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Finally, WHO thanks Health Canada of the Government of Canada, especially the Natural Health Products Directorate, Health Products and Food Branch, which hosted and supported the WHO Informal Meeting on Methodologies for Quality Control of Finished Herbal Products, in Ottawa, Canada in July 2001. It was at this meeting that the idea of developing these guidelines was first discussed and recommended.
The use of medicinal plants for treating diseases is probably the oldest existing method that humanity has used to try to cope with illness.

For this reason, medicinal plants have been used therapeutically all around the world, being an important aspect of various traditional medicine systems. From Ayurveda to Chinese traditional medicine, from Unani to Tibetan Medicine, from Amazonian to African Medicine, all systems of traditional medicine, although based on different theoretical and cultural models, integrate phytotherapy into their doctrine.

In high-income countries, the widespread use of phytotherapy declined at the end of the first part of the twentieth century, due to the development and production of synthetic medicines. During the past few decades, however, phytotherapy has started to be increasingly used even in industrialized countries. In low- and middle-income countries, phytotherapy never stopped being important, often representing the only therapeutic system to which certain people could refer.

In facing this challenge, it is fundamental that the conditions for the correct and appropriate use of phytotherapy methods are in place. If correctly applied, these methods can contribute to protecting and improving citizens’ health and well-being. The correct use of such methods should follow the criteria of safety, efficacy and quality. These principles characterize modern medical practice and are at the basis of consumer protection.

The work of the Regional Government of Lombardy in the field of traditional and complementary and alternative medicine (TM/CAM) has been guided by the above-mentioned criteria. TM/CAM was included in the Regional Health Plan (2002–2004). Along these lines, a series of governmental provisions has defined a framework for the protection of consumers and providers. The keystone of this process is the Quadrennial Cooperation Plan between the Regional Government of Lombardy and the World Health Organization (WHO) on the use and evaluation of TM/CAM. Furthermore, the promotion of clinical and observational studies on Lombardy territory has been a crucial step in the evaluation of the efficacy of TM/CAM.

As mentioned above, phytotherapy is highly diffused in high-income countries, but the scientific medical model is more diffused in the developing countries. This contact between the two models has raised the urgent need to compare the immense background of traditional knowledge with the scientific procedures of research and validation. This process is aimed at demonstrating the safety and efficacy of single or combined medicines that have been used in the past millennia. In this respect, the Regional Government of Lombardy shares WHO’s concern to prove not only the safety but also the efficacy of phytotherapeutic medicines. This is especially true for those countries where the primary or prevalent system is conventional medicine, and where phytotherapy could substitute medicines.
whose efficacy has already been verified. In these cases, the physician should conduct a careful evaluation of the correlation of the efficacy and safety of the herbal medicine compared with the chemical medicines that are to be substituted by the herbal medicine.

For all these reasons, it is evident that the diffusion of phytotherapy in developed countries requires a series of extended studies aimed at establishing the safety and the efficacy of commonly used herbal medicines. Concerted action by different specialists is needed in order to translate the use, often safe, of the traditional recipes to industrial societies.

However, the use of phytotherapy according to the efficacy and safety criteria is not sufficient to guarantee the quality of both the herbal medicine and its use. Products with high quality standards are needed to allow the patient to make safe use of phytotherapeutic products. Nowadays, as a consequence of market globalization, many of the medicines used in the phytotherapeutic systems do not come from the country of origin but from third countries.

A set of regulations shared at the global level is crucial to guarantee high quality standards for medicinal plants. The legislative framework should establish the basic parameters to guarantee quality and safety in the use of phytotherapeutic products.

Led by this vision, experts from around the world participated in the WHO Consultation on Contaminants and Residues held from 12–14 July 2004 in Laveno di Menaggio and produced the present document. This work could be a model for other regulations on the quality of medicinal plants, and should be a reference point for providers, and for political and administrative authorities that desire that phytotherapy be of help in promoting citizens’ health according to the safety and efficacy criteria.

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With the ever-increasing use of herbal medicines and the global expansion of the herbal medicines market, safety has become a major concern for both health authorities and the public in many Member States. The World Health Organization (WHO) developed a strategy on traditional medicines for the period 2002–2005 (WHO Traditional Medicine Strategy: 2002–2005), and this was subsequently implemented under the WHO Medicines Strategy covering the period 2004–2007. One of the major objectives is to promote the safety, efficacy and quality of traditional medicines.

The quality of herbal medicines has a direct impact on their safety and efficacy. There are many control measures for herbal medicines, and the first important step is to control the quality of medicinal plants and herbal materials. However, this is a very complicated and difficult task as it involves many different areas, such as the environment and agricultural practices.

It is well known that there are many contaminants and residues that may cause harm to the consumers of herbal medicines. Many are natural, such as naturally occurring radionuclides, toxic metals or bacteria. Some arise from past or present use of agents or materials that pollute the environment and subsequently medicinal plants, such as emissions from factories or the residues of certain pesticides. Recent research has also demonstrated that herbs may absorb heavy metals during growth. For these reasons, there is currently a potential global danger to the health and well-being of people. This risk can be reduced by ensuring that herbal medicines with harmful contaminants and residues do not reach the public, by assessing the quality of the medicinal plants, herbal materials and finished herbal products before they reach the market.

WHO has developed a series of technical guidelines and documents relating to the safety and quality assurance of medicinal plants and herbal materials. These include, Guidelines on good agricultural and collection practices (GACP) for medicinal plants and Quality control methods for medicinal plant materials.

The above-mentioned WHO technical documents have introduced some commonly used methods to determine biological, chemical and radionuclear contaminants, as well as pesticide residues. However these documents tend to focus on technical issues related to the quality control of herbal materials and medicinal plants, and although they mention methods for the determination of contaminants, they do not go into great detail. The document on Quality control methods for medicinal plant materials was developed a long time ago, at a time when there was a lack of appropriate test methods and a lack of national and regional quality specification standards on specific contaminants and residues. Therefore, it has become necessary, and also possible, to develop these new guidelines, which focus on providing technical guidance on the assessment of quality of herbal medicines, related to both major and common contaminants and residues, based on countries’ efforts, technical advancement and recent developments.
Herbal materials and medicinal plants are also often used as food, functional food, nutritional or dietary supplements. The results of the WHO global survey on national policy and regulation of herbal medicines (2005) showed that in more than 100 responding countries, the regulatory status most often given to herbal medicines is as over-the-counter medicines, prescription medicines or as dietary supplements and health food. Hence, in some countries, there is a saying: “medicines and foods have the same origin”. It is clear that quality control of herbal materials and medicinal plants is very important, not only for the safety of the herbal medicines themselves, but also for food safety. Therefore, much of the work undertaken in the area of food safety is relevant to the safety of herbal medicines. Hence, the development of these guidelines was initiated as a joint effort between the teams of Traditional Medicine and Food Safety, in collaboration with the Food and Agriculture Organization of the United Nations and the WHO Joint Programme: Codex Alimentarius.

Here it should be emphasized that due to lack of research data and technical limitations at present, many of the accepted limits in the guidelines are referenced and extrapolated from work done in the food area. It should be borne in mind that although controlling the contaminants and residues in food and in herbal medicines has certain similarities, there may also be many differences. Therefore, more research is needed in order to establish the scientific criteria for herbal medicines.

The Traditional Medicine team has not only cooperated with the WHO Food Safety Programme, but also with other relevant WHO departments and technical units such as the Department of Protection of the Human Environment and the Department of Policy and Standards of Medicines at WHO Headquarters in Geneva. The validated test methods and standards related to the determination of contaminants and residues described in these guidelines were adopted, after extensive technical review, from some existing WHO guidelines and technical documents in the field of herbal medicines and traditional medicine, particularly from the "Quality control methods for medicinal plant materials" and also from documents in the areas of food safety, chemical safety, control of communicable diseases, and radiation and environmental health as well as from some national, regional and international pharmacopoeias. These recommended analytical methods, procedures and standards were finally reviewed during the meetings of the WHO Expert Committee on Pharmaceutical Specifications in 2004, 2005 and 2006.

Although the guidelines include many new validated analytical methods involving new technologies, they do not cover every complicated situation and technical demand for controlling contaminants and residues of herbal medicines. WHO will continue to cooperate with Member States and other relevant technical programmes within WHO to update the guidelines to take into account the development of new technologies and methods, to promote quality control of herbal medicines and to ensure the safety and efficacy of the use of traditional medicines.

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1. Introduction

1.1 Background

With the ever-increasing use of herbal medicines worldwide and the rapid expansion of the global market for these products, the safety and quality of medicinal plant materials and finished herbal medicinal products have become a major concern for health authorities, pharmaceutical industries and the public.

National regulation and registration of herbal medicines varies from country to country. Where herbal medicines are regulated, they are categorized either as prescription medicines or non-prescription medicines. Within a country, a group of herbal products categorized other than as medicines may coexist. Herbal products categorized other than as medicines and foods, are becoming increasingly popular and there is potential for adverse events due to lack of regulation, weak quality control systems and loose distribution channels (including mail order and Internet sales).

A resolution on traditional medicine (WHA56.31) adopted by the 56th session of the World Health Assembly in May 2003, urges Member States, where appropriate, to ensure safety, efficacy and quality of herbal medicines by determining national standards for, or issuing monographs on, herbal raw materials and traditional medicine formulae. It also requested the Director-General of the World Health Organization (WHO) to provide technical support for the development of methodology to monitor or ensure product quality, efficacy and safety, preparation of guidelines and promotion of exchange of information.

The International Conference of Drug Regulatory Authorities (ICDRA) at its 9th, 10th and 11th meetings and the Meeting of the National Centres Participating in the WHO Drug Monitoring Programme requested WHO to develop and constantly update the technical guidelines on quality, safety and efficacy of herbal medicines.

The participants at the WHO informal meeting on methodologies for quality control of finished herbal products, held in Ottawa, Canada, 20–21 July 2001, also reviewed the entire production process of herbal medicines, from raw materials to distribution and supply of finished herbal products. Recommendations from this meeting led to the development of these general guidelines addressing the important issue of safety and quality of herbal medicines with special reference to contaminants and residues.

In ensuring the quality and safety of herbal medicines, the national authorities in many Member States as well as other stakeholders in provision of herbal medicines are likely to encounter numerous challenges, including the setting of standards for quality, their adoption, monitoring and enforcement. The national policy and regulations that are likely to be developed using these WHO guidelines, should also take into account all local and other special needs. Methods, both general
and specific, for the determination of the various standards and values are also suggested in this document. In the absence of relevant and appropriate national standards, there is a risk that these herbal medicines may be lost to traditional users and become unavailable to new users for many reasons. These reasons include: their failing to meet various trade, registration, import and export requirements; loss of confidence in these products due to the presence of real or perceived health risks; and increased reporting of adverse events involving use of these herbal medicines.

From time to time, undesirable and/or undeclared substances have been present or have been purported to be present in herbal medicines or medicinal plants in many parts of the world. These substances have included pesticides, radioactive particles, and microbes including pathogens, mycotoxins, heavy metals and arsenic.

In order to reduce the risk of adverse events attributable to unsafe and poor-quality herbal medicines, WHO has committed to developing a series of new technical guidelines relating to the safety and quality assurance of herbal medicines, and to updating existing technical documents in this field. These actions implement parts of the WHO Traditional Medicine Strategy: 2002–2005 (1).

Within the overall context of quality control of herbal medicines, WHO has also developed general global guidelines for assessing the safety of potentially hazardous substances in herbal medicines, with particular reference to biological, chemical, and radioactive contaminants, and pesticide residues.

1.2 Purpose and objectives

Within the overall context of quality assurance, these guidelines are primarily intended to provide general technical guidance to Member States in the assessment of quality related to safety of herbal medicines with regard to both major and common contaminants and residues. These guidelines may need to be adjusted according to each country’s situation.

The objectives of these guidelines are to provide:

- guiding principles for assessing the quality in relation to the safety of herbal medicines, with specific reference to contaminants and residues;
- model criteria for use in identifying possible contaminants and residues;
- examples of methods and techniques; and
- examples of practical technical procedures for controlling the quality of finished herbal products.

In the pursuit of the above-mentioned objectives, these guidelines should be read together with the other WHO documents and publications (including future versions) relating to the quality assurance of herbal medicines with regard to safety, for example (for details see reference list):

- Quality control methods for medicinal plant materials (2)
- Good agricultural and collection practices (GACP) for medicinal plants (3)
- International pharmacopoeia, 4th ed. (4, 5)
• Good manufacturing practices: main principles for pharmaceutical products (6)
• Good manufacturing practices: supplementary guidelines for the manufacture of herbal medicinal products (7)
• Guide to good storage practices for pharmaceuticals (8)
• Good trade and distribution practices (GTDP) for pharmaceutical starting materials (9)
• General guidelines for methodologies on research and evaluation of traditional medicine (10)
• Guidelines for assessment of herbal medicines (11)
• WHO monographs on selected medicinal plants (12, 13).

Also, as much of the work in the field of food safety is relevant to the safety of herbal medicines, these guidelines should be reviewed together with the relevant guidelines and codes of practice developed by the Joint Food and Agriculture Organization of the United Nations (FAO)/WHO Codex Alimentarius Commission, particularly those of its Committees on Pesticide Residues and on Food Additives and Contaminants, which are often reflected in national legislation. Thus medicinal plants may be subject to the general requirements for foods. Examples of Codex texts, which may be applicable to medicinal plants, include:

• Codex Alimentarius code of practice, general principles of food hygiene (14)
• Codex Alimentarius guidelines for the production, processing, labelling and marketing of organically produced foods (15)
• Codex Alimentarius code of practice for spices and dried aromatic plants (16).

The scope of these guidelines does not cover issues of adulteration of herbal medicines and/or counterfeit products, which should be dealt with in separate guidelines. WHO has produced guidelines on counterfeits in general (8, 9, 17–20).

1.3 Use of the document

A rapid increase in the global use of herbal medicines over the last few years has led to concerns over the safety and quality of herbal materials and herbal products.

Currently, there is considerable variation from country to country in the quality control of such materials and products and this variation not only impacts on public health, as contaminants in herbal medicines may represent avoidable risks for patients and consumers, but also has effects on international trade. Thus implementation of testing for contaminants in the national and/or regional quality control of herbal medicines is highly recommended and it is the intention of this document to provide guidance on which aspects should be considered when implementing such tests.

In particular:

• Consideration should be given to potential risk factors that might have an impact on possible contamination. For example, testing for radioactive contaminants will be necessary only if there are special reasons for concern. However, it should be noted that even if a herb is organically or “biologically” grown, contaminants from the soil or other environmental sources may still be present.
WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues

• Not all tests have to be performed on every production batch. For example, if it has been shown that pesticides will not concentrate during an extraction process, it will be sufficient to test the herbal material.

• Some analytical methods require significant investment in instruments and reagents.

However, economic constraints should not prevent the implementation of testing for contaminants. If resources are insufficient, the establishment of a national or regional pesticide control laboratory could be a suitable solution and cooperation with laboratories from academia or research centres, or with specialized laboratories working in food-related areas should be considered.

These guidelines mention several potential major contaminants and residues and Member States are advised to prioritize their testing programmes according to local agricultural practice and, in the case of imports, the agricultural practices of the source country.

The annexes to these guidelines present several examples of suitable methodologies found in national or regional pharmacopoeias and in WHO documents. It should be noted that these methods need to be validated for the material that is to be tested, e.g. roots, seeds, leaves, plant species and also for each type of instrument, e.g. for gas chromatography (GC), high-performance liquid chromatography (HPLC) or atomic absorption (AA) spectrophotometry. This process should include validation of detection limits, as these may depend on the instrument and type of sample. It is recommended that Member States and WHO work together to improve these methods and to harmonize requirements with a view to including validated standard methods in the International pharmacopoeia and regional pharmacopoeias.

Many of the accepted limits are extrapolated from work done in the food area, therefore more research is needed on the establishment of the scientific basis (e.g. for maximum residue limit (MRL) values for herbal medicines or herbal materials in general). The guidelines are a living document and will be revised and updated in the light of further developments and new technologies as they become available in the future.

It is important that the WHO Member States adopt the document as a working document in their regulatory controls.

The Member States are encouraged to make free use of this document and incorporate it into their legal framework.

1.4 Glossary

The following terms are used in the guidelines. The numbers in parentheses following a term refer to the number of the publication as given in the reference list; sometimes it was necessary to adapt the definitions so that they would apply properly to herbal medicines. Where references are given, they identify the source document from which the term has been abstracted or derived.

