specialized subject or an aspect of it

EXAMPLE Description of a herb in a pharmacopoeia.

3.6
raw material
substance going into or involved in the *manufacturing* (3.4) of a bulk product

[SOURCE: ISO 22716:2007, 2.28]

3.7
Rf-value
distance travelled by a given component divided by the distance travelled by the solvent front in thin layer chromatography

3.8
starting material
material received by a manufacturer to be commercially processed, manufactured or packaged

Note 1 to entry: This includes *raw materials* (3.6) and other materials, for example solvents, excipients and capsule shells.

4 Minimum requirements for the testing of the identity of starting materials and finished products

General quality control methods shall include the following identification tests and, if applicable, an "assay" as established in typical monographs:

a) macroscopic description test;

b) microscopic description test;

c) organoleptic description test;

d) test with HPLC;

e) test with thin layer chromatography (TLC).

The use of the methods depends on the material being tested (see Table 1).

Table 1 — Overview of identity testing methods for different materials

	Starting material with monograph	Starting material without monograph	Mixture of starting materials	Finished product
a) **macroscopic description test**	According to monograph	According to scientific literature	Not applicable	Not applicable
b) **microscopic description test**	According to monograph	According to scientific literature	According to scientific literature	Not applicable
c) **organoleptic description test**	According to monograph	According to scientific literature	Not applicable	Not applicable
d) **HPLC test**	According to monograph or alternative Clause 6	Clause 6	Clause 6	Clause 6
e) **TLC test**	According to monograph or alternative Clause 6	Clause 6	Clause 6	Clause 6

5 Requirements for macroscopic, microscopic and organoleptic description tests

5.1 General

For sample collection see ISO 19609-1.

Visual and organoleptic examinations include the following three methods:

a) Macroscopic description test for identification of herbal materials.

This description consists of the form, size, colour, surface characters and texture (including cut surface or fracture characters) of the crude materials and prepared slices.

— "Form" refers to the shape of crude materials and prepared slices. Wrinkled herbs, leaves or flowers can be moistened, softened and spread.

— "Size" refers to the length, diameter and thickness of crude materials and prepared slices. In general, a millimetre ruler is used for the measurement.

— "Colour" refers to the colour and glossiness of crude materials and prepared slices observed in daylight. If the colour is described in a combination of two colours, the main colour is the latter.

— "Surface characters", texture and cut surface of crude materials or prepared slices is described without pretreatment.

b) Microscopic description test for identification of herbal materials.

Microscopic identification is a method where the application of a microscope is used to identify the characters of tissues, stomata and stomata index, cells or cell contents in sections, powders, disintegrated tissues or surface slides of prepared slices of crude materials and dosage form, including powders of prepared slices of crude materials. Representative samples are chosen to be identified and slides are prepared to meet the requirements of identification for each crude material.

If the samples being examined are pulverised or ground (e.g. in the case of decoction pieces), the resulting material should conform with the requirements in the monograph and ISO/TS 21310, as well as the general criteria.

c) Organoleptic description test for identification of herbal materials.

— Odour

— Taste

NOTE Historically, the exact visual examination of the herbs was the only criterion for identification. This is still used in addition to the described modern testing methods for starting materials. For uncut, cut and powdered herbal materials, microscopic, macroscopic and organoleptic tests are established.

5.2 Macroscopic description test

5.2.1 Application

Macroscopic description tests are applicable for starting materials. Mixtures of starting materials and finished products cannot be described typically by macroscopic description tests.

5.2.2 Macroscopic examination

The macroscopic examination is normally done without any apparatus for the description. The use of a magnifying glass or binoculars is appropriate for the macroscopic examination of test samples. The magnification shall be sufficient to allow adequate characterization of the smallest characteristics to be classified in the test sample. The use of an appropriate lamp is recommended.

5.2.3 Assessment

The examinations shall be compared with authenticated reference data from monographs or other reliable scientific data. If the results are identical with the reference data this part of the identity can be confirmed.