1.4.1 Terms relating to herbal medicines

The terms and their definitions have been selected and adopted from other WHO documents and guidelines that are widely used by WHO Member States. Definitions
of the terms may differ from those adopted in regulations and/or in common usage in some Member States. However, one of the purposes of these definitions is to provide consistency in terminology with other relevant WHO documents in this field, such as the WHO General guidelines for methodologies on research and evaluation of traditional medicine (10) and WHO Good manufacturing practices (6, 7). It should also be noted that these definitions have been developed to meet the demand for the establishment of standard, internationally acceptable definitions to be used in the evaluation and research of herbal medicines (10).

**Herbal medicines (10)**
These include herbs, herbal materials, herbal preparations and finished herbal products:

**Herbs (10)**
Herbs include crude plant material such as leaves, flowers, fruit, seeds, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered.

**Herbal materials (10)**
Herbal materials are either whole plants or parts of medicinal plants in the crude state. They include herbs, fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs. In some countries, these materials may be processed by various local procedures, such as steaming, roasting, or stir baking with honey, alcoholic beverages or other materials.¹

**Herbal preparations (10)**
Herbal preparations are the basis for finished herbal products and may include comminuted or powdered herbal materials, or extracts, tinctures and fatty oils, expressed juices and processed exudates of herbal materials. They are produced with the aid of extraction, distillation, expression, fractionation, purification, concentration, fermentation or other physical or biological processes. They also include preparations made by steeping or heating herbal materials in alcoholic beverages and/or honey, or in other materials.

**Finished herbal products or herbal medicinal products (10)**
Medicinal products containing as active substances exclusively herbal drugs or herbal drug preparations. They may consist of herbal preparations made from one or more herbs. If more than one herb is used, the term *mixed herbal product* can also be used. They may contain excipients in addition to the active ingredients. In some countries herbal medicines may contain, by tradition, natural organic or inorganic active ingredients, which are not of plant origin (e.g. animal materials and mineral materials). Generally however, finished products or mixed products to which chemically defined active substances have been added, including synthetic compounds and/or isolated constituents from herbal materials, are not considered to be herbal.

¹ The wording of this definition has been modified from the original which appeared in reference (10).
Medicinal plant materials: see Herbal materials

Medicinal plant (7)
A plant, either growing wild or cultivated, used for its medicinal purposes.

1.4.2 Terms relating to contaminants and residues in herbal medicines

In general the following terms and their explanations as they relate to contaminants and residues in herbal medicines have been adopted verbatim or where necessary adapted from the definitions for pesticide residues in foods, developed by the Codex Alimentarius Commission (21) and the Joint FAO/WHO Meeting on Pesticide Residues. Thus when Member States consider the terms relevant to their individual needs, these documents should be consulted. The reason for this suggestion is that in future the Joint FAO/WHO Meetings on Pesticide Residues (JMPR) will probably continue as the group mandated to evaluate the safety of pesticides and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for contaminants in herbal medicines and in foods.

The definitions contained in this glossary were originally quoted from various documents listed above; however, they were modified at the WHO Consultation on Contaminants and Residues to adapt them to the scope of this document.

In general when countries are setting standards for their herbal medicines they should take into account the differences in dosages, quantities and frequency of use, and methods of preparation of herbal medicines relative to those of foods.

Contamination (6)
The undesired introduction of impurities of a chemical or microbiological nature, or of foreign matter into or onto a starting material, intermediate product or finished herbal product during production, sampling, packaging or repackaging, storage or transport.

Cross-contamination (6)
The contamination of a starting material, intermediate product or finished product with another starting material or product during production.

Foreign matter (2)
Material consisting of any or all of the following:

- parts of the medicinal plant material or materials other than those named with the limits specified for the plant material concerned;
- any organism, part or product of an organism, other than that named in the specification and description of the plant material concerned;
- mineral admixtures such as soil, stones, sand, and dust; and glass, metal and plastics or any other extraneous materials. These may be loose or adhering to these medicinal plant materials.

Acceptable daily intake (ADI) of a chemical (21)
The estimated maximum amount of an agent, expressed on a body mass basis, to which an individual in a (sub)population may be exposed daily over his or
her lifetime without appreciable health risk. ADIs are normally determined for substances that are deliberately added (to foods) or are residues that are present as a result of approved uses of the agent.

A daily intake, which, during an entire lifetime, appears to be without appreciable risk to the health of the consumer, on the basis of all the known facts at the time of the evaluation of the chemical by the Joint FAO/WHO Meeting on Pesticide Residues. It is expressed in milligrams of the chemical per kilogram of body weight.1

**Acceptable residue level (ARL) (21)**
The ARL is given in mg of pesticide per kg of medicinal plant material and can be calculated from the maximum acceptable daily intake (ADI) of the pesticide for humans, as recommended by FAO and WHO, and the mean daily intake (MDI) of the medicinal plant material.

**Acute reference dose (ARD) (21)**
The acute reference dose of a chemical is an estimate of the amount of a substance, normally expressed on a body-weight basis, that can be ingested in a period of 24 hours or less without appreciable health risk to the consumer on the basis of all known facts at the time of the evaluation.

ARD is the amount of pesticide to which a person is exposed, usually, at one day’s regimen of herbal medicines and which results in acute effects on the human body. ARD estimations include a safety factor to ensure that the elderly, infants, children and those whose systems are under stress because of illness, are protected.

**Extraneous maximum residue limit (EMRL) (21)**
A pesticide residue or a contaminant arising from environmental sources (including former agricultural uses) other than the use of a pesticide or contaminant substance directly or indirectly on the herbal medicine. The concentration is expressed in milligrams of pesticide residue or contaminant per kilogram of the herbal medicine.

**Maximum residue limit (MRL) (21)**
The MRL is the maximum concentration of a pesticide residue (expressed as mg/kg) recommended by the Codex Alimentarius Commission to be legally permitted (in food commodities and animal feeds). MRLs are based on good agricultural practices (GAP) data established for foods, and foods derived from commodities that comply with the respective MRLs are intended to be toxicologically acceptable.

Such MRL values might be used by analogy for herbal medicines.

MRLs which are primarily intended to apply in international trade are derived from estimations made by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) following:

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1 For additional information on ADIs relative to pesticide residues refer to the Report of the 1975 Joint FAO/WHO Meeting on Pesticide Residues, FAO Plant Production and Protection Series No. 1 or WHO Technical Report Series No. 592.
• Toxicological assessment of the pesticide and its residue and review of residue data from supervised trials and supervised uses including those reflecting national food agricultural practices. Data from supervised trials conducted at the highest nationally recommended, authorized or registered uses are usually included in the review.

• Consideration of the various dietary residue estimates and determinations both at the national and international levels in comparison with the ADI, should indicate that herbal medicines complying with MRLs proposed by the Codex Alimentarius Commission are safe for human consumption.

• In order to accommodate variations in national pest control requirements, Codex MRLs take into account the higher levels shown to arise in such supervised trials, which are considered to represent effective pest control practices. For herbal medicines, the levels recommended by the Codex Alimentarius Commission to be legally permitted in food commodities or in animal feeds could be applicable to herbal materials/preparations. Generally MRLs would be based on GAP data and are intended to be toxicologically acceptable.

GAP includes the nationally authorized safe uses of pesticides under actual conditions necessary for effective and reliable pest control. It encompasses a range of levels of pesticide applications up to the highest authorized use, applied in a manner which leaves a residue that is the smallest amount practicable. Authorized safe uses are determined at the national level and include nationally registered or recommended uses, which take into account public and occupational health and environmental safety considerations. GAP applies at all stages of production, storage, transport, distribution and processing of herbal medicines.

Permitted daily exposure
The term “permitted daily exposure” (PDE) is defined, in the ICH guidelines, as a pharmaceutically acceptable intake of residual solvents to avoid confusion of differing ADIs for the same substance (22).1

Pesticide
For the purpose of these guidelines, pesticides are defined as any substance intended for preventing, destroying, attracting, repelling, or controlling any pest including unwanted species of plants or animals during production, storage, transport, distribution and processing. The term includes substances intended for use as a plant-growth regulator, defoliant, desiccant, fruit thinning agent, or sprouting inhibitor and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport. The term normally excludes fertilizers and plant nutrients.

Pesticide residue (21)
Pesticide residues are any specified substance in food, agricultural commodities or animal feed resulting from the use of a pesticide. The term includes any derivatives of a pesticide, such as conversion products, metabolites, reaction products and impurities considered to be of toxicological significance.

1 http://www.ich.org/cache/compo/363-272-1.html#Q3C
**Persistent organic pollutants (POPs)**

Persistent organic pollutants (POPs) are chemical substances that persist in the environment, bioaccumulate through the food web and pose a risk of causing adverse effects to human health and the environment. With the evidence of long-range transport of these substances to regions where they have never been used or produced and the consequent threats they pose to the environment of the whole globe, the international community has, on several occasions, called for urgent global action to reduce and eliminate releases of these chemicals.¹

**Tolerable intake (TI) – general definition**

Tolerable intake is defined as an estimate of the intake of a substance over a lifetime that is considered to be without appreciable health risk (23).²

**TI of a contaminant**

In the context of the present guidelines, the TI is defined as the estimated amount of a contaminant, expressed on a body mass basis, to which each individual in a (sub)population may be exposed over a specified period without appreciable risk. The term “tolerable” is used for agents which are not deliberately added, such as contaminants.

**TI of pesticide as a contaminant in herbal products**

The estimated amount of pesticide consumed as a contaminant in herbal products, together with other sources over a period of time, ranging from daily to lifetime, without causing harm to humans.

**Residual solvents**

These are residues of organic solvents that are used or produced in the manufacture of and processing of herbal preparations/products. Solvents are classified by the ICH (CPMP/ICH 283/95) according to their potential risk into:

- class 1 (solvents to be avoided such as benzene);
- class 2 (limited toxic potential such as methanol or hexane); and
- 3 (low toxic potential such as ethanol).

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² [http://www.inchem.org/documents/ehc/ehc/ehc170.htm#SectionNumber:2.2](http://www.inchem.org/documents/ehc/ehc/ehc170.htm#SectionNumber:2.2)
2. Potentially hazardous contaminants and residues in herbal medicines

2.1 General considerations

Herbal medicines are defined as herbal products in the medicines category in a national drug regulatory framework, and may include “herbs”, “herbal materials”, “herbal preparations” and “finished herbal products” / “herbal medicinal products”. In some countries, certain herbs and herbal materials may also be used as foods or as ingredients of foods. For this reason, the following terms have been adapted accordingly to address both regulatory categories of herbal medicines and food.

Table 1 shows examples of potentially hazardous contaminants and residues that may occur in herbal medicines. The summary table includes information on possible sources of contaminants and residues, as well as the manufacturing stages at which they may be detectable. Some of them are considered as unavoidable contaminants or residues of herbal medicines.

Contaminants in herbal medicines are classified into physicochemical contaminants and biological contaminants. A variety of agrochemical agents and some organic solvents may be important residues in herbal medicines.

Contamination should be avoided and controlled through quality assurance measures such as good agricultural and collection practices (GACP) for medicinal plants, and good manufacturing practices (GMP) for herbal medicines. Chemical and microbiological contaminants can result from the use of human excreta, animal manures and sewage as fertilizers. As noted in the WHO guidelines on GACP for medicinal plants (3), human excreta must not be used as a fertilizer, and animal manures should be thoroughly composted. Toxic elements and other chemical contaminants, including solvents originating from products intended for use in households and industrial chemicals, can be concentrated in composted sewage. Therefore, care should also be exercised with sewage management in agricultural areas.

Foreign matter should be controlled.

By far the majority of potentially hazardous contaminants and residues are found in the herbs and herbal materials. This results in their presence in the products, such as herbal preparations and finished herbal medicines. The level of some contaminants and residues present at the stage of the medicinal plant may change as a result of post-harvest processing (e.g. drying), in herbal preparations such as extracts, and in finished herbal products during the manufacturing process.

Each contaminant and residue is described in the following two subsections. Some concerns have been expressed in connection with the advancement of biotechnology, which, in the future, might be applied to medicinal plants produced using DNA technologies. This is an area that requires continuous monitoring for probable modification and new policy development. This subject, however, is beyond the scope of these guidelines.
Table 1. Classification of major contaminants and residues in herbal medicines

<table>
<thead>
<tr>
<th>Contaminants</th>
<th>General classification</th>
<th>Group</th>
<th>Subgroup</th>
<th>Specific examples</th>
<th>Possible sources</th>
<th>Stage of production at which detectable*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical contaminants</td>
<td></td>
<td>Toxic and hazardous materials</td>
<td>Toxic metals and non-metals</td>
<td>Lead, cadmium, mercury, chromium (arsenic, nitrite)</td>
<td>Polluted soil and water, during cultivation/growth, manufacturing process</td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Persistent organic pollutants</td>
<td>Dioxin aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex</td>
<td>Polluted air, soil and water, during cultivation/growth</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Radionuclides</td>
<td>Cs-134, Cs-137</td>
<td>Air, soil, water during cultivation/growth</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td>Biological toxins</td>
<td></td>
<td>Mycotoxins</td>
<td></td>
<td>Post-harvest processing, transportation and storage</td>
<td>2, 3, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacterial endotoxins</td>
<td></td>
<td>Post-harvest processing, transportation and storage</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td>Biological contaminants</td>
<td>Micro-organisms</td>
<td>Bacteria</td>
<td>Stephtylococcus aureus, Pseudomonas aeruginosa, Salmonella species, Shigella species, Escherichia coli</td>
<td>Soil, post-harvest processing, transportation and storage</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fungi</td>
<td>Yeast, moulds</td>
<td>Post-harvest processing, transportation and storage</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Animals</td>
<td>Protozoa – amoebae, Helminths – nematoda</td>
<td>Soil, excreta; organic farming/cultivation, manufacturing process</td>
<td>1, 3, 4</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Insects</td>
<td>Cockroach and its parts</td>
<td>Post-harvest processing, transportation and storage</td>
<td>1, 2, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Others</td>
<td>Mouse excreta, earthworms, acarus</td>
<td>Post-harvest processing, transportation and storage</td>
<td>1, 2, 4</td>
<td></td>
</tr>
<tr>
<td>Solvents</td>
<td></td>
<td>Organic solvents</td>
<td>Acetone, methanol, ethanol, butanol</td>
<td>Soil and water, during cultivation/growth, manufacturing process</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td>Residues</td>
<td></td>
<td>Agrochemical residues</td>
<td>Pesticides</td>
<td>Carbamate, chlorinated hydrocarbons, organophosphorus</td>
<td>Air, soil, water, during cultivation/growth, post-harvest processing</td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Insecticides</td>
<td>2,4-D, 2,4,5-T</td>
<td>Air, soil, water, during cultivation/growth, post-harvest processing</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Herbicides</td>
<td></td>
<td>Air, soil, water, during cultivation/growth, post-harvest processing</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fungicides</td>
<td>Dithiocarbamate</td>
<td>Air, soil, water, during cultivation/growth, post-harvest processing</td>
<td>1, 2, 3, 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fumigants</td>
<td>Chemical agents</td>
<td>Ethylene oxide, phosphine, methyl bromide, sulfur dioxide</td>
<td>Post-harvest processing</td>
<td>2, 3, 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Disease control agents</td>
<td>Antiviral agents</td>
<td>Thiamethoxam</td>
<td>During cultivation</td>
<td>1, 2, 3, 4</td>
</tr>
<tr>
<td>Residual solvents</td>
<td></td>
<td>Organic solvents</td>
<td>Acetone, methanol, ethanol, butanol</td>
<td>Manufacturing process</td>
<td>3, 4</td>
<td></td>
</tr>
</tbody>
</table>

* Stage of production at which detectable: 1, medicinal plants; 2, herbal materials; 3, herbal preparations; 4, finished herbal products.
2.2 Chemical contaminants

2.2.1 Toxic metals and non-metals

Contamination of herbal materials with toxic substances such as arsenic can be attributed to many causes. These include environmental pollution (i.e. contaminated emissions from factories and leaded petrol and contaminated water including runoff water which finds its way into rivers, lakes and the sea, and some pesticides), soil composition and fertilizers. This contamination of the herbal material leads to contamination of the products during various stages of the manufacturing process.

Pesticides containing arsenic and mercury were widely used until a few years ago and they are still being used in some countries.

As toxic substances are likely to be present in many foods, due to their abundance in nature, it is important to note that concomitant ingestion of herbal products would add to the total concentration of toxic metals consumed by people, even if best practice guidelines are followed.

2.2.2 Persistent organic pollutants

POPs include organic chemicals, such as the synthetic aromatic chlorinated hydrocarbons, which are only slightly soluble in water and are persistent or stable in the presence of sunlight, moisture, air and heat. In the past, they were extensively used in agriculture as pesticides. They are still generated inadvertently as by-products of combustion or industrial processes.

The use of persistent pesticides, such as DDT and benzene hexachloride (BHC), in agriculture has been banned for many years in many countries. However they are still found in the areas where they were previously used and often contaminate medicinal plants growing nearby. Also many of these substances are still being used for public health purposes, for example the control of disease vectors such as malaria-carrying mosquitoes, and are often applied near agricultural fields. The pesticide residues can then drift through the air on to the medicinal plant crops growing in nearby fields resulting in their contamination.

Thus care should be exercised with checking the quality of the medicinal plants grown in areas where these persistent pesticides are still being used.

The Stockholm Convention on Persistent Organic Pollutants\(^1\) currently includes DDT and 11 other POP’s including dioxin (a potent carcinogen), aldrin, chlordane, dieldrin, endrin, heptachlor, mirex, toxaphene and hexachlorobenzene.\(^2\)

2.2.3 Radioactive contamination

A certain amount of exposure to ionizing radiation is unavoidable because many sources, including of radionuclides occur naturally in the ground and the atmosphere (24).

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\(^1\) http://www.unep.org/themes/chemicals

\(^2\) http://www.pops.int/documents/pops/default.htm
Dangerous contamination may be the consequence of a nuclear accident or may arise from other sources. WHO, in close collaboration with several other international organizations, has developed guidelines for use in the event of widespread contamination by radionuclides resulting from a major nuclear accident (25). Examples of such radionuclides include long-lived and short-lived fission products, actinides and activation products. In general the nature and the intensity of these radionuclides may differ markedly and depends on factors such as the source, which could be a reactor, reprocessing plant, fuel fabrication plant, isotope production unit or other (26).

These guidelines emphasize that the health risks posed by herbal medicines accidentally contaminated by radionuclides depend not only on the specific radionuclide and the level of contamination, but also on the dose and duration of use of the product consumed. An important consideration in the testing for radioactive substances in herbal materials and products is the availability of the appropriate methodology and equipment. Member States would probably benefit from collaboration with countries where these facilities are available.

Cross-contamination of radionuclide-free herbal materials should be totally avoided during all the stages of production, transportation and storage.

2.2.4 Mycotoxins and endotoxins

Mycotoxins
The presence of mycotoxins in plant material can pose both acute and chronic risks to health. Mycotoxins are usually secondary metabolic products which are nonvolatile, have a relatively low molecular weight, and may be secreted onto or into the medicinal plant material. They are thought to play a dual role, firstly, in eliminating other microorganisms competing in the same environment and secondly, helping parasitic fungi to invade host tissues. Mycotoxins produced by species of fungi including *Aspergillus*, *Fusarium* and *Penicillium* are the most commonly reported.

Mycotoxins comprise four main groups, namely, aflatoxins, ochratoxins, fumonisins and tricothecenes, all of which have toxic effects. Aflatoxins have been extensively studied and are classified as Group 1 human carcinogens by the International Agency for Research on Cancer (27).

Endotoxins
Endotoxins are found mainly in the outer membranes of certain Gram-negative bacteria and are released only when the cells are disrupted or destroyed. They are complex lipopolysaccharide molecules that elicit an antigenic response, cause altered resistance to bacterial infections and have other serious effects. Thus tests for their presence on herbal medicines should be performed in dosage forms for parenteral use, in compliance with the requirements of national, regional or international pharmacopoeias.

2.2.5 Solvents occurring as contaminants
Solvents used in industries other than the manufacturing of herbal medicines, are often detected as contaminants in water used in irrigation, for drinking and for
industrial purposes and thus they find their way into medicinal plants and herbal materials at various stages of growth and processing.

2.3 Biological contaminants

2.3.1 Microbiological contaminants

Herbs and herbal materials normally carry a large number of bacteria and moulds, often originating in soil or derived from manure. While a large range of bacteria and fungi form the naturally occurring microflora of medicinal plants, aerobic spore-forming bacteria frequently predominate. Current practices of harvesting, production, transportation and storage may cause additional contamination and microbial growth. Proliferation of microorganisms may result from failure to control the moisture levels of herbal medicines during transportation and storage, as well as from failure to control the temperatures of liquid forms and finished herbal products. The presence of *Escherichia coli*, *Salmonella* spp. and moulds may indicate poor quality of production and harvesting practices.

Microbial contamination may also occur through handling by personnel who are infected with pathogenic bacteria during harvest/collection, post-harvest processing and the manufacturing process. This should be controlled by implementing best practice guidelines such as GACP and GMP.

2.3.2 Parasitic contamination

Parasites such as protozoa and nematoda, and their ova, may be introduced during cultivation and may cause zoonosis, especially if uncomposted animal excreta are used. Contamination with parasites may also arise during processing and manufacturing if the personnel carrying out these processes have not taken appropriate personal hygiene measures.

2.4 Agrochemical residues

The main agrochemical residues in herbal medicines are derived from pesticides and fumigants.

Pesticides may be classified on the basis of their intended use, for example as follows:

- insecticides;
- fungicides and nematocides;
- herbicides; and
- other pesticides (e.g. ascaricides, molluscicides and rodenticides).

Examples of fumigants include ethylene oxide, ethylene chlorohydrin, methyl bromide and sulfur dioxide.

2.4.1 Pesticide residues

Medicinal plant materials may contain pesticide residues, which accumulate as a result of agricultural practices, such as spraying, treatment of soils during cultivation and administration of fumigants during storage. It is therefore recommended that
every country producing medicinal plant materials should have at least one control laboratory capable of performing the determination of pesticides using a suitable method.

2.4.1.1 Classification of pesticides

Different classifications of pesticides exist (28, 29). A classification based on the chemical composition or structure of the pesticide is the most useful for analytical chemists, for example:

- chlorinated hydrocarbons and related pesticides: hexachlorocyclohexane (HCH) or benzene hexachloride (BHC), lindane, methoxychlor
- chlorinated phenoxyalkanoic acid herbicides: 2,4-D, 2,4,5-T
- organophosphorus pesticides: carbophenothion (carbofenotion), chlorpyrifos and methylchlorpyrifos, coumaphos (coumafos), demeton, dichlorvos, dimethoate, ethion, fenchlorphos (fenclfos), malathion, methyl parathion, parathion
- carbamate insecticides: carbaryl (carbaril)
- carbamoyl benzimidazoles: benomyl, carbendazim
- dithiocarbamate fungicides: ferbam, maneb, nabam, thiram, zineb, ziram
- amino acid herbicides: glyphosate
- inorganic pesticides: aluminium phosphide, calcium arsenate
- miscellaneous: bromopropylate, chloropicrin, ethylene dibromide, ethylene oxide, methyl bromide, sulfur dioxide
- pesticides of plant origin: tobacco leaf extract, pyrethrum flower, and pyrethrum extract; derris and Lonchocarpus root and rotenoids.

Only the chlorinated hydrocarbons and related pesticides (e.g. HCH) and a few organophosphorus pesticides (e.g. carbophenothion) have a long residual action. Although the use of many persistent pesticides has been widely discontinued, residues may still remain in the environment (e.g. DDT (see section 2.2.2)). Thus the recording of all pesticide usage in countries should be strongly encouraged so as to enable cost-effective quality control of medicinal plants and of their products.

Pesticides based upon copper as the active agent e.g. copper sulfate and mixtures of copper sulfate and hydrated lime were often used in the past and are still popular with farmers today. Such compounds are effective fungicides. Although copper is an essential nutrient for plants its levels must be controlled because if ingested at high levels, around 70 mg/day, it does have serious adverse effects on health. The likelihood of exposure to copper is also heightened by the fact that copper is strongly bioaccumulated in nature and therefore it is likely to persist in herbal materials, similarly to the heavy metals.

Most other pesticides have very short residual actions. Therefore it is suggested that, where the length of exposure to pesticides is unknown, the herbal materials should be tested for the presence of organically bound chlorine and phosphorus as a preliminary screening method (see also Annex 6, section 1), which can be useful in predicting where a pesticide might be used.
2.4.2. Extraneous pesticide residues (see section 2.2.2)

2.5 Residual solvents

A range of organic solvents are used for manufacturing herbal medicines, and can be detected as residues of such processing in herbal preparations and finished herbal products. They should be controlled through GMP and quality control.

Solvents are classified by ICH (CPMP/ICH 283/95), according to their potential risk, into:

- class 1 (solvents to be avoided such as benzene);
- class 2 (limited toxic potential such as methanol or hexane); and
- class 3 (low toxic potential such as ethanol).
3. Guiding principles for assessing safety of herbal medicines with reference to contaminants and residues

3.1 General approach – compliance with good practice guidelines

Compliance with GACP and GMP is crucial for the production of good quality herbal medicines. The entire production process, starting from cultivation and ending with the sale of the products, must adhere rigorously to these two sets of practices. The contents of these guidelines should therefore be read in conjunction with GACP and GMP in an effort to produce quality products for the local and international markets.

3.2 Foreign matter

Foreign matter found in a sample of herbs and herbal materials should not exceed limits set in national, regional or international pharmacopoeias. Foreign matter includes insects and other animal contamination including animal excreta, as well as other species of plants. In general, any substance other than the acceptable sample of good quality medicinal plant material is regarded as foreign matter. A pure sample is seldom found and there is always some foreign matter present. However no poisonous, dangerous or otherwise harmful foreign matter should be allowed. Thus following the GACP should help to ensure that contamination is kept to a minimum.

Removal of larger pieces of foreign matter from whole and cut plants is often done by hand-sorting after macroscopic examination. Finished products should also be examined for foreign materials.

3.3 Contaminants

3.3.1 Arsenic and toxic metals

The maximum amounts of toxic metals and non-metals in medicinal plant materials can be given based on the provisional tolerable intake (PTI) values. These values should be established on a regional or national basis. Examples are given in Annex 3 under Section A.4.

The use of herbal medicinal products is not generally expected to contribute significantly to the exposure of the population to heavy metal contaminants. However, it should be understood that the heavy metal content of herbal medicines adds to the burden originating from food so it is recommended that heavy metal contamination is minimized.
In general it would be desirable to harmonize limits for toxic metals and standards, as this would have many benefits including the facilitation of global trade.

### 3.3.2 Persistent organic pollutants

POPs comprise hundreds of chemicals that are not soluble in water and are persistent or stable in the environment. They are often transported globally because of their resistance to breakdown and they have the potential to cause harm to humans and wildlife that ingest them. They will not disappear from our environments in the short term because some are still being produced and used in many countries.

Internationally, through the Stockholm Convention on Persistent Organic Pollutants, efforts are being made to control their production and emission, and to substitute them with other less problematic pesticides.

### 3.3.3 Radioactive contaminants

The amount of exposure to radiation depends on the intake of radionuclides and its significance depends in turn on other variables such as the age, metabolic kinetics and weight of the individual who ingests them (also known as the dose conversion factor).

The level of contamination might be reduced during the manufacturing process. Therefore, no limits for radioactive contamination are proposed in these guidelines and herbal materials should be tested on a case-by-case basis according to national and regional standards if there are concerns. In such a process, national regulation on the limits could be set based on risk management, but no risk assessment.¹

### 3.3.4 Microbial toxins

Mycotoxins and, when appropriate, endotoxins should be tested for using an appropriately validated and sensitive method, and amounts should be below the limits set in national or regional standards. It is recommended that in performing analyses for mycotoxins particular care should be taken, in line with good practice guidelines such as WHO good practices for national pharmaceutical control laboratories (30).

### 3.3.5 Microbiological contaminants

#### 3.3.5.1 Bacteria

*Salmonella* and *Shigella* species must not be present in herbal medicines intended for internal use, at any stage. Other microorganisms should be tested for and should comply with limits set out in regional, national or international pharmacopoeias.

Different pharmacopoeias have different testing requirements and these should be consulted when making the appropriate choice for the selected herbal materials and herbal product.

¹ Office for Official Publications of the European Communities, Council Regulation (EEC). No. 737/90 of 22 March 1990 on the conditions governing import of agricultural products originating in third countries following the accident at the Chernobyl nuclear power station.
3.4 Residues

3.4.1 Pesticide residues

Limits for pesticide residues should be established following the recommendations of the JMPR which have already been established for food and animal feed (26). These recommendations include an ADI and the analytical methodology for the assessment of specific pesticide residues. Currently however there are no standard procedures for assignment of these MRLs in the medicinal plant area and thus the methods used for foods could probably be used for the preparation of a model. This approach would apply in the case where a botanically identical medicinal plant is used as food.

Historically the FAO and WHO have established MRLs, based on supervised trials and establishment of GAP (21) for the use of pesticides for a variety of food commodities and combinations of pesticides and food commodities. If the listed food commodity (name of the original plant and the part of it used as food) is botanically identical to the medicinal plant part concerned and has an established MRL for a specific pesticide, the relevant MRL could be regarded as the MRL for the specific raw medicinal plant material. In this case, the MRL for the medicinal plant material in question could be further elaborated by establishing an appropriate formula to factor in drying of the plant material.

The Codex Alimentarius Commission has adopted a list of approved pesticides for spices and their MRLs as shown in Table 4 (see section 4.5) (31).1

If the medicinal plant in question is identical to the plant, but the part concerned is different to that listed for the food commodity, or it is not identical to the plant on the food commodity list, elaboration of MRLs for medicinal plant materials could be attempted by similar approaches to those described below.

3.4.1.1 Maximum limit of pesticide residues for herbal materials

The toxicological evaluation of pesticide residues in herbal materials should be based on the likely intake of the material by patients. In the absence of a full risk assessment and for practical reasons, it is recommended that, in general, the intake of residues from herbal materials should account for no more than 1% of total intake from all sources, including food and drinking-water (2). Since the level of pesticide residues may change during the production process, it is vital to determine the actual quantity of residues consumed in the final dosage form.

Because herbal medicines may be used for treatment of chronic diseases or for prophylactic reasons, it is suggested that the approach of the FAO in determining MRLs should be followed.

3.4.1.2 Acceptable residue level

An ARL (in mg of pesticide per kg of medicinal plant material) can be calculated on the basis of the maximum ADI of the pesticide for humans, as, recommended by FAO and WHO, and the mean daily intake (MDI) of the medicinal plant material.

1 http://www.codexalimentarius.net/download/report/644/al28_41e.pdf
Some countries and/or regions have established national requirements for residue limits in medicinal plant materials. If no such requirements exist, other references can be consulted, such as other pharmacopoeias or published documents. The appropriateness of risk assessment using the ARL needs further investigation and research. Examples are given in Annex 6 under Section 4. Where such requirements do not exist, the following formula that is based on the acceptable daily intake (ADI) determined by FAO and WHO, may be used:

\[
\text{ARL} = \frac{\text{ADI} \times E \times 60}{\text{MDI} \times 100}
\]

where:
\[
\text{ADI} = \text{maximum acceptable daily intake of pesticide (mg/kg of body weight)}
\]
\[
E = \text{extraction factor, determined experimentally, which determines the transfer rate of the pesticide from the medicinal plant material into the dosage form}
\]
\[
\text{MDI} = \text{mean daily intake in kilograms of medicinal plant material}
\]
60: this number represents a mean adult body weight of 60 kg; it may need to be adjusted for certain patient groups, nationalities, etc.
100: this number is a consumption factor of 100 reflecting the requirement that no more than 1% of the total pesticide residue consumed should be derived from medicinal plant material.

### 3.4.2 Extraneous pesticide residues

According to the Codex Commission Committee on Pesticide Residues, residues of DDT and BHC have been found in some spices. It is suggested that for these compounds, EMRLs be established instead of MRLs in the same manner as for other pesticides for food commodities.\(^1\)

### 3.4.3 Residual solvents

The term “permitted daily exposure” (PDE) is proposed as defining a pharmaceutically acceptable intake of residual solvents to avoid confusion resulting from differing values for ADIs of the same substance (22).\(^2\)

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\(^1\) Codex Alimentarius Commission, Food and Agriculture Organization of the United Nations and World Health Organization CLPR 03/15.

4. Recommended analytical methods

The test methods described here are presented as examples of suitable methods for the detection of selected contaminants and residues in herbal medicines, mainly for herbal materials. Where available, test methods applicable to different products and stages of herbal medicines, such as extracts and finished herbal products, are also described. In addition to the test methods, some examples of national experience regarding general limits for contaminants and residues are included, where applicable. Both should be considered as the basis for establishing national and regional requirements for limits and methodologies. WHO is currently not able to recommend limits for contaminants and residues because they are too diverse and there is a lack of consensus. Also the test procedures cannot take into account all possible impurities, but where impurities are known to occur, validated methods should be developed.

The test methods should be used where appropriate, only at certain stages, and on a case-by-case basis.

The analysis of herbal medicines is not restricted to those methods discussed or recommended here and other techniques are also available. Details of analytical methods, such as volumetric analysis, are described in international pharmacopoeias (4, 5).