5.3 Microscopic description test

5.3.1 Application

Microscopic description tests are applicable for starting materials. Mixtures of starting materials and finished products cannot be described typically by microscopic description tests.

5.3.2 Microscopic examination

The sample collection shall be done in accordance with ISO 19609-1:2021, 6.2.

If the samples being examined are pulverised or ground (e.g. in the case of decoction pieces) the resulting material should conform with the requirements in the monograph.

The microscopic examination should be done in accordance with ISO/TS 21310 and can be applied to other materials, such as powdered herbals.

5.3.3 Assessment

The examinations shall be compared with authenticated reference data from monographs or other reliable scientific data. If the results are identical with the reference data this part of the identity can be confirmed.

5.4 Organoleptic description test

5.4.1 Application

Organoleptic description tests are applicable for starting materials. Mixtures of starting materials and finished products cannot be described typically by an organoleptic description test.

NOTE Organoleptic tests of odour and taste of raw materials are useful, but are often unsuitable for finished products because of the use of tasteless "dosage forms" or corresponding odourless and taste-neutralizing additives.

5.4.2 Organoleptic examination

When examining the odour, smell directly or, if necessary, fracture, crush or rub the samples. When necessary, moisten the sample with hot water before examination.

When examining the taste, use a small quantity of the sample directly. In some cases it can be appropriate to macerate the sample in hot water and taste the extract.

Specific requirements of pharmacopoeias should be taken into account.

WARNING — Be cautious when tasting poisonous, hazardous or toxic materials.

5.4.3 Assessment

The examinations shall be compared with authenticated reference data from monographs or other reliable scientific data. If the results are identical to the reference data, this part of the identity can be confirmed.

6 Chromatographic identification tests with HPLC and TLC

6.1 General

Chromatographic identification tests shall be applied only for the determination of the identity of starting materials and finished products based on herbal raw materials and ingredients, as well as materials after advanced processing (e.g. boiling, steaming, roasting).

Gas chromatographic tests can be additionally applied for the detection of volatile compounds, such as essential oils, and other components with characteristic odour, such as fatty acids, after hydrolysis of lipids and others.

Chromatographic methods for hydrophilic to slightly lipophilic compounds are suitable for the analysis of identity. In this case specific and non-specific separation processes shall be used.

Specific separation methods such as TLC detect, with a certain specificity, classical active component groups in the field of herbal secondary products (e.g. alkaloids, flavonoids, saponins, essential oils and other classes of natural compounds).

Non-specific separation methods such as HPLC are suitable to show the total spectrum of ingredients of a herbal or a related product in its entirety and complexity.

In order to perform a chromatographic separation, the test samples (dosage forms) have to be transferred into an analysable test solution in a connected upstream sample preparation step.