When considering the choice of method, the level of detection and the plant product matrix used for the testing, e.g. seeds containing oils or finished products, must be taken into account, and the method modified if required. The method of determination should be validated for the relevant matrix.

Although selected methods are described in detail in the annexes to this document, they may not necessarily be the most modern or state-of-the-art methods. They do offer some options and guidance, but the available technology and resources, including human and financial, may influence their use in particular countries.

In the event of limitations precluding the required analytical services for herbal products in a particular district(s) in a country, it is recommended that at least other national or regional official laboratories be made available for such purposes.

The guidance of good practices for national pharmaceutical control laboratories, including quality assurance measures, should be followed when methods are chosen for all analyses. All methods chosen should be properly validated in accordance with these good practices (30).

The annexes cover the following:

- Annex 3 – determination of arsenic and toxic metals
- Annex 4 – determination of aflatoxins
- Annex 5 – determination of microorganisms
4.1 Determination of arsenic and toxic metals (Annex 3)

In general, quantitative tests and limit tests accurately determine the concentrations of toxic metals in the form of impurities and contaminants. The latter are unavoidably present in the samples being tested i.e. herbal medicines and their herbal products. Member States can elect to use either quantitative tests or limit tests and their choices will be influenced by the nature of the sample and the contaminants or residues, assessed on a case-by-case basis. Another factor would be that the method(s) identified, and chosen to be applied to control heavy metals, should be relevant and should meet the requirements at a regional and national level.

Some examples of proposed national limits for arsenic and toxic metals in various types of herbal products are shown in Table 2. Country figures are based on information provided by national health authorities.

Table 2. Examples of national limits for arsenic and toxic metals in herbal medicines and products

<table>
<thead>
<tr>
<th></th>
<th>Arsenic (As)</th>
<th>Lead (Pb)</th>
<th>Cadmium (Cd)</th>
<th>Chromium (Cr)</th>
<th>Mercury (Hg)</th>
<th>Copper (Cu)</th>
<th>Total toxic metals as lead</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For herbal medicines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canada raw herbal materials</td>
<td>5 ppm</td>
<td>10 ppm</td>
<td>0.3 ppm</td>
<td>2 ppm</td>
<td>0.2 ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>China herbal materials</td>
<td>2 ppm</td>
<td>10 ppm</td>
<td>1 ppm</td>
<td>0.5 ppm</td>
<td>20 ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malaysia finished herbal products</td>
<td>5 mg/kg</td>
<td>10 mg/kg</td>
<td></td>
<td>0.5 mg/kg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Republic of Korea herbal materials</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30 ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singapore finished herbal products</td>
<td>5 ppm</td>
<td>20 ppm</td>
<td></td>
<td>0.5 ppm</td>
<td>150 ppm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thailand herbal material, finished herbal products</td>
<td>4 ppm</td>
<td>10 ppm</td>
<td>0.3 ppm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WHO recommendations (2)</td>
<td>10 mg/kg</td>
<td>0.3 mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>For other herbal products</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>National Sanitation Foundation draft proposal (Raw Dietary supplement)</td>
<td>5 ppm</td>
<td>10 ppm</td>
<td>0.3 ppm</td>
<td>2 ppm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>National Sanitation Foundation draft proposal (Finished Dietary Supplement)</td>
<td>0.01 mg/day</td>
<td>0.02 mg/day</td>
<td>0.006 mg/day</td>
<td>0.02 mg/day</td>
<td>0.02 mg/day</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Dietary supplement – for further information see ref. (32).*
The metals are widely distributed throughout nature and occur freely in soil and water and are often components of certain pesticides (see also section 4.5).

In general, during analysis of metals, one should always aim to use the best and latest methods whenever possible. However it is crucial to ensure that all methods are fully validated, not forgetting the need to validate the integrity of the starting matrix of plant product. This imperative should apply both to governments and companies/applicants submitting these methods as part of their applications to the national regulatory authority for market authorization.

Limit tests find wide application in the area of pharmaceutical medicines where it is common to test for substances such as chlorides, sulfates, arsenic and heavy metals. Thus they will be very useful in the testing of herbal medicines and their products. Limit tests can also be modified in many instances to function as true limit tests where the actual amount of toxic metal can be estimated with great accuracy.

The need for the inclusion of tests for toxic metals and acceptance criteria should be studied at the various developmental stages of the plant and based on knowledge of the medicinal plant species, its growth and or cultivation and the manufacturing process. The choice of procedures for control should take account of this information: the choice between a limit test and a specific quantitative method will depend on the level of control required for a particular material, for example starting material, and should be justified.

In general, if the heavy metals burden of the herbal material is unknown, it is suggested that it be determined qualitatively and quantitatively on several batches, preferably collected over several years. These data should be used to establish acceptance limits that should be checked by appropriate limit tests.

### 4.2 Determination of radioactive contaminants

#### 4.2.1 Method of measurement

Following a severe nuclear accident, the environment may be contaminated with airborne radioactive materials. These may deposit on the leaves of medicinal plants. Their activity concentration and the type of radioactive contamination can be measured by the radiation monitoring laboratories of most of the WHO Member States. The activity concentration of radioisotopes in herbs should be assessed by the competent national radiohygiene laboratories taking into account the relevant recommendations of international organizations, such as Codex Alimentarius, the International Atomic Energy Agency (IAEA), FAO and WHO.

Since radionuclides from accidental discharges vary with the type of facility involved, a generalized method of measurement is not yet available. However, should such contamination be a concern, suspect samples can be analysed by a competent laboratory. Details of laboratory techniques are available from the IAEA.\(^1\)

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\(^1\) International Atomic Energy Agency (IAEA), Analytical Quality Control Services, Laboratory Seibersdorf, PO Box 100, Vienna, Austria.
4.3 Determination of aflatoxins (Annex 4)

Determination of aflatoxins should take place after using a suitable clean-up procedure, during which great care should be taken not to become exposed or to expose the working or general environment to these dangerous and toxic substances. Thus Member States should adapt their good practices for national pharmaceutical control laboratories and GMP accordingly. Only products that have a history of aflatoxin contamination need to be tested.

There are specific sampling problems especially of aflatoxins due to the way in which contamination spreads, as described for some food commodities, such as nuts and corn. This may need to be taken into consideration when sampling, for example in terms of sample selection and sample size, and when the analysis is made (33, 34).

Tests for aflatoxins are designed to detect the possible presence of aflatoxins B₁, B₂, G₁, and G₂, which are highly toxic contaminants in any material of plant origin.

Some examples of proposed national limits for aflatoxin in various types of herbal products are presented in Box 1 below. Country figures are based on information provided by national health authorities.

Box 1. Herbal materials, preparations and products

<table>
<thead>
<tr>
<th>Country</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>Determination method: HPLC-based technique using a monoclonal antibody immunoaffinity column.</td>
</tr>
<tr>
<td></td>
<td>• For herbs, herbal materials and herbal preparations used for herbal tea infusions</td>
</tr>
<tr>
<td></td>
<td>20 μg/kg for aflatoxins B₁ + B₂ + G₁ + G₂ with the condition that aflatoxin B₁ ≤ 5 μg/kg</td>
</tr>
<tr>
<td></td>
<td>• For finished herbal products for internal or topical use</td>
</tr>
<tr>
<td></td>
<td>Absence per 1 gram</td>
</tr>
<tr>
<td>Germany</td>
<td>Any materials used in manufacture of medicinal products (including medicinal herbal products)</td>
</tr>
<tr>
<td></td>
<td>2 μg/kg&lt;sup&gt;c&lt;/sup&gt; for aflatoxin B₁ or 4 μg/kg&lt;sup&gt;c&lt;/sup&gt; for total sum of aflatoxins B₁, B₂, G₁, and G₂.</td>
</tr>
<tr>
<td></td>
<td>(The determination of the level of aflatoxin content has to be based on sampling procedures that take into account a potential heterogeneous distribution in the material.)</td>
</tr>
</tbody>
</table>

<sup>c</sup> Calculated on at least 88% of dry weight.
4.4 Determination of microbiological contaminants (Annex 5)

4.4.1 Microbial contamination limits in herbal materials, preparations and finished products

Different limits are set according to the intended use of the herbal material and the medicines themselves. Some examples are given here:

4.4.1.1 Raw medicinal plant and herbal materials intended for further processing

For contamination of raw medicinal plant and herbal materials intended for further processing (including additional decontamination by a physical or chemical process) the limits, adapted from the provisional guidelines established by an international consultative group (35), are given for untreated herbal material harvested under acceptable hygienic conditions:

- *Escherichia coli*, maximum $10^4$ per gram
- mould propagules, maximum $10^5$ per gram
- shigella, absence per gram or ml.

4.4.1.2 Herbal materials that have been pretreated

For herbal materials that have been pretreated (e.g. with boiling water as used for herbal teas and infusions) or that are used as topical dosage forms, the limits are:

- aerobic bacteria, maximum $10^7$ per gram
- yeasts and moulds, maximum $10^4$ per gram
- *Escherichia coli*, maximum $10^5$ per gram
- other enterobacteria, maximum $10^4$ per gram
- clostridia, absence per 1 gram
- salmonellae, absence per 1 gram
- shigella, absence per 1 gram.

4.4.1.3 Other herbal materials for internal use

For other herbal materials for internal use, the limits are:

- aerobic bacteria, maximum $10^5$ per gram
- yeasts and moulds, maximum $10^3$ per gram
- *Escherichia coli*, maximum 10 per gram
- other enterobacteria, maximum $10^3$ per gram
- clostridia, absence per 1 gram
- salmonellae, absence per 1 gram
- shigella, absence per 1 gram.

4.4.1.4 Herbal medicines to which boiling water is added before use

For herbal medicines to which boiling water is added before use, the limits are:

- aerobic bacteria, maximum $10^7$ per gram
- yeasts and moulds, maximum $10^4$ per gram
- *Escherichia coli*, maximum 10 per gram
- other enterobacteria, maximum $10^3$ per gram
- clostridia, absence per 1 gram
- salmonellae, absence per 1 gram
- shigella, absence per 1 gram.
4.4.1.5 **Other herbal medicines**

For other herbal medicines, the limits are:

- aerobic bacteria, maximum $10^5$ per gram
- yeasts and moulds, maximum $10^3$ per gram
- *Escherichia coli*, absence per 1 gram
- other enterobacteria, maximum $10^3$ per gram
- clostridia, absence per 1 gram
- salmonellae, absence per 1 gram
- shigella, absence per 1 gram.

4.5 **Determination of pesticide residues (Annex 6)**

Some examples of national and regional limits set for various types of pesticide residues are shown in Table 3. As a reference, the list of approved pesticides for spices and their MRLs adopted by the Codex Alimentarius Commission is given in Table 4.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Limit EPa (mg/kg)</th>
<th>Japanb (ppm)</th>
<th>USPc and FAi (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acephate</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alachlor</td>
<td>0.05</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>aldrin and dieldrin (sum of)</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>azinphos-methyl</td>
<td>1</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>bromine</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bromophos-ethyl</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bromophos-methyl</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bromopropylate</td>
<td>3</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>chlordane (sum of cis-, trans- and oxythlordane)</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>chlorfenvinphos</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>chlorpyrifos</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chlorpyrifos-ethyl</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>chlorpyrifos-methyl</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>chlorthal-dimethyl</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyfluthrin, sum</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ramda-cyhalothrin</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cypermethrin (and isomers)</td>
<td>1</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>DDT (sum of 0,p'-DDE, p,p'- DDE, o,p'-DDT, p,p'-DDT, o,p'-TDE and p,p'-TDE)</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DDT (sum of p,p'- DDT o,p'-DDT p,p'- DDD and p,p'- DDE)</td>
<td></td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>DDT (sum of p,p'- DDT, o,p'-DDT, p,p'- DDE, and p,p'- TDE)</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>deltamethrin</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>diazinon</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Substances</td>
<td>Limit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------------------------------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EP&lt;sup&gt;a&lt;/sup&gt; (mg/kg)</td>
<td>Japan&lt;sup&gt;b&lt;/sup&gt; (ppm)</td>
<td>USP&lt;sup&gt;c&lt;/sup&gt; and FA&lt;sup&gt;d&lt;/sup&gt; (mg/kg)</td>
</tr>
<tr>
<td>dichlofluanid</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dichlorvos</td>
<td>1</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>dicofol</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dimethoate and omethoate (sum of)</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dithiocarbamate (as CS&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>2</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>endosulfan (sum of isomers and endosulfan sulfate)</td>
<td>3</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>endrin</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>ethion</td>
<td>2</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>fenchlorophos (sum of fenchlorophos and fenchlorophos-oxon)</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fenitrothion</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>fenpropathrin</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fensulfothion (sum of fensulfothion, fensulfothion-oxon, fensulfothion-oxonsulfon and fensulfothion-sulfon)</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fenvalerate</td>
<td>1.5</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>fluclorinate</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-fluvalinate</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fonofos</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>heptachlor (sum of heptachlor, cis-heptachlorepoxide and trans-heptachlorepoxide)</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>heptachlor (sum of heptachlor and heptachlorepoxide)</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexachlorobenzene</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>hexachlorocyclohexane (sum of isomers, α-,β-,γ-, and ε- hexachlorocyclohexane)</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexachlorocyclohexane isomers (sum of α,β,γ and δ)</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hexachlorocyclohexane isomers (other than γ)</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lindane (γ-hexachlorocyclohexane)</td>
<td>0.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>malathion</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>malathion and malaoxon (sum of)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mecarbam</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>methacrifos</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>methamidophos</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>methidathion</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>methoxychlor</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mirex</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>monocrotophos</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parathion</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parathion-ethyl and paraoxon-ethyl (sum of)</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parathion-methyl</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>parathion-methyl and paraoxon-methyl (sum of)</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pendimethalin</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pentachloranisol</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>permethrin (and isomers) (sum of)</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>permethrin</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>phosalone</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. The list of approved pesticides for spices\textsuperscript{a} and their maximum residue limits (MRLs) (Codex Alimentarius Commission, 2005)\textsuperscript{b}

<table>
<thead>
<tr>
<th>Pesticide (CCPR-number)</th>
<th>Group or sub-group of spices</th>
<th>MRL (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetamiprid (095)</td>
<td>Entire group 028\textsuperscript{c}</td>
<td>0.2 (*)</td>
</tr>
<tr>
<td>Azinphos-methyl (002)</td>
<td>Entire group 028\textsuperscript{c}</td>
<td>0.5 (*)</td>
</tr>
<tr>
<td>Chlorpyrifos (017)</td>
<td>Seeds</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>1</td>
</tr>
<tr>
<td>Chlorpyrifos-methyl (090)</td>
<td>Seeds</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fruits</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>5</td>
</tr>
<tr>
<td>Cypermethrin (118)</td>
<td>Fruits or berries</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.2</td>
</tr>
<tr>
<td>Diazinon (22)</td>
<td>Seeds</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Fruits</td>
<td>0.1 (*)</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.5</td>
</tr>
<tr>
<td>Dichlorvos (025)</td>
<td>Entire group 028\textsuperscript{c}</td>
<td>0.1 (*)</td>
</tr>
<tr>
<td>Dicofol (026)</td>
<td>Seeds</td>
<td>0.05 (*)</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Applicable to medicinal plant materials included in the \textit{European pharmacopoeia}, 5th ed, unless otherwise indicated in the applicable monograph. Reference: \textit{PHARMEUROP A} Volume 18, No. 4, October 2006.

\textsuperscript{b} Currently applicable only to five medicinal plant materials (ginseng root, powdered ginseng root, red ginseng root, senna leaf and powdered senna leaf) included in \textit{Japanese pharmacopoeia}, XIV.