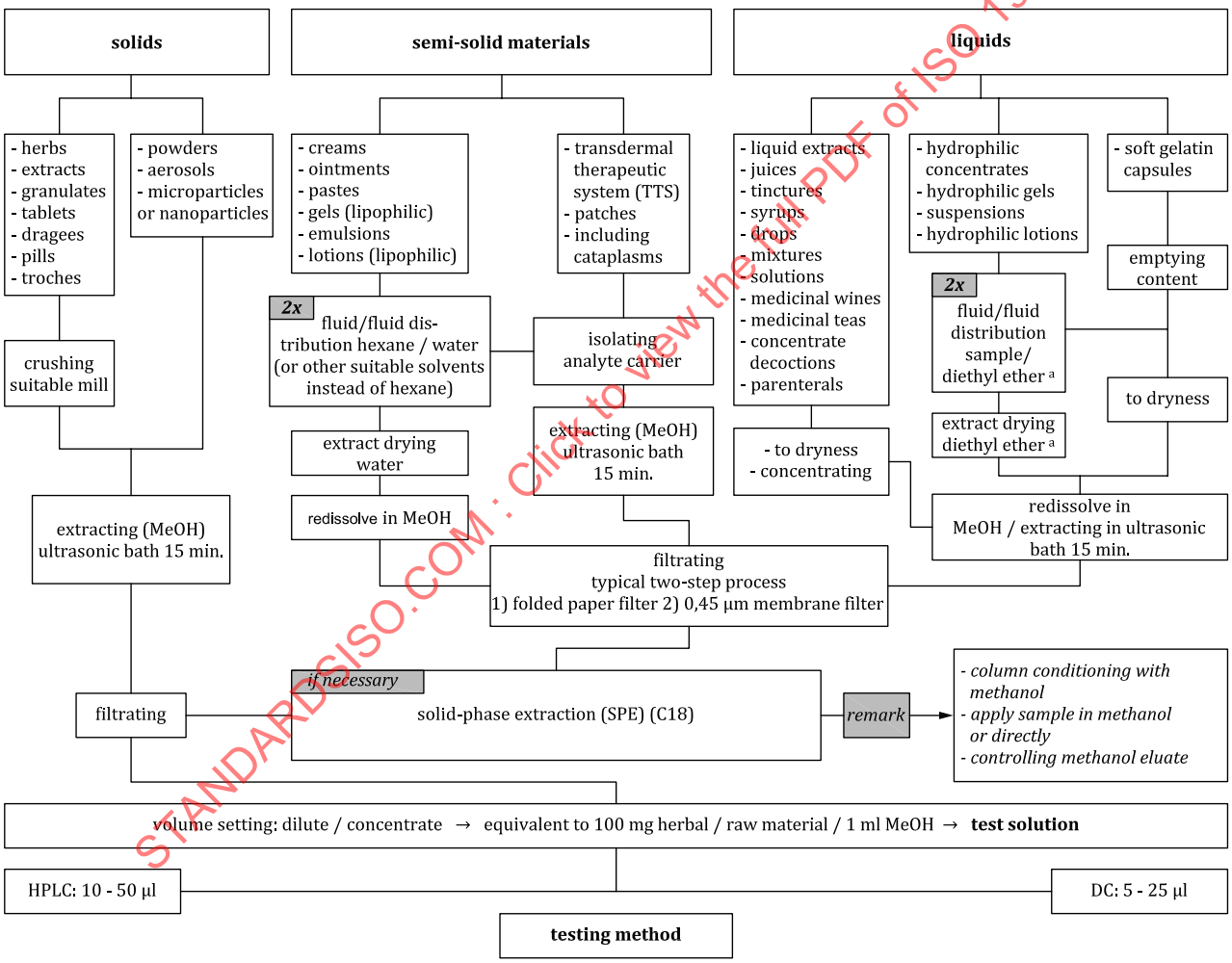
6.2 Sample preparation

6.2.1 General

For a classical analysis with chromatographic methods, it is necessary to dissolve the ingredients or active principles, which shall be tested by suitable methods. Chromatographic techniques separate the components, followed by an appropriate method of detection. This separation pattern can be used for both determination of the identity and a possible quantification (assay) of relevant compounds.

For a sufficient analysis, sample preparation with appropriate methods shall ensure that neither solids, particles nor emulsions are used.

Figure 2 gives an overview of the sample preparation of solids, semi-solid materials and liquids. Less common dosage forms such as chewing gums, ear preparations, foams, suppositories, tampons, poultices, sticks, medicinal distillates, liniments, smeared films, sprays, pessaries and others are not included in the sample preparation procedure. For these products a suitable connected upstream sample preparation method has to be adapted individually or newly developed and validated.



a Alternatively, chloroform can be used.

Figure 2 — Sample preparation procedure

6.2.2 Reagents

6.2.2.1 Methanol gradient grade/HPLC grade (MeOH).

6.2.2.2 Chloroform gradient grade/HPLC grade ($CHCl_3$).

WARNING — Work with chloroform can have critical effects on the health of staff.

6.2.2.3 Hexane gradient grade/HPLC grade.

6.2.2.4 Water gradient grade/HPLC grade.

6.2.2.5 Ethyl acetate gradient grade/HPLC grade.

6.2.2.6 Diethyl ether gradient grade/HPLC grade.

6.2.3 Apparatus

Use the usual laboratory equipment and, in particular, an **ultrasonic bath**.

6.2.4 Sample preparation method for solids

For the examination of solid testing materials there are two processing methods for further handling. Materials in powder form, such as powders, aerosols, microparticles and nanoparticles, can be used directly for extracting.

Coarse materials such as granules and dry extract particles, as well as herbs and ready dosage forms such as tablets, dragees, pills, troches and hard capsules, shall be crushed to increase the possibility of extracting relevant secondary products by enhancing the amount of surface.

A sieving step shall be applied if necessary.

Both types of crushed materials shall be extracted with methanol of appropriate quality in an ultrasonic bath for minimum 15 min at room temperature. A solvent amount 10 times higher than the solid shall be used.

EXAMPLE In most cases, 10 g powdered or pulverized solid is dissolved in 100 ml methanol of laboratory quality or better.

In some cases higher amounts of solvents can be used.

A filtration step eliminates particles in the test solution.

The filtration shall be done with an appropriate folded paper filter. A further rinsing is not necessary. The filtered methanolic extract should be used for analysis or filtered again with a fine membrane filter of about 0,45 µm.

If there are compounds which are not elutible from reversed phase materials, a solid-phase extraction (SPE) step should be applied if necessary (see 6.2.7).

6.2.5 Sample preparation method for semi-solid materials

Semi-solid materials often contain high amounts of lipids or other lipophilic compounds which lead to problems in classic analysis. To conduct a valid and reproducible analysis of products such as creams, ointments, pastes, gels, emulsions and lotions, the reduction of the lipids shall be realized with a two-time liquid-liquid distribution step.

As solvents, equal amounts of hexane and water shall be used. (If necessary, other suitable solvents instead of hexane can be used.) Typically, the amount of liquids should be 10 times higher than the amount of tested material.

EXAMPLE For 10 g of semi-solids, about 100 ml hexane and 100 ml water of appropriate quality are used.

The procedure of phase separation after sufficient shaking can be done as established in typical laboratories.

Next, the water extract shall be concentrated to dryness and redissolved in methanol (100 ml).

Other semi-solid materials such as transdermal therapeutic systems, patches and cataplasms can be used directly for extraction.

After the isolation of the carrier of the active principles, this material shall be extracted in methanol of appropriate quality for 15 min in an ultrasonic bath at room temperature.

The amount of extracting solvent shall be 10 times higher than the weight of analyte in matrix.

A filtration step shall eliminate particles in the test solution.

The filtration should be done with an appropriate folded paper filter. A further rinsing is not necessary. The filtered methanolic extract shall be filtered again with a fine membrane filter of about 0,45 µm.

If there are compounds which are not elutable from reversed phase materials, an SPE step should be applied if necessary (see 6.2.7).

6.2.6 Sample preparation method for liquids

Liquid products such as liquid extracts, juices, tinctures, syrups, drops, mixtures, solutions, medicinal wines, medicinal teas, concentrate decoctions and parenterals shall be concentrated to dryness.

The concentrated materials shall be extracted with methanol of appropriate quality in an ultrasonic bath for a minimum 15 min at room temperature. A solvent amount 10 times higher than the dried material shall be used.

EXAMPLE 1 10 g dried liquid material (solid) is dissolved in 100 ml methanol.

Hydrophilic concentrates and gels, suspensions and hydrophilic lotions also need liquid-liquid distribution steps similar to semi-solids.

In this case a separation between diethyl ether and the waterish sample shall be done twice.