\textsuperscript{c} Values in \textit{United States pharmacopoeia} 28 and \textit{Argentina pharmacopoeia}, Vol. 1.
<table>
<thead>
<tr>
<th>Pesticide (CCPR-number)</th>
<th>Group or sub-group of spices</th>
<th>MRL (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethoate (027)</td>
<td>Seeds</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.1 (*)</td>
</tr>
<tr>
<td>Disulfoton (074)</td>
<td>Entire group 028c</td>
<td>0.05 (*)</td>
</tr>
<tr>
<td>Endosulfan (032) (total)</td>
<td>Seeds</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.5</td>
</tr>
<tr>
<td>Ethion (034)</td>
<td>Seeds</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.3</td>
</tr>
<tr>
<td>Fenitrothion (037)</td>
<td>Seeds</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.1 (*)</td>
</tr>
<tr>
<td>Iprodion (111)</td>
<td>Seeds</td>
<td>0.05 (*)</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.1 (*)</td>
</tr>
<tr>
<td>Malathion (049)</td>
<td>Seeds</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.5</td>
</tr>
<tr>
<td>Metalaxyl (138)</td>
<td>Seeds</td>
<td>5</td>
</tr>
<tr>
<td>Methamidophos (100)</td>
<td>Entire group 028c</td>
<td>0.1 (*)</td>
</tr>
<tr>
<td>Parathion (058)</td>
<td>Seeds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.2</td>
</tr>
<tr>
<td>Parathion-methyl (059)</td>
<td>Seeds</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>3</td>
</tr>
<tr>
<td>Permethrin (120)</td>
<td>Entire group 028c</td>
<td>0.05 (*)</td>
</tr>
<tr>
<td>Phenthoate (128)</td>
<td>Seeds</td>
<td>7</td>
</tr>
<tr>
<td>Phorate (112)</td>
<td>Seeds sub-group</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>0.1 (*)</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>0.1 (*)</td>
</tr>
<tr>
<td>Phosalone (060)</td>
<td>Seeds</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Fruits</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>3</td>
</tr>
<tr>
<td>Pirimicarb (101)</td>
<td>Seeds</td>
<td>5</td>
</tr>
<tr>
<td>Pirimiphos-methyl (086)</td>
<td>Seeds sub-group</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Fruits sub-group</td>
<td>0.5</td>
</tr>
<tr>
<td>Quintozene (064)</td>
<td>Seeds sub-group</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Fruits or berries</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Roots or rhizomes</td>
<td>2</td>
</tr>
<tr>
<td>Vinlozolin (159)</td>
<td>Entire spice group</td>
<td>0.05 (*)</td>
</tr>
</tbody>
</table>

(*) At or about the limit of determination.

a The residue definitions remain the same as those recommended for the given pesticide in other plant commodities (http://www.codexalimentarius.net/mrls/pestdes/pest_ref/MRLs_Spices_e.pdf).


c The Group of A28 as modified by the 36th Session of CCPR.
5. References


**National and regional pharmacopoeias**


Annex 1: List of participants in the WHO consultation on contaminants and residues in herbal medicines, Milan-Loveno di Menaggio, Italy, 12–14 July 2004

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Annex 2: General technical notices

General considerations

The metric system is used throughout the text. All temperatures are expressed in degrees Celsius (°C).

Tests are normally carried out at room temperature (between 15 and 25 °C, or up to 30 °C in some climatic zones), unless otherwise indicated.

Any glassware used in the tests should be of suitable quality. Graduated and volumetric vessels should be calibrated at room temperature.

When a water-bath is referred to in the text, a bath containing boiling water (about 100 °C) is to be used, unless a specific water temperature is given.

Unless otherwise specified, all solutions indicated in the tests are prepared with distilled or demineralized water of adequate purity.

Reagents and solutions

Reagents and solutions used must conform to the requirements specified in annex 8 “Reagents and solutions”, and are designated as follows: reagent, R; test solution, TS; volumetric solution, VS.

Precision of measurement

Quantities and volumes

The quantities and volumes of the materials and reagents used in the tests must be measured with adequate precision, which is indicated in the following way:

A value of: 20.0 means not less than 19.5 and not more than 20.5
          2.0 means not less than 1.95 and not more than 2.05
          0.20 means not less than 0.195 and not more than 0.205.

Temperature

Temperature measurement is indicated in a manner similar to that given for quantities and volumes.

Storage conditions given in general terms refer to the following equivalent temperatures:

<table>
<thead>
<tr>
<th>Condition</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>In a refrigerator</td>
<td>0–6 °C</td>
</tr>
<tr>
<td>Cold or cool</td>
<td>6–15 °C</td>
</tr>
<tr>
<td>Room temperature</td>
<td>15–25 °C,</td>
</tr>
<tr>
<td></td>
<td>or up to 30 °C in some climatic zones.</td>
</tr>
</tbody>
</table>
pH values

Precision in the measurement of pH values is indicated in a manner similar to that for quantities and volumes.

Calculation of results

The results of tests and assays should be calculated to one decimal place more than indicated in the requirement and then rounded up or down as follows:

- if the last figure calculated is 5 to 9, the preceding figure is increased by 1;
- if the last figure calculated is 4 or less, the preceding figure is left unchanged.

Other calculations, for example in the standardization of volumetric solutions, should be carried out in a similar manner.

If the material has to be dried before it can be reduced to a powder for use in a determination, a correction must be made to take into account the loss on drying, and the amount of active principle calculated with reference to the undried sample.

Establishment of limits

Reasonable limits may be established using simple statistical methods, e.g. control chart techniques (1, 2). Analytical results from about 20 successive batches are pooled together, and the grand average and “three sigma limits” (±3 standard deviations from the grand average) are calculated. (Such calculations are applicable when more than one individual or independent sample per batch is analysed (3, 4).)

Solubility

Unless otherwise specified in the test procedure for the plant material concerned, the approximate solubility of medicinal plant materials should be determined at 20 °C. Solubility is expressed in terms of “parts”, representing the number of millilitres (ml) of the solvent, in which 1 g of the solid is soluble. Descriptive terms are sometimes used to indicate the solubility of a substance, with the following meanings:

- very soluble: less than 1 part
- freely soluble: 1–10 parts
- soluble: 10–30 parts
- sparingly soluble: 30–100 parts
- slightly soluble: 100–1000 parts
- very slightly soluble: 1000–10 000 parts
- practically insoluble: more than 10 000 parts

Storage

Medicinal plant materials must be stored under specified conditions in order to avoid contamination and deterioration.
Containers
The container and its closure must not interact physically or chemically with the material within in any way that would alter its quality. The following descriptive terms are used to indicate general requirements for the permeability of containers:

Well-closed containers must protect the contents from extraneous matter or from loss of the material under normal conditions of handling, shipment or storage.

Tightly closed containers must protect the contents from extraneous matter, from loss of the material, and from efflorescence, deliquescence, or evaporation under normal conditions of handling, shipment or storage. If the container is intended to be opened on several occasions, it must be designed to be airtight after reclosure.

Hermetically closed containers must protect the contents from extraneous matter and from loss of the substance, and be impervious to air or any other gas under normal conditions of handling, shipment or storage.

In addition, a tamper-evident container is one that is fitted with a device that reveals clearly whether it has ever been opened.

Protection from light
Medicinal plant materials requiring protection from light should be kept in a light-resistant container that – either by reason of the inherent properties of the material from which it is made or because a special coating has been applied to it – shields the contents from the effects of light. Alternatively, the container may be placed inside a suitable light-resistant (opaque) covering and/or stored in a dark place.

Temperature
Materials that need to be stored at temperatures other than room temperature (15 to 25 °C or, depending on the climate conditions, up to 30 °C) should be labelled accordingly.

Humidity
Low humidity may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided. Care must be taken when the container is opened in damp or humid conditions.

Size of cut
Medicinal plant materials are used either whole, or in cut or powdered form.

Cut medicinal plant materials are prepared by cutting or crushing the plant into small pieces. The cut is graded according to the aperture size of the mesh of the sieve through which the material will pass, and is indicated as follows:

<table>
<thead>
<tr>
<th>Aperture size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>coarse cut</td>
</tr>
<tr>
<td>medium cut</td>
</tr>
<tr>
<td>fine cut</td>
</tr>
</tbody>
</table>
Powder fineness and sieve size

**Powders**
The coarseness or fineness of a powder is classed according to the nominal aperture size expressed in micrometres of the mesh of the sieve through which the powder will pass, and is indicated as follows:

<table>
<thead>
<tr>
<th>Descriptive term</th>
<th>Particle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coarse (2000/355)</td>
<td>All the particles will pass through a No. 2000 sieve, and not more than 40% through a No. 355 sieve</td>
</tr>
<tr>
<td>Moderately coarse (710/250)</td>
<td>All the particles will pass through a No. 710 sieve, and not more than 40% through a No. 250 sieve</td>
</tr>
<tr>
<td>Moderately fine (355/180)</td>
<td>All the particles will pass through a No. 355 sieve, and not more than 40% through a No. 180 sieve</td>
</tr>
<tr>
<td>Fine (180)</td>
<td>All the particles will pass through a No. 180 sieve</td>
</tr>
<tr>
<td>Very fine (125)</td>
<td>All the particles will pass through a No. 125 sieve</td>
</tr>
</tbody>
</table>

**Sieves**
The wire sieves used to sift powdered medicinal plant materials are classified by numbers that indicate their nominal aperture size expressed in μm. The sieves are made of wire of uniform circular cross-section, and have the following specifications:

<table>
<thead>
<tr>
<th>Number of sieve (μm)</th>
<th>Nominal size of aperture (mm)</th>
<th>Nominal diameter of wire (mm)</th>
<th>Approximate screening area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>2.00</td>
<td>0.90</td>
<td>48</td>
</tr>
<tr>
<td>710</td>
<td>0.710</td>
<td>0.450</td>
<td>37</td>
</tr>
<tr>
<td>500</td>
<td>0.500</td>
<td>0.315</td>
<td>38</td>
</tr>
<tr>
<td>355</td>
<td>0.355</td>
<td>0.224</td>
<td>38</td>
</tr>
<tr>
<td>250</td>
<td>0.250</td>
<td>0.160</td>
<td>37</td>
</tr>
<tr>
<td>212</td>
<td>0.212</td>
<td>0.140</td>
<td>36</td>
</tr>
<tr>
<td>180</td>
<td>0.180</td>
<td>0.125</td>
<td>35</td>
</tr>
<tr>
<td>150</td>
<td>0.150</td>
<td>0.100</td>
<td>36</td>
</tr>
<tr>
<td>125</td>
<td>0.125</td>
<td>0.090</td>
<td>34</td>
</tr>
<tr>
<td>90</td>
<td>0.090</td>
<td>0.063</td>
<td>35</td>
</tr>
<tr>
<td>75</td>
<td>0.075</td>
<td>0.050</td>
<td>36</td>
</tr>
<tr>
<td>45</td>
<td>0.045</td>
<td>0.032</td>
<td>34</td>
</tr>
</tbody>
</table>

The sieves recommended here have been selected from among those conforming to ISO standard 565, 1990.

**Units of measurement**
The names and symbols for units of measurement used in this manual conform with those used in *The international pharmacopoeia* (5) and those of the International System of units (SI), developed by the General Conference of Weights and Measures (CGPM) in collaboration with other international organizations (6, 7).
General advice on sampling

The reliability of any conclusions drawn from the analysis of a sample will depend upon how well the sample represents the whole batch. General recommendations for the sampling of pharmaceutical materials in connection with quality control are provided in the thirty-ninth report of the WHO Expert Committee on Specifications for Pharmaceutical Preparations (3).

Because of the specific characteristics of medicinal plant materials, in particular their lack of homogeneity, special handling procedures are required in relation to sampling. The following procedures should be observed when selecting and preparing an average sample from a batch of material.

Recommended procedures

Sampling of material in bulk
Inspect each container or packaging unit for conformity with pharmacopoeia monographs or other requirements regarding packaging and labelling. Check the condition of the package and note any defects that may influence the quality or stability of the contents (physical damage, moisture, etc.). Sample damaged containers individually.

If initial inspection indicates that the batch is uniform, take samples as follows. When a batch consists of five containers or packaging units, take a sample from each one. From a batch of 6–50 units, take a sample from five. In the case of batches of over 50 units, sample 10%, rounding up the number of units to the nearest multiple of ten. For example, a batch of 51 units would be sampled as for 60, i.e. take samples from six packages.

After opening, inspect the contents of the units selected for sampling for:

- organoleptic characteristics (colour, texture and odour);
- presentation of the material (raw, cut, crushed, compressed);
- the presence of admixtures, foreign matter (sand, glass particles, dirt), mould or signs of decay;
- the presence of insects;
- the presence of packaging material originating from poor or degraded containers.

From each container or package selected, take three original samples, taking care to avoid fragmentation. Samples should be taken from the top, middle and bottom of the container. In the case of sacks and packages, the three samples should be taken by hand, the first from a depth of not less than 10 cm from the top and the second and third from the middle and bottom after cutting into the side of the package. Samples of seeds should be withdrawn with a grain probe. Material in boxes should first be sampled from the upper layer; then approximately half of the contents should be removed and a second sample taken. Finally after further removal of material, another sample should be taken from the bottom. Samples should be as uniform as possible in mass. The three original samples should then be combined into a pooled sample which should be mixed carefully.
The average sample is obtained by quartering. Form the pooled sample, adequately mixed, into an even and square-shaped heap, and divide it diagonally into four equal parts. Take two diagonally opposite parts and mix carefully. Repeat the process as necessary until the required quantity, to within ± 10%, is obtained (100–200 g for flowers and up to 10 kg for certain roots). Any remaining material should be returned to the batch.

Using the same quartering procedure, divide the average sample into four **final samples**, taking care that each portion is representative of the bulk material. The final samples are tested for the following characteristics:

- degree of fragmentation (sieve test);
- identity and level of impurities;
- moisture and ash content;
- level of active ingredients, where possible.

A portion of each final sample should be retained to serve as reference material, which may also be used for re-test purposes, if necessary.

**Sampling of material in retail packages**

From each wholesale container (boxes, cartons, etc.) selected for sampling, take at random two consumer packages. From small batches (1–5 boxes), take ten consumer packages. Prepare the **pooled sample** by mixing the contents of the selected consumer packages and proceed as described above to obtain the **final sample**.

**References**

Annex 3: Determination of arsenic and toxic metals

A. Limit tests

A.1 Limit test for arsenic

Arsenic is abundant in nature and its presence in herbal medicines should be no different to its wide occurrence in foods. A popular test method relies on the digestion of the plant matrix followed by subjection of the digestate to a comparative colorimetric test in a special apparatus.

The test method described below uses colorimetry and does not use toxic mercuric bromide paper. The method uses N,N-diethylmethylthiocarbamate in pyridine and it reacts with hydrogen arsenide to afford a red–purple complex. The limit is expressed in terms of arsenic (III) trioxide (As$_2$O$_3$).

**Apparatus**

Use the apparatus illustrated in Fig. A3.1.

**Figure A3.1 Apparatus for arsenic limit test**

A, generator bottle (capacity up to the shoulder: approximately 70 ml)
B, exit tube
C, glass tube (inside diameter: 5.6 mm, the tip of the part to be inserted in the absorber tube D is drawn out to 1 mm in diameter)
D, absorber tube (inside diameter: 10 mm)
E, small perforation
F, glass wool (about 0.2 g)
G, mark at 5 ml
H and J, rubber stopper

B. Determination of specific toxic metals

B.1 Detection of cadmium, copper, iron, lead, nickel and zinc
B.2 Detection of arsenic and mercury
Place glass wool F in the exit tube B up to about 30 mm in height, moisten the glass wool uniformly with a mixture of an equal volume of lead (II) acetate TS and water, and apply gentle suction to the lower end to remove the excess mixture. Insert the tube vertically into the centre of the rubber stopper H, and attach the tube to the generator bottle A so that the small perforation E in the lower end of B extends slightly below the stopper. At the upper end of B, attach the rubber stopper J to hold the tube C vertically. Make the lower end of the exit tube of C level with that of the rubber stopper J.

**Preparation of the test solution**

Unless otherwise specified, proceed as follows.

*Examples for ginseng, powdered ginseng and red ginseng*

Prepare the test solution with 1.0 g of pulverized ginseng (or red ginseng) according to the method described below, and perform the test using the apparatus described above.

**Method**

Weigh the amount of the sample as directed in the monograph, and place it in a crucible of platinum, quartz or porcelain. Add 10 ml of a solution of magnesium nitrate hexahydrate in ethanol (95) (1 in 10), burn the ethanol, heat gradually, and ignite to incinerate. If carbonized material still remains after this procedure, moisten with a small quantity of nitric acid, and ignite again to incinerate in the same manner. After cooling, add 3 ml of hydrochloric acid, heat in a water bath to dissolve the residue, and designate it as the test solution.

**Standard solutions**

- *Absorbing solution for hydrogen arsenide.* Dissolve 0.50 g of silver N,N-diethyl-dithiocarbamate in pyridine to make 100 ml. Preserve this solution in a glass-stoppered bottle protected from light, in a cold place.
- *Standard arsenic stock solution.* Weigh accurately 0.100 g of finely powdered arsenic (III) trioxide standard reagent dried at 105 °C for 4 hours, and add 5 ml of sodium hydroxide solution (1 in 5) to dissolve. Add dilute sulfuric acid to neutralize, add a further 10 ml of dilute sulfuric acid, and add freshly boiled and cooled water to make exactly 1000 ml.
- *Standard arsenic solution.* Pipette 10 ml of standard arsenic stock solution, add 10 ml of dilute sulfuric acid, and add freshly boiled and cooled water to make exactly 1000 ml. Each ml of the solution contains 1 μg of arsenic (III) trioxide (As₂O₃). Prepare standard arsenic solution just before use and preserve in a glass-stoppered bottle.