EXAMPLE 2 100 ml diethyl ether is added to 100 ml of sample and shaken vigorously before waiting for phase distribution. After separation of the organic phase 100 ml of diethyl ether is again added to the aqueous solution and the process is repeated. The two organic solutions (2 × 100 ml) are combined and concentrated to dryness. This residue is redissolved with 100 ml methanol.

Soft gelatine capsules and similar products shall be analysed as follows.

The first step shall be emptying the content, washing the capsule envelope and afterwards combining both.

If the content is oily, the processing step is the same as described before a liquid-liquid distribution.

Otherwise, if the content is hydrophilic the resulting solution or emulsion shall be concentrated to dryness and afterwards 10 g redissolved in 100 ml methanol using an ultrasonic bath for 15 min at room temperature.

A filtration step shall eliminate particles in the test solution. The filtration should be done with an appropriate folded paper filter. A further rinsing is not necessary. The filtered methanolic extract shall be filtered again with a fine membrane filter of about 0,45 µm.

If there are compounds which are not elutible from reversed phase materials, an SPE step should be applied if necessary (see 6.2.7).

If the use of diethyl ether is not appropriate, chloroform can be used.

6.2.7 Solid-phase extraction (SPE)

SPE is necessary for the stability and increase of the life period of chromatographic columns. Strong lipophilic compounds cannot be eluted from reverse phase chromatographic columns (RP) completely. In this case an elimination step of non-elutible compounds (such as chlorophyll) is appropriate.

Typically, ready SPE columns are used with a stationary phase made of RP-C18 material. Depending on the volume and the adsorption capacity the application, the washing step and the elution of the secondary compounds should be adapted.

The resulting solution from step 6.2.4, 6.2.5 and 6.2.6 in methanol can be used.

The SPE shall be done following the conditions in Table 2.

Table 2 — Conditions for SPE processes

Column ready to use for syringe combination with 2 luer locks	About 0,8 ml C18-material
Activating step (conditioning)	Press about 5 ml pure MeOH through the dry column and afterwards press air through for eliminating the MeOH residues
Separation step	Fill the syringe with 2,5 ml of filtrate and press it through the SPE column
Eluate	Use the resulting clear solution of about 1,5 ml to 2 ml

6.2.8 Test solution

After the filtration step and, if necessary, the SPE step, the resulting solutions in methanol shall be used as test solutions after a volume-setting step.

With concentration steps or dilutions at the end a solution of an equivalent of about 100 mg herbal or raw material at 1 ml methanol should be produced.

This methanolic solution is the test solution for further analytical determinations.

6.3 Identification of constituents with HPLC and diode array detection

6.3.1 General

This proposed method can be applied to all herbs without official monographs.

The main focus of this method is the application of herb mixtures and other related products. With this proposed method (HPLC analysis) a high variety of different test materials can be analysed with only one main method. In some cases an optimization step can be used to adapt this method to the specific problems for a small number of complex samples. But this method is in all cases an easy basis for modification processes.

6.3.2 HPLC fingerprint testing method

6.3.2.1 Application

This HPLC fingerprint testing method is applicable for starting materials, mixtures of starting materials and finished products.

The used HPLC fingerprint method is an attempt to determine the complete ingredient spectra of a herb in terms of distribution of hydrophilic to lipophilic characteristics, and also the intensities of the single peaks and their individual spectra (from 195 nm to 650 nm in the UV and visible range). To ensure the identity of a product it is necessary to detect the whole distribution pattern of the used raw materials and to compare it with those from the product. The fingerprints from raw material testing and the

fingerprints from the finished product shall show a high similarity. In this case the identity can be seen as fulfilled.

6.3.2.2 Reference materials

For the identification of the single constituents of a product, it is necessary in opposition to many monographs from pharmacopoeias to use the whole fingerprints of the starting materials instead of single marker compounds. In the case of a product made from only one herbal material, marker fingerprints can also be used.

For all other materials an appropriate reference material is needed.

6.3.2.3 Reagents

6.3.2.3.1 Acetonitrile (ACN) gradient grade.

6.3.2.3.2 0,05 % aqu. phosphoric acid.

6.3.2.4 Apparatus

The usual laboratory equipment and, in particular, the following.

6.3.2.4.1 HPLC apparatus with gradient system (low-pressure or high-pressure systems) with photodiode array-detection.

6.3.2.5 Procedure of HPLC fingerprint testing method

The liquid chromatography with photodiode array detector (DAD/PDA) shall be done following the conditions in Table 3.

Table 3 — Conditions for liquid chromatographic HPLC analysis

Apparatus	HPLC apparatus with gradient system and DAD-detector (low-pressure or high-pressure systems)			
Column	Type:	Octadecyl RP-phase column 100 Å C18 5 µm		
	X	Pre-column	4 × 4 mm	
	X	Column	250 mm × 4,0 mm or 4,6 mm	
Detection	X	Diode-array-detection	195 nm to 650 nm	
Flowrate	1,0 ml / min			
Temperature	25 +\– 2 °C			
Record interval	90 min			
Inject volume	Typical 20 µl			
Mobile phases	A	ACN gradient grade		
	B	0,05 % aqu. phosphoric acid		
Gradient program	Time		A	B
	0 min		5 %	95 %
	10 min		5 %	95 %
	70 min		98 %	2 %
	85 min		98 %	2 %
	85,1 min		5 %	95 %
	100 min		5 %	95 % if necessary

6.3.2.6 Assessment

The analytical data shall be compared with data produced with authentic reference material (see 6.3.2.2).

If the analytical data are comparable with the reference data, this part of the identity can be confirmed. Comparability means that the fingerprint of the test sample shall conform with the fingerprint of a qualified reference solution in the main peaks according to relative retention time and spectra.

6.3.2.7 Validity

The validation of this HPLC fingerprint testing method is done according to the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines for identity test methods. In this case the "specificity" of this test method is fulfilled.

The validity of complex mixtures such as finished products should be demonstrated. Other appropriate and validated methods can be used.

6.4 Identification of typical constituents with TLC

6.4.1 Group selected identification tests with TLC

6.4.1.1 Application

Group selected identification tests with TLC are applicable for starting materials, mixtures of starting materials and finished products.

This methodology works with a combination of only five solvent systems and an additional five specific derivatization reagents (with one additional non-specific).

6.4.1.2 Reference materials

For the identification of the single constituents of a product, it is necessary, in opposition to many monographs from pharmacopoeias, to use the whole fingerprints of the starting materials instead of single marker compounds. In the case of a product made from only one herbal material marker, fingerprints can also be used.

For all other materials an appropriate reference material is needed.

6.4.1.3 Reagents

6.4.1.3.1 Ethyl acetate.

6.4.1.3.2 Formic acid.

6.4.1.3.3 Acetic acid.

6.4.1.3.4 Water.

6.4.1.3.5 Toluene.

NOTE Work with toluene can have critical effects on the health of staff.

6.4.1.3.6 Diethylamine.

6.4.1.3.7 Methanol.

6.4.1.3.8 Chloroform.

NOTE Work with chloroform can have critical effects on the health of staff.

6.4.1.3.9 Naturstoff reagent.

6.4.1.3.10 Dragendorff reagent.

6.4.1.3.11 Molybdatophosphoric acid reagent.

6.4.1.3.12 Bornträger reagent.

6.4.1.3.13 Antimony (III)-chloride reagent.

6.4.1.3.14 Anisaldehyde-sulfuric acid reagent.

6.4.1.4 Apparatus

Use the usual laboratory equipment and, in particular, the following.