**Procedure**

Unless otherwise specified, proceed using the above-mentioned apparatus. Carry out the preparation of the standard colour at the same time.

Place the test solution in the generator bottle A and, if necessary, wash down the solution in the bottle with a small quantity of water. Add 1 drop of methyl orange TS, and after neutralizing with ammonia TS, ammonia solution (NH₄OH), or diluted hydrochloric acid, add 5 ml of diluted hydrochloric acid (1 in 2) add 5 ml of potassium iodide TS, and allow to stand for 2 to 3 minutes. Add 5 ml of acidic tin (II) chloride TS, and allow to stand for 10 minutes. Then add water to make 40 ml, add 2 g of zinc for arsenic analysis, and immediately connect the rubber stopper H fitted
with B and C with the generator bottle A. Transfer 5 ml of the absorbing solution for hydrogen arsenide to the absorber tube D, insert the tip of C to the bottom of the absorber tube D, then immerse the generator bottle A up to the shoulder in water maintained at 25 °C, and allow to stand for 1 hour. Disconnect the absorber tube, add pyridine to make 5 ml, if necessary, and observe the colour of the absorbing solution: the colour produced is not more intense than the standard colour.

*Preparation of standard colour.* Measure accurately 2 ml of Standard Arsenic Solution into the generator bottle A. Add 5 ml of diluted hydrochloric acid (1 in 2) and 5 ml of potassium iodide TS, and allow to stand for 2 to 3 minutes. Add 5 ml of acidic tin (II) chloride TS, allow to stand at room temperature for 10 minutes, and then proceed as directed above. The colour produced corresponds to 2 μg of arsenic (III) trioxide (As₂O₃) and is used as the standard.

**Notes:** Apparatus, reagents and test solutions used in the test should contain little or no arsenic. If necessary, perform a blank determination.

Details of the reagents and solutions are given in Annex 8.

### A.2 Limit test for cadmium and lead

**Procedure**

**Apparatus**

The equipment comprises a digestion vessel, consisting of a vitreous silica crucible (DIN 12904), “tall form”, height 62 mm, diameter 50 mm, capacity 75 ml, with a vitreous silica cover.

The materials used are:

- *digestion mixture:* 2 parts by weight of nitric acid (~1000 g/l) TS and 1 part by weight of perchloric acid (~1170 g/l) TS.
- *reference materials:* olive leaves (*Olea europaea*)¹ and hay powder.²

Clean scrupulously with nitric acid (~1000 g/l) TS the digestion vessel and all other equipment to be used for the determination, rinse thoroughly several times with water and dry at 120 °C.

**Preparation of the sample**

For the wet digestion method in an open system, place 200–250 mg of air-dried medicinal plant material, accurately weighed, finely cut and homogeneously mixed, into a cleaned silica crucible. Add 1.0 ml of the digestion mixture, cover the crucible without exerting pressure and place it in an oven with a controlled temperature and time regulator (computer-controlled, if available).

Heat slowly to 100 °C and maintain at this temperature for up to 3 hours; then heat to 120 °C and maintain at this temperature for 2 hours. Raise the temperature very

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¹ BCR reference material CRM No. 62 Community Bureau of Reference, obtainable from BCR, Directorate-General XII, Commission of the European Communities, 200 rue de la Loi, B-1049 Brussels, Belgium.
² Obtainable from IAEA/V-10, International Atomic Energy Agency, Analytical Quality Control Services, Laboratory Geibersdorf, PO Box 100, A-Vienna, Austria.
slowly to 240 °C, avoiding losses due to possible violent reactions, especially in the temperature range of 160–200 °C, and maintain at this temperature for 4 hours. Dissolve the remaining dry inorganic residue in 2.5 ml of nitric acid (~1000 g/l) TS and use for the determination of heavy metals.

Every sample should be tested in parallel with a blank.

**Method**
The contents of lead and cadmium may be determined by inverse voltammetry or by atomic absorption spectrophotometry.

**A.3 Limit test for total toxic metals as lead**

In this method, the heavy metals are the metallic inclusions that are darkened with sodium sulfide TS in acidic solution; their quantity is expressed in terms of the quantity of lead (Pb).

**Preparation of sample solution and blank solution**

*Test solution.* Place an amount of the sample, as directed in the monograph, in a quartz or porcelain crucible, cover loosely with a lid, and carbonize by gentle ignition. After cooling, add 2 ml of nitric acid and 5 drops of sulfuric acid, heat cautiously until white fumes are evolved, and incinerate by ignition between 500 °C and 600 °C. Cool, add 2 ml of hydrochloric acid, evaporate to dryness in a water-bath, moisten the residue with 3 drops of hydrochloric acid, add 10 ml of hot water, and warm for 2 minutes. Then add 1 drop of phenolphthalein TS, add ammonia TS drop by drop until the solution develops a pale red colour, add 2 ml of dilute acetic acid, filter, if necessary, and wash with 10 ml of water. Transfer the filtrate and washings to a Nessler tube, and add water to make 50 ml. Designate this as the test solution.

*Control solution.* Evaporate a mixture of 2 ml of nitric acid, 5 drops of sulfuric acid, and 2 ml of hydrochloric acid on a water-bath, further evaporate to dryness on a sand-bath, and moisten the residue with 3 drops of hydrochloric acid. Hereinafter, proceed as directed above for the test solution, and then add the volume of standard lead solution as directed in the monograph and sufficient water to make 50 ml.

**Procedure**

Add 1 drop of sodium sulfide TS both to the test solution and to the control solution, mix thoroughly, and allow to stand for 5 minutes. Then compare the colours of the two solutions by viewing the tubes downwards or transversely against a white background. The test solution has no more colour than the control solution.

**A.4 Limit test for total toxic metals as lead in extracts**

*Test solution.* Ignite 0.3 g of extracts to ash, warm with 3 ml of dilute hydrochloric acid, and filter. Wash the residue with two 5 ml portions of water. Neutralize the combined filtrate and washings by adding ammonia TS, filter, if necessary, and add 2 ml of dilute acetic acid and water to make 50 ml. Perform the heavy metals limit test using this solution as the test solution.

*Control solution.* Proceed with 3 ml of dilute hydrochloric acid in the same manner as directed above for the preparation of the test solution, and add 3 ml of standard lead solution 1 ppm, and water to make 50 ml.
Procedure
Add 1 drop of sodium sulfide TS to both the test solution and to the control solution, mix thoroughly, and allow to stand for 5 minutes. Then compare the colours of the two solutions by viewing the tubes downwards or transversely against a white background. The test solution has no more colour than the control solution.

B. Determination of specific toxic metals

Atomic absorption spectrometry (AA) is used for the determination of the amount or concentration of specific heavy metals. AA uses the phenomenon that atoms in the ground state absorb light of a specific wavelength, characteristic of the particular atom, when the light passes through an atomic vapour layer of the element to be determined.

Caution must be exercised when using the recommended closed high-pressure digestion vessels and microwave laboratory equipment, and the operators should be fully familiar with the safety and operating instructions given by the manufacturer.

Procedure

Apparatus
Usually the apparatus consists of a light source, a sample atomizer, a spectroscope, a photometer and a recording system, together with the following:

- A digestion flask: a polytetrafluoroethylene flask with a volume of about 120 ml, fitted with an airtight closure, a valve to adjust the pressure inside the container and a polytetrafluoroethylene tube to allow release of gas. A good example is the Digestion Vessel Assembly P/N ZZ 1000.
- A system for making flasks airtight, using the same torsional force. One example is the CEM Capping station calibration.
- A microwave oven, with a magnetron frequency of 2450 MHz, with a selectable output from 0 to 630 ± 70 W in 1% increments, a programmable digital computer, a polytetrafluoroethylene-coated microwave cavity with a variable speed exhaust fan, a rotating turntable drive system and exhaust tubing to vent fumes.
- An atomic absorption spectrometer, equipped with an appropriate lamp for each element as source of radiation and a deuterium lamp as background corrector; the system is fitted with a sample atomiser of which there are three types: the flame type, the electrochemical type and the cold-vapour type.
- a graphite furnace (electrochemical type) is used as an atomisation device for cadmium, copper, iron, lead, nickel and zinc. One example is the Vapour Generation Accessory.
- an automated continuous-flow hydride vapour generation system is used for arsenic and mercury.¹

¹ The Vapour Generation Accessory is suitable.
Method
Clean all the glassware and laboratory equipment with a 10 g/l solution of nitric acid R in water, carbon-dioxide free R before use.

Test solution. In a digestion flask, place the prescribed quantity of the substance to be examined (about 0.5 g of powdered drug or 0.5 g of fatty oil). Add 3 ml of nitric acid R, 1 ml hydrogen peroxide R and 1 ml of hydrochloric acid R. Seal the flask so that it is airtight. Place the digestion flasks in the microwave oven. Carry out the digestion in 3 steps according to the following procedure, for 7 flasks each containing the test solution: 80% power for 15 min; 100% power for 5 min; then 80% power for 20 min. At the end of the cycle allow the flasks to cool in air and to each add 4 ml of sulfuric acid R. Repeat the digestion programme. After cooling in air, open each digestion flask and introduce the clear, colourless solution obtained into a 50-ml volumetric flask. Rinse each digestion flask with 2 quantities, each of 15 ml, of water R and collect the rinsings in the volumetric flask. Add 1 ml of 10 g/l solution of magnesium nitrate R and 1 ml of 100 g/l solution of ammonium dihydrogen phosphate R and dilute to 50 ml with water R.

Blank solution. Mix 3 ml of nitric acid R, 1 ml hydrogen peroxide R (30%) and 1 ml of hydrochloric acid R in a digestion flask. Carry out the digestion in the same manner as for the test solution.

B.1 Detection of cadmium, copper, iron, lead, nickel and zinc
Measure the content of (Cd), copper (Cu), iron (Fe), lead (Pb), nickel (Ni) and zinc (Zn) by the standard additions method using reference solutions of each heavy metal. Suitable instrumental parameters are listed in Table A3.1.

The absorbance value of the compensation liquid (blank solution) is subtracted from the value obtained with the test solution.

| Table A3.1. Instrumental parameters for heavy metals |
|---------------------------------|-----|-----|-----|-----|-----|-----|
|                                | Cd  | Cu  | Fe  | Ni  | Pb  | Zn  |
| Wavelength                     | nm  | 228.8| 324.8| 248.3| 232 | 283.5| 213.9|
| Slit width                     | nm  | 0.5 | 0.5 | 0.2 | 0.2 | 0.5 | 0.5 |
| Hollow-cathode lamp current    | mA  | 6   | 7   | 5   | 10  | 5   | 7   |
| Ignition temperature          | °C  | 800 | 800 | 800 | 800 | 800 | 800 |
| Atomization temperature       | °C  | 1800| 2300| 2300| 2500| 2200| 2000|
| Background corrector          |     | on  | on  | on  | on  | on  | on  |
| Nitrogen flow                 | Liter/min | 3   | 3   | 3   | 3   | 3   | 3   |

B.2 Detection of arsenic and mercury
Measure the content of arsenic (As) and mercury (Hg) in comparison with reference solutions containing these elements at a known concentration by direct calibration using an automated continuous-flow hydride vapour generation system.

The absorbance value of the compensation liquid (blank solution) is automatically subtracted from the value obtained with the test solution.
**Arsenic**

*Sample solution.* To 19 ml of the test solution or of the blank solution as described above, add 1 ml of a 200 g/l solution of potassium iodide R. Allow the test solution to stand at room temperature for about 50 min or at 70 °C for about 4 min.

*Acid reagent.* Heavy metal-free hydrochloric acid R.

*Reducing reagent.* A 6 g/l solution of sodium tetrahydroborate R in a 5 g/l solution of sodium hydroxide R.

The instrumental parameters in Table A3.2 may be used.

**Mercury**

*Sample solution.* Test solution or blank solution, as described above.

*Acid reagent.* A 515 g/l solution of heavy metal-free hydrochloric acid R.

*Reducing reagent.* A 10 g/l solution of stannous chloride R or sodium tetrahydroborate in dilute hydrochloric acid R.

The instrumental parameters in Table A3.2 may be used.

**Table A3.2. Instrumental parameters for determination of arsenic and mercury**

<table>
<thead>
<tr>
<th></th>
<th>As</th>
<th>Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength  nm</td>
<td>193.7</td>
<td>253.7</td>
</tr>
<tr>
<td>Slit width  nm</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Hollow-cathode lamp current mA</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Acid reagent flow rate ml/min</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Reducing reagent flow rate ml/min</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Sample solution flow rate ml/min</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Absorption cell</td>
<td>Quartz (heated)</td>
<td>Quartz (unheated)</td>
</tr>
<tr>
<td>Background corrector</td>
<td>on</td>
<td>on</td>
</tr>
<tr>
<td>Nitrogen flow rate litre/min</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Heating °C</td>
<td>800</td>
<td>100</td>
</tr>
</tbody>
</table>

Annex 3
Annex 4: Determination of aflatoxins

Whenever testing for aflatoxins is required this should be done after using a suitable clean-up procedure during which great care should be taken not to expose any personnel or the working or general environment to these dangerous and toxic substances. Thus Member States should adapt their good practices for national pharmaceutical control laboratories and GMP accordingly. Only products that have a history of aflatoxin contamination need to be tested.

Tests for aflatoxins

These tests are designed to detect the possible presence of aflatoxins B₁, B₂, G₁, and G₂, which are highly toxic contaminants in any material of plant origin.

Test method

The method described below does not require the use of toxic solvents, such as chloroform and dichloromethane. It uses a multifunctional column, which contains lipophilic and charged active sites, and high-performance liquid chromatography (HPLC) using fluorescence detection to determine aflatoxins B₁, B₂, G₁, and G₂. The advantages of employing a multifunctional column are:

- high total recoveries of aflatoxins B₁, B₂, G₁, and G₂ (more than 85%); and
- the column can be kept (stocked) at room temperature and for a fairly long time prior to use.

Standard solutions of aflatoxin B₁, B₂, G₁ and G₂ (2.5 ng/ml)

Stock standard solution: weigh exactly 1.0 mg each of crystalline material of aflatoxins B₁, B₂, G₁, and G₂ and dissolve in 50 ml of toluene-acetonitrile (9:1) solution by shaking vigorously in a glass flask to obtain a standard stock solution (20 μg/ml). This standard solution should be kept in a tightly sealed container, covered with aluminium foil, and kept in a refrigerator at 4 °C in the dark.

Working standard solution: 0.5 ml of stock standard solution is added to toluene-acetonitrile (9:1) solution to give 200 ml (working standard solution (50 ng/ml).

Standard solution: Take 1.0 ml of working standard solution and add to toluene-acetonitrile (9:1) solution to give 20 ml (final standard solution (2.5 ng/ml).

Standard solution for liquid chromatography analysis

Transfer 0.25 ml of the final standard solution (as described above) into a glass centrifuge tube and evaporate to dryness at 40 °C or by using a nitrogen air stream. To derivatize aflatoxins B₁ and G₁ (precolumn derivatization), add 0.1 ml

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1 BCR reference material CRM No. 62 Community Bureau of Reference, obtainable from BCR, Directorate-General XII, Commission of the European Communities, 200 rue de la Loi, B-1049 Brussels, Belgium.
of trifluoroacetic acid (TFA) solution to the residue in the tube, tightly seal the tube and shake vigorously. Allow the tube to stand at room temperature for 15 min in the dark. Add 0.4 ml of acetonitrile-water (1:9) solution to the tube. A 20-μl portion of the sample solution in the tube is subjected to liquid chromatography analysis.

**Preparation of sample**

Grind the medicinal plant material for testing to a uniform consistency using a coffee mill, and extract a 50-g test sample with 400 ml of acetonitrile-water (9:1) by shaking vigorously in a glass flask fitted with a stopper for 30 min or by using a mechanical blender for 5 min. Filter the solution through a filter paper or centrifuge. Transfer a 5-ml portion of the filtrate, or the top clean layer, to a multifunctional column (such as a MultiSep #228 cartridge column (Romer Labs) or an Autoprep MF-A (Showa-denko)) and pass through at a flow rate of 1 ml/min. The aflatoxins present in a sample are passed through the column as the first eluate. Obtain the first 1-ml of the eluate as the test solution.

Evaporate 0.5 ml of the test solution in a glass centrifuge tube to dryness at 40 °C or by using a nitrogen air stream to remove solvent.

To derivatize aflatoxins B<sub>1</sub> and G<sub>1</sub> (precolumn derivatization), add 0.1 ml of trifluoroacetic acid (TFA) solution to the residue in the tube, tightly seal the tube and shake vigorously. Allow the tube to stand at room temperature for 15 min in the dark. Add 0.4 ml of acetonitrile-water (1:9) solution to the tube. Subject a 20-μl portion of the sample solution in the tube to liquid chromatography analysis.