6.4.1.4.1 Chromatography tank system (vertical separation).

6.4.1.4.2 Oven at 105 °C.

6.4.1.5 Procedure of TLC testing method

From each product a suitable test solution (see 6.2) is produced; afterwards this mixture is separated with TLC with five basic TLC separation systems. These five methods are typical for the five main groups of secondary products in herbal materials. For comparison, qualified reference extracts or reference solutions (tested and validated according to the ICH guidelines) individually for each herbal constituent, in addition to the test solution of the products, are chromatographically separated on an individual TLC line. Additionally, on one line of the TLC plate the pure solvent (used for the reference and test solutions) is also spotted (validity requirement). At the first step in parallel the described five basic TLC separations are executed to select the most suitable method. As an auxiliary criterion, visualizing with UV, wavelengths 254 nm (extinction with silica gel 60 F254 TLC plates) and 365 nm are used for intrinsic fluorescent substances. This is already evident in many cases as the basic suitability of separation systems.

NOTE The specificity of TLC is fulfilled if in the line of pure solvent no signal appears and in the other lines appropriate separation is reached.

Then the associated derivatization from Table 5 shall be used.

The TLC with derivatization shall be done following the conditions in Table 4.

Table 4 — Conditions for TLC analysis

TLC apparatus	Chromatography tank system (vertical separation)	
TLC plate	Type	Silica gel 60 F_{254}
	Size	10 × 10 cm, 10 × 20 cm or 20 × 20 cm
Precondition	Only if necessary, ready-to-use commercial TLC plates need no precondition	
Separation conditions	Chamber saturation	
NOTE In some cases an addition of, for example, formic acid can be appropriate to improve the chromatographic separation of TLC systems 3, 4 and 5.		

Table 4 *(continued)*

Sample application	Minimum 10 mm over the bottom of the plate					
	Manual			or	Mechanical	
	As a spot			or	As a line	
Removal of solvent	Drying in a warm air stream			or	Direct air stream	
TLC separation systems for TLC fingerprinting	1	solvent	Ethyl acetate	Formic acid	Acetic acid	Water
		mixture	100	11	11	27
	2	solvent	Toluene	Ethyl acetate	Diethylamine	—
		mixture	70	20	10	—
	3	solvent	Toluene	Ethyl acetate	—	—
		mixture	93	7	—	—
	4	solvent	Ethyl acetate	Methanol	Water	—
		mixture	100	17	13	—
	5	solvent	Chloroform	Methanol	Water	—
		mixture	64	50	10	—
Detection	Visual detection in daylight					
	Visual detection at 254 nm in UV-light					
	Visual detection at 365 nm in UV-light					
NOTE In some cases an addition of, for example, formic acid can be appropriate to improve the chromatographic separation of TLC systems 3, 4 and 5.						

The use of the group-specific derivatization reagents for visual detection show more individual criteria of individual compounds and their recognition in starting materials and finished products. It can be detected under UV light if a separation system is suitable in terms of its selectivity.

The derivatization reagents in Table 5 lead to reliable results.

Table 5 — Selected derivatization reagents

Derivatisation (see also Figure 3)	Reagent A	Naturstoff reagent
	Reagent B	Dragendorff-Reagent
	Reagent C	Molybdatophosphoric acid reagent
	Reagent D	Bornträger reagent
	Reagent E	Antimony (III)-chloride reagent
	Reagent S1	Anisaldehyde-sulfuric acid reagent
Drying of plate	Removal of solvents from derivatization in an air stream	
	If necessary for the specific derivatization, use an oven at 105 °C under regular monitoring over the time needed	
Detection	Visual detection in daylight	
	Visual detection at 365 nm in UV-light	

6.4.1.6 Selection of optimized test system

The starting point of TLC analysis is the methanolic test solution after sample preparation (see 6.2).

In most herbs more than one main group of active constituents are present. In combination products a lot of different secondary products can be detected, but for an easy and cost-effective analysis it should be possible to reduce the tests to only one.

In some countries for each herb a specific (focused on one marker) TLC system is needed for the product release as a remedy. The following system can simplify this complicated procedure. In many cases the