**Method**

**Liquid chromatography conditions**

The mobile phase is acetonitrile-methanol-water (1:3:6). De-gas the mobile phase by sonication. Connect an octadecyl-silica gel (ODS) column (4.6 mm inner diameter (ID) × 250 mm, 3–5 μm), such as Inertsil ODS-3 (4.6 mm ID × 250 mm, 3 μm) as the liquid chromatography column. Maintain the column at 40 °C with a flow rate of 1 ml/min. The aflatoxin and its derivatives are detected at the excitation and emission wavelengths of 365 nm and 450 nm, respectively. The injection volume is 20 μl.

If an impurity peak overlaps the peaks corresponding to aflatoxins, the alternative liquid chromatography conditions, described below, are recommended.

**Alternative liquid chromatography conditions**

The mobile phase is methanol-water (3:7). De-gas the mobile phase by sonication. Connect a fluorocarbonated column, such as Wako-pack Fluofix 120E (4.6 mm ID × 250 mm, 5 μm) as the liquid chromatography column. Maintain the column at 40 °C with a flow rate of 1 ml/min. The aflatoxin and its derivatives are detected at the excitation and emission wavelengths of 365 nm and 450 nm, respectively. The injection volume is 20 μl.

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1 If the sample solution contains a lot of impurity, the column should be washed by acetonitrile for 5–10 min and reconditioned with the mobile phase for 10 min before the next analysis.
**Interpretation of the results**

Compare the retention time of peak area or peak heights of the aflatoxin under study in the chromatograms: if they are bigger or higher than those obtained in a standard solution of the aflatoxin under investigation, it should be regarded as a positive result for the presence of aflatoxin in the sample solution.
Annex 5: Determination of microorganisms

A. Total viable aerobic count

The total viable aerobic count (TVC) of the herbal material being examined is determined, as specified in the test procedure below, using one of the following methods: membrane-filtration, plate count or serial dilution. Aerobic bacteria and fungi (moulds and yeasts) are determined by the TVC.

Usually a maximum permitted level is set for certain products, but when the TVC exceeds this level then it is unnecessary to proceed with determination of specific organisms; the material should be rejected without being subjected to further testing.

A.1 Pretreatment of the test herbal material

Depending on the nature of the crude medicinal plant material, grind, dissolve, dilute, suspend or emulsify it using a suitable method and eliminate any antimicrobial properties by dilution, neutralization or filtration. Either phosphate buffer pH 7.2; buffered sodium chloride-peptone solution, pH 7.0; or fluid medium, used for the test, is used to suspend or dilute the test specimen.

Some materials have special requirements, which have to be met for acceptable pretreatment to be performed. Some examples are as follows:
A.1.1 Materials containing tannins, antimicrobial substances
Some herbal preparations present difficulties in determining levels of microbes, e.g., those containing high contents of tannins or essential oils. When test specimens have antimicrobial activity or contain antimicrobial substances, any such antimicrobial properties are removed as mentioned above. For the test, use a mixture of several portions selected at random from the bulk or from the contents of a sufficient number of containers. If the test specimens are diluted with fluid medium, the test should be performed quickly.

A.1.2 Water-soluble materials
Dissolve or dilute 10 g or 10 ml of plant material, unless otherwise specified in the test procedure for the material concerned, in lactose broth or another suitable medium proven to have no antimicrobial activity under the conditions of the test. Adjust the volume to 100 ml with the same medium. (Note that some materials may require the use of larger volumes.) If necessary, adjust the pH of the suspension to about 7.

A.1.3 Non-fatty materials insoluble in water
Suspend 10 g or 10 ml of the plant material, unless otherwise specified in the test procedure for the material concerned, in lactose broth or another suitable medium proven to have no antimicrobial activity under the conditions of the test; dilute to 100 ml with the same medium. (Note that some materials may require the use of a larger volume.) If necessary, divide the material and homogenize the suspension mechanically. A suitable surfactant, such as a solution of polysorbate 20 R or 80 R containing 1 mg per ml may be added to aid dissolution. If necessary, adjust the pH of the suspension to about 7.

A.1.4 Fatty materials
Homogenize 10 g or 10 ml of material, unless otherwise specified in the test procedure for the material concerned, with 5 g of polysorbate 20 R or 80 R. If necessary, heat to a temperature not exceeding 40 °C. (Occasionally, it may be necessary to heat to a temperature of up to 45 °C, for the shortest possible time.) Mix carefully while maintaining the temperature in a water-bath or oven. Add 85 ml of lactose broth or another suitable medium proven to have no antimicrobial activity under the conditions of the test, if necessary heated to a temperature not exceeding 40 °C. Maintain this temperature for the shortest time necessary until an emulsion is formed and, in any case, for not more than 30 minutes. If necessary, adjust the pH of the emulsion to about 7.

A.2 Test procedures
Plate count
For bacteria use Petri dishes 9–10 cm in diameter. To one dish add a mixture of 1 ml of the pre-treated plant material and about 15 ml of liquefied casein-soybean digest agar at a temperature not exceeding 45 °C. Alternatively, spread the material on the surface of the solidified medium in a Petri dish. If necessary, dilute the material to obtain an expected colony count of not more than 300. Prepare at least two dishes using the same dilution, invert them and incubate them at 30–35 °C for 48–72 hours, unless a more reliable count is obtained in a shorter period of time. Count the number of colonies formed and calculate the results using the plate with the largest number of colonies, up to a maximum of 300.
For fungi use Petri dishes 9–10 cm in diameter. To one dish add a mixture of 1 ml of the pretreated material and about 15 ml of liquefied Sabouraud glucose agar with antibiotics (also used is potato dextrose agar with antibiotics) at a temperature not exceeding 45 °C. Alternatively, spread the pretreated material on the surface of the solidified medium in a Petri dish. If necessary, dilute the material as described above to obtain an expected colony count of not more than 100. Prepare at least two dishes using the same dilution and incubate them upright at 20–25 °C for 5 days, unless a more reliable count is obtained in a shorter period of time. Count the number of colonies formed and calculate the results using the dish with not more than 100 colonies.

Membrane filtration
Use membrane filters with a nominal pore size of not greater than 0.45 μm, and with a proven effectiveness at retaining bacteria, e.g. cellulose nitrate filters are used for aqueous, oily and weakly alcoholic solutions, whereas cellulose acetate filters are better for strongly alcoholic solutions. The technique below uses filter discs of about 50 mm in diameter. Where filters of a different diameter are used, adjust the volumes of the dilutions and washings accordingly. Sterilize, by appropriate means, the filtration apparatus and the membrane, as the solution is introduced, filtered and examined under aseptic conditions, and the membrane is then transferred to the culture medium.

The detailed method
Transfer 10 ml or a solution containing 1g of the material to each of two membrane filter apparatuses and filter immediately. If necessary, dilute the pretreated material to obtain an expected colony count of 10–100. Wash each membrane, filtering three or more successive quantities of approximately 100 ml of a suitable liquid such as buffered sodium chloride-peptone solution at pH 7.0. For fatty materials, a suitable surfactant may be added, such as Polysorbate 20 R or 80 R. Transfer one of the membrane filters, intended primarily for the enumeration of bacteria, to the surface of a plate with soybean-casein digest agar and the other, intended primarily for the enumeration of fungi, to the surface of a plate with Sabouraud glucose agar with antibiotics. Incubate the plates for 5 days, unless a more reliable count can be obtained otherwise, at 30–35 °C for the detection of bacteria and at 20–25 °C for the detection of fungi. Count the number of colonies formed. Calculate the number of microorganisms per gram or per ml of the material tested, if necessary counting bacteria and fungi separately.

Serial dilution
Prepare a series of 12 tubes each containing 9–10 ml of soybean-casein digest medium. To each of the:

- first group of three tubes, add 1 ml of the 1:10 dilution of dissolved, homogenized material (containing 0.1 g or 0.1 ml of specimen) prepared as described later in these guidelines (see section B.2);
- second group of three tubes, add 1 ml of a 1:100 dilution of the material;
- third group of three tubes, add 1 ml of a 1:1000 dilution of the material; and to the
- last three tubes, add 1 ml of the diluent.

Incubate the tubes at 30–35 °C for at least 5 days. No microbial growth should appear in the last three tubes. If the reading of the results is difficult or uncertain,
owing to the nature of the material being examined, prepare a subculture in a liquid or a solid medium, and evaluate the results after a further period of incubation. Determine the most probable number of microorganisms per gram or per ml of the material using Table A5.1.

If, for the first column, the number of tubes showing microbial growth is two or less, the most probable number of microorganisms per g or per ml is less than 100 (Table A5.1).

Table A5.1. Determination of total viable aerobic count

<table>
<thead>
<tr>
<th>Number of tubes with microbial growth*</th>
<th>Most probable number of microorganisms per g or ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg or 0.1 ml per tube</td>
<td>10 mg or 0.01 ml per tube</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

* Amounts in mg or ml are quantities of original plant material.

A.3 Effectiveness of the culture medium, confirmation of antimicrobial substances and validity of the counting method

The following strains are normally used (see also Annex 7):

- **Staphylococcus aureus** NCIMB 8625 (ATCC 6538-P, CIP 53.156) or NCIMB 9518 (ATCC 6538, CIP 4.83, IFO 13276)
- **Bacillus subtilis** NCIMB 8054 (ATCC 6633, CIP 52.62, IFO 3134)
- **Escherichia coli** NCIMB 8545 (ATCC 8739, CIP 53.126, IFO 3972)
- **Candida albicans** ATCC 2091 (CIP 1180.79, IFO 1393) or ATCC 10 231 (NCPF 3179, CIP 48.72, IFO 1594)
- **Clostridia botulinum** ATCC 19297 (NCTC 7272)
- **Clostridium perfringens** ATCC 13124 (NCTC 8239)
- **Clostridium tetani** ATCC e19406 (NCTC 279)

Allow the test strains to grow separately in tubes containing soybean-casein digest medium at 30–35 °C for 18–24 hours for aerobic bacteria and between 20–25 °C for...
Candida albicans, for 48 hours. (Antibiotics are often added to the culture medium to attain a particular selectivity.)

Dilute portions of each of the cultures using buffered sodium chloride-peptone solution, pH 7.0 or phosphate buffer, pH 7.2 to prepare test suspensions containing 50–200 viable colony forming units (cfu) (microorganisms) per ml. Growth-promoting qualities are tested by inoculating 1 ml of each microorganism into each medium. The test media are satisfactory if clear evidence of growth appears in all the inoculated media after incubation at the indicated temperature for 5 days. When a count of test organisms with a test specimen is less than one-fifth of that without the test specimen, any such effect must be eliminated by dilution, filtration, neutralization or inactivation.

To confirm the sterility of the medium and of the diluent and the aseptic performance of the test, follow the TVC method using sterile buffered sodium chloride-peptone solution, pH 7.0, or phosphate buffer, pH 7.2, as a control. There should be no growth of microorganisms.

To validate the method, a count for the test organism should be obtained differing by not more than a factor of 10 from the calculated value for the inoculum.

B. Tests for specific microorganisms

Microbial tests should be applied to starting plant materials, intermediate and finished products where necessary. Enterobacteria and certain other Gram-negative bacteria Escherichia coli, Salmonella and Staphylococcus aureus are included as target strains of the test.

The conditions of the tests for microbial contamination are designed to minimize accidental contamination of the materials being examined and the precautions taken must not adversely affect any microorganisms that could be revealed.

B.1 Pretreatment of the material being examined

Refer to the sampling and preparation of the test solution in TVC (see section A.2), including the elimination of any antimicrobial substances which may be present.

B.2 Test procedure for the Enterobacteriaceae and certain other Gram-negative bacteria

Detection of bacteria

Homogenize the pretreated material appropriately and incubate at 30–37 °C for a length of time sufficient for revivification of the bacteria, but not sufficient for multiplication of the organisms (usually 2–5 hours). Shake the container, transfer aliquots equivalent to 1 g or 1 ml of the homogenized material to 100 ml of Enterobacteriaceae enrichment broth Mossel and incubate at 35–37 °C for 18–48 hours. Prepare a subculture on a plate with violet-red bile agar with glucose and lactose. Incubate at 35–37 °C for 18–48 hours. The material passes the test if no growth of colonies of Gram-negative bacteria is detected on the plate.
**Quantitative evaluation**

Inoculate a suitable amount of *Enterobacteriaceae enrichment broth Mossel* with quantities of homogenized material prepared as described under the above section on “Detection of bacteria”, appropriately diluted as necessary, to contain 1 g, 0.1 g and 10 μg, or 1 ml, 0.1 ml and 10 μl, of the material being examined. Incubate at 35–37 °C for 24–48 hours. Prepare a subculture of each of the cultures on a plate with *violet-red bile agar with glucose and lactose* in order to obtain selective isolation. Incubate at 35–37 °C for 18–24 hours. The growth of well-developed colonies, generally red or reddish in colour, of Gram-negative bacteria constitutes a positive result. Note the smallest quantity of material that gives a positive result. Determine the probable number of bacteria using Table A5.2.

**Table A5.2. Determination of Enterobacteriaceae and certain other Gram-negative bacteria**

<table>
<thead>
<tr>
<th>Result for each quantity or volume</th>
<th>Probable number of bacteria per g of material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 g or 1.0 ml</td>
<td>0.1 g or 0.1 ml</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Escherichia coli**

Transfer a quantity of the homogenized material in *lactose broth*, prepared and incubated as described above, and containing 1 g or 1 ml of the material being examined, to 100 ml of *MacConkey broth* and incubate at 43–45 °C for 18–24 hours.

Prepare a subculture on a plate with *MacConkey agar* and incubate at 43–45 °C for 18–24 hours. Growth of red, generally non-mucoid colonies of Gram-negative rods, sometimes surrounded by a reddish zone of precipitation, indicates the possible presence of *E. coli*. This may be confirmed by the formation of indole at 43.5–44.5 °C or by other biochemical reactions. The material passes the test if no such colonies are detected or if the confirmatory biochemical reactions are negative.

**Salmonella spp.**

Incubate the solution, suspension or emulsion of the pretreated material prepared as described above at 35–37 °C for 5–24 hours, as appropriate for enrichment.

**Primary test**

Transfer 10 ml of the enrichment culture to 100 ml of *tetrathionate bile brilliant green broth* and incubate at 42–43 °C for 18–24 hours. Prepare subcultures on at least two of the following three agar media: *deoxycholate citrate agar; xylose, lysine, deoxycholate agar;* and *brilliant green agar*. Incubate at 35–37 °C for 24–48 hours. Carry out the secondary test if any colonies are produced that conform to the description given in Table A5.3.

**Secondary test**

Prepare a subculture of any colonies showing the characteristics described in Table A5.3 on the surface of *triple sugar iron agar* using the deep inoculation technique. This is done by first inoculating the inclined surface of the culture medium, followed by a stab culture with the same inoculating needle and then,
incubating at 35–37 °C for 18–24 hours. The test is positive for the presence of Salmonella spp. if a change of colour from red to yellow is observed in the deep culture (but not in the surface culture), usually with the formation of gas with or without production of hydrogen sulfide in the agar. Confirmation is obtained by appropriate biochemical and serological tests.

The material being examined passes the test if cultures of the type described do not appear in the primary test, or if the confirmatory biochemical and serological tests are negative.

Table A5.3. Description of Salmonella colonies appearing on different culture media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Description of colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxycholate citrate agar</td>
<td>Well-developed, colourless</td>
</tr>
<tr>
<td>Xylose, lysine, deoxycholate agar</td>
<td>Well-developed, red, with or without black centres</td>
</tr>
<tr>
<td>Brilliant green agar</td>
<td>Small, transparent and colourless, or opaque, pink or white (frequently surrounded by a pink to red zone)</td>
</tr>
</tbody>
</table>

*Pseudomonas aeruginosa*

Pretreat the material being examined as described under A.1, but using buffered sodium chloride-peptone solution, pH 7.0, or another suitable medium shown not to have antimicrobial activity under the conditions of the test, in place of lactose broth. Inoculate 100 ml of *soybean-casein digest medium* with a quantity of the solution, suspension or emulsion thus obtained containing 1 g or 1 ml of the material being examined. Mix and incubate at 35–37 °C for 24–48 hours. Prepare a subculture on a plate of *cetrimide agar* and incubate at 35–37 °C for 24–48 hours. If no growth of microorganisms is detected, the material passes the test. If growth of colonies of Gram-negative rods occurs, usually with a greenish fluorescence, apply an oxidase test and test the growth in *soybean-casein digest medium* at 42 °C. The following method may be used. Place 2 or 3 drops of a freshly prepared 0.01 g/ml solution of *N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride R* on a filter paper and apply a smear of the suspected colony; the test is positive if a purple colour is produced within 5–10 seconds. The material passes the test if cultures of the type described do not appear or if the confirmatory biochemical test is negative.

*Staphylococcus aureus*

Prepare an enrichment culture as described for *Pseudomonas aeruginosa*. Prepare a subculture on a suitable medium such as *Baird-Parker agar*. Incubate at 35–37 °C for 24–48 hours. The material passes the test if no growth of microorganisms is detected. Black colonies of Gram-positive cocci often surrounded by clear zones may indicate the presence of *Staphylococcus aureus*. For catalase-positive cocci, confirmation may be obtained, for example, by coagulase and deoxyribonuclease tests. The material passes the test if cultures of the type described do not appear or if the confirmatory biochemical test is negative.

*Clostridium spp.*

Add 10 g (10 ml) of the herbal materials, preparation or product to be examined to two suitable vessels, each containing 100 ml of *cooked-meat medium*, heated just prior to use, to 100 °C for a few minutes and cooled to 37 °C. To distinguish between sporing and non-sporing organisms, immediately seal one vessel with
a layer of sterile paraffin or agar, heat the other vessel at 65 °C for 30 minutes, and then similarly seal.

Incubate both vessels at 35–37 °C and examine every 24 hours for up to 4 days. Growth of sporing organisms occurs in the vessel which was heated after inoculation.

If no growth occurs in either of the vessels, the sample passes the test for absence of Clostridia and other anaerobic bacteria.

If sporing anaerobic organisms (Table A5.4) are found, inoculate the cultures, each in duplicate, on one half of the surface of plates containing 5% defibrinated sheep blood agar medium. Incubate at 37 °C for 48 hours, one plate anaerobically and the other aerobically, to check that the organisms will not grow under aerobic conditions.

**Table A5.4. Characteristics of Clostridium species on cooked-meat medium**

<table>
<thead>
<tr>
<th>Clostridium botulinum</th>
<th>Clostridium perfringens</th>
<th>Clostridium tetani</th>
</tr>
</thead>
<tbody>
<tr>
<td>No digestion of meat; much gas, white sediment</td>
<td>No digestion of meat; meat turns pink colour</td>
<td>No digestion of meat; burnt organic smell</td>
</tr>
</tbody>
</table>

After 24 and 48 hours examine the appearance of the colonies together with the type and extent of haemolysis, and also examine microscopically for spore formation using Gram stain or spore stain techniques. Match the result with the description in Table A5.5, for further identification of specified clostridia.

**Table A5.5. Characteristics of Clostridium species on 5% defibrinated sheep blood agar medium**

<table>
<thead>
<tr>
<th></th>
<th>Clostridium botulinum</th>
<th>Clostridium perfringens</th>
<th>Clostridium tetani</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonies</td>
<td>Irregular, translucent with a granular surface and indefinite fimbriated spreading edge</td>
<td>Large, circular, convex, semitranslucent, smooth with an entire edge</td>
<td>Transparent with long feathery spreading projections</td>
</tr>
<tr>
<td>Haemolysis</td>
<td>+</td>
<td>Double zone</td>
<td>+</td>
</tr>
<tr>
<td>Spores</td>
<td>Oval, central, subterminal distended bacilli</td>
<td>Absent</td>
<td>Spherical and terminal (drum stick)</td>
</tr>
</tbody>
</table>

**Shigella**

The method described below is adapted from the WHO guidelines for the control of epidemics due to *Shigella dysenteriae* type 1.

**Direct inoculation of agar plates**

Use 2 or 3 loopfuls of the herbal materials, preparations or products to be tested. Incubate plates at 35–37 °C for 18–24 hours.

Inoculate a general purpose plating medium of low selectivity and one of moderate or high selectivity. *MacConkey agar* is recommended as a medium of low selectivity. *MacConkey agar* with 1 mcg/ml of potassium tellurite has been reported to be particularly useful for *S. dysenteriae* type 1 (Sd1). Use a small inoculum. Incubate at 35–37 °C for 18–24 hours.
Xylose-lysine-desoxycholate (XLD) agar is recommended as a medium of moderate or high selectivity for isolation of *Shigella*. Desoxycholate citrate agar (DCA) is a suitable alternative.

*Note:* Do not use salmonella–shigella (SS) agar, as it often inhibits growth of Sd1.

Each new batch of medium should be controlled for quality before routine use by inoculating it with known reference strains and observing their growth and colony characteristics.

**Identification of colonies on plating media**
Colonies suspicious for *Shigella* will appear as follows:

- MacConkey agar: convex, colourless, 2–3 mm
- XLD agar: red, smooth, 1–2 mm
- DCA agar: colourless, translucent, 2–3 mm

Identify well-separated colonies of typical appearance to be transferred from each of the plating media for further testing by making a mark on the bottom of the Petri dish.

Whenever possible a person experienced in the identification of *Shigella* should train laboratory workers who are unfamiliar with its identification.

**Inoculation of Kligler iron agar**
Pick three characteristic colonies from the plating media and inoculate into Kligler iron agar (KIA) as follows: stab the butt and then streak the slant with a zigzag configuration. Pay attention to proper labelling of the tubes. If screw-cap KIA tubes are used, make sure that the caps are loose. Incubate overnight. On the following morning, examine the reactions in the KIA tubes. Tubes suspicious for *Shigella* will have an acid (yellow) butt and an alkaline (red) slant. They will not produce gas (no bubbles or cracks in the agar) and will not produce hydrogen sulfide (no black along the stab line).

**Triple sugar iron agar** (TSI) can also be used for the identification of *Shigella*. It will give the same reactions as KIA.

**B.3 Validation of the tests for specific microorganisms**
If necessary, grow separately the test strains listed in Table A5.6 on the culture media indicated, at 30–35 °C for 18–24 hours. Dilute portions of each of the cultures using buffered sodium chloride-peptone solution pH 7.0 so that the test suspensions contain about 103 microorganisms per ml. Mix equal volumes of each suspension and use 0.4 ml (approximately 102 microorganisms of each strain) as an inoculum in tests for *Escherichia coli*, *Salmonella* spp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*, in the presence of the material being examined, if necessary. The test method should give a positive result for the respective strain of microorganism.
Table A5.6. Validation of tests for detection of specific microorganisms in the herbal sample

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Strain number*</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>e.g. NCIMB 8545 (ATCC 8739, CIP 53.126, IFO 3972)</td>
<td>lactose broth</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>e.g. NCIMB 8626 (ATCC 9027, CIP 82.118)</td>
<td>soybean-casein digest medium</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>No strain number is recommended. Species not pathogenic for humans, such as <em>Salmonella abony</em> (NCTC 6017, CIP 80.39), may be used</td>
<td>lactose broth</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>e.g. ATCC 19297 (NCTC 7272)</td>
<td>cooked-meat medium</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>e.g. ATCC 13124 (NCTC 8239)</td>
<td>cooked-meat medium</td>
</tr>
<tr>
<td><em>Clostridium tetani</em></td>
<td>e.g. ATCC e19406 (NCTC 279)</td>
<td>cooked-meat medium</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>e.g. NCIMB 8625 (ATCC 6538 P, CIP 53.156) or NCIMB 9518 (ATCC 6538, CIP 4.83, IFO 13276)</td>
<td>soybean-casein digest medium</td>
</tr>
</tbody>
</table>

* See Annex 7.

For other microorganisms see overview under section A.3.
Annex 6: Determination of pesticide residues

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General

Methods for the determination of pesticide residues

Chromatography (mostly column and gas) is recommended as the principal method for the determination of pesticide residues. These methods may be coupled with mass spectrometry (MS). Samples are extracted by a standard procedure, impurities are removed by partition and/or adsorption, and the presence of a moderately broad spectrum of pesticides is measured in a single determination. However, these techniques are not universally applicable. Some pesticides are satisfactorily carried through the extraction and clean-up procedures, others are recovered with a poor yield, and some are lost entirely. Following chromatography, the separations may not always be complete, pesticides may decompose or metabolize, and many of the metabolic products are still unknown. Consequently, as a result of limitations in the analytical technique and incomplete knowledge of pesticide interactions with the environment, it is not yet possible to apply an integrated set of methods that will be satisfactory in all situations.

Generally the methodology should be adapted to the type of herbal material being tested and modifications may be necessary for different samples including seeds, leaves, oils, extracts, finished products and for samples containing different quantities of moisture. Also the spectrum of pesticides to be tested for is dependent on the specific pesticides used on the plant material and the history of use of persistent pesticides in the region.

It is, therefore, desirable to test plant materials of unknown history for broad groups of compounds rather than for individual pesticides. A variety of methods are suitable for this purpose. Pesticides containing chlorine in the molecule, for
example, can be detected by the measurement of total organic chlorine; insecticides containing phosphate can be measured by analysis for total organic phosphorus, whereas pesticides containing arsenic and lead can be detected by measurement of total arsenic or total lead, respectively. Similarly, the measurement of total bound carbon disulfide in a sample will provide information on whether residues of the dithiocarbamate family of fungicides are present.

Importantly, where such general methods are employed, care must be taken to ensure that results are not adversely affected by contributions from certain plant constituents containing the targeted elements.

If the pesticide to which the plant material has been exposed is known or can be identified by suitable means, an established method for the determination of that particular pesticide residue should be employed.

**General aspects of analytical methodology**

The samples should be tested as quickly as possible after collection, before any physical or chemical changes occur. If prolonged storage is envisaged, the samples should preferably be stored in airtight containers under refrigeration.

The water content of samples can also be problematic and in some official pharmacopoeias, content is limited to 15% and below for the determination of organochlorine and pyrethroid insecticides.

Light can cause degradation of many pesticides, and it is therefore advisable to protect the samples and any extracts or solutions from undue exposure to light.

The type of container or wrapping material used should not interfere with the sample or affect the analytical results.

Solvents and reagents used in the analytical method should be free from substances that may interfere with the reaction, alter the results or provoke degradation of the pesticide residue in the sample. It is usually necessary to employ specially purified solvents or to distil them freshly in an all-glass apparatus. Blank determinations with the solvents should be carried out, concentrating and testing them as specified in the test procedure for the plant material concerned.

The simplest and quickest procedure should be used to separate unwanted material from the sample (clean-up procedure) in order to save time when many samples have to be tested.

The process of concentrating solutions should be undertaken with great care, especially during the evaporation of the last traces of solvent, to avoid losses of pesticide residues. For this reason, it is often not advisable to remove the last traces of solvent. Agents, such as mineral oil or other oils of low volatility that may help to preserve the solution could be added to retard the loss of the relatively volatile pesticides, especially when the last traces of solvent are being evaporated. However, these agents, while satisfactory in colorimetric procedures, are usually not desirable in gas chromatographic methods. It may be necessary to evaporate heat-labile compounds using a rotary vacuum apparatus.
1. Determination of total chlorine and phosphorus

Most pesticides contain organically bound chlorine or phosphorus.

Procedure

Preparation of samples
Reduce the plant material to a fine powder, and extract with a mixture of water and acetonitrile. Most pesticides are soluble in this mixture, while most cellular constituents (e.g. cellulose, proteins, amino acids, starch, fats and related compounds) are sparingly soluble and are thus removed. A number of polar and moderately polar compounds may also be dissolved; it is therefore necessary to transfer the pesticides to light petroleum. For pesticides containing chlorine, further purification is seldom required, but for those containing phosphorus, further purification by column chromatography may be necessary, eluting with mixtures of light petroleum and ether.

Preparation of the column
Use Florisil grade 60/100 PR (or equivalent), activated at 650 °C, as the support. If this material is obtained in bulk, transfer it immediately after opening to a 500-ml glass jar or bottle with a glass stopper or foil-lined, screw-top lid. Store in the dark. Before use, heat at not less than 130 °C, cool in a desiccator to room temperature and heat once again to 130 °C after 2 days.

Prepare a Florisil column (external diameter, 22 mm), which contains, after settling, 10 cm of activated Florisil topped with about 1 cm of anhydrous sodium sulfate. Pre-wet the column with 40–50 ml of light petroleum. Place a graduated flask under the column to receive the eluate.

Method
Grind the material to allow it to pass through a sieve no. 710 or 840 and mix thoroughly. Place 20–50 g of the ground sample into a blender, add 350 ml of acetonitrile with a water content of 35% (to 350 ml of water add sufficient acetonitrile to produce 1000 ml). Blend for 5 minutes at a high speed. Filter under vacuum through an appropriate funnel, diameter 12 cm, fitted with filter paper, into a 500-ml suction flask.

Transfer the filtrate to a 250-ml measuring cylinder and record the volume. Transfer the measured filtrate to a 1-litre separating funnel and carefully add 100 ml of light petroleum. Shake vigorously for 1–2 minutes, add 10 ml of sodium chloride (400 g/l) TS and 600 ml of water. Hold the separating funnel in a horizontal position and mix vigorously for 30–45 seconds. Allow to separate, discard the aqueous layer and gently wash the solvent layer with two 100-ml portions of water. Discard the washings, transfer the solvent layer to a 100-ml glass-stoppered cylinder, and record the volume. Add about 15 g of anhydrous sodium sulfate and shake vigorously. The extract must not remain in contact with this reagent for longer than 1 hour. Transfer the extract directly to a Florisil column; if necessary, reduce the volume first to 5–10 ml. Allow it to pass through the column at a rate of not more than 5 ml per minute. Carefully rinse the cylinder with two portions, each of 5 ml, of light petroleum, transfer them to the column, rinse with further small portions of light petroleum if necessary, and then elute at the same rate with 200 ml of ether/light petroleum TS1. Change the receiver and elute with 200 ml
of ether/light petroleum TS2. Again change the receiver and elute with 200 ml of ether/light petroleum TS3. Evaporate each eluate to a suitable volume, as required, for further testing.

- The first eluate contains chlorinated pesticides (aldrin, DDE, TDE (DDD), o,p'- and p,p'-DDT, HCH, heptachlor, heptachlor epoxide, lindane, methoxychlor), polychlorinated biphenyls (PCB), and phosphated pesticides (carbophenothion, ethion and fenchlorphos).
- The second eluate contains chlorinated pesticides (dieldrin and endrin) and phosphated pesticides (methyl parathion and parathion).
- The third eluate contains phosphated pesticide (malathion).

Combustion of the organic matter
Combustion of the organic matter in oxygen is the preparatory step for the determination of chlorine and phosphorus. The pesticide is extracted from the sample and purified if necessary. The extract is concentrated, evaporated to dryness, transferred to a sample holder, and burned in a suitable conical flask flushed with oxygen. The gases produced during combustion are then absorbed in a suitable solution. The absorbed chlorine is determined as chloride and the absorbed phosphorus as orthophosphate, both using colorimetry.

Apparatus
The combustion is carried out in a 1-litre conical flask made of borosilicate glass, into the stopper of which is fused one end of a piece of platinum wire about 1 mm in diameter. To the free end of the wire is attached a piece of platinum gauze measuring about 1.5 × 2 cm to provide a means of holding the sample clear of the absorbing liquid during combustion.

Sample holder for chlorine-containing residues
For a small quantity of solid material, use a sample holder made from a piece of halide-free filter-paper about 5 cm long and 3 cm wide; for a small volume of liquid, it is preferable to use a sample holder in the form of a cone made from cellulose acetate film. Prepare the cone as follows: wearing cloth gloves and using a suitable cardboard template, cut from the film a circle of radius 4 cm. Manually pin the two edges together to form a cone. Seal the joined edges using heat to form a seam about 5 mm wide. Immerse the seam in acetone R to about one half of its width for 10 seconds. Remove and dry it immediately in a stream of hot air. Using forceps, wash the cone by dipping in a 1-litre beaker containing warm sodium hydroxide (~240 g/l) TS for 10 seconds at a temperature of about 60 °C. Rinse the cone thoroughly with water and allow to drain dry on a piece of aluminium foil. Place each cone in a clean funnel (diameter 65 mm).

Sample holder for phosphorus-containing residues
Use a piece of halide-free filter-paper about 4 cm square as the sample holder.

Combustion of chlorine-containing residues
Transfer an aliquot of the extract as prepared above onto the sample holder, which is placed in a funnel using a solvent that will not dissolve the sample holder. Allow the solvent to evaporate. Wearing rubber gloves, remove the sample holder and its dry contents from the funnel, and fold it over and up to form a small packet, about 1 cm² in area, and secure it in the centre of the platinum gauze. Insert a narrow strip of filter
paper, about 1 × 3 cm, as a fuse into the top of the holder, between the folds of the packet. Add 30 ml of water to the combustion flask. Moisten the neck of the flask with water. Fill the flask thoroughly with oxygen by means of a tube with its end just above the liquid. Ignite the free end of the paper strip and immediately insert the stopper. Hold the stopper firmly in place. When vigorous burning has begun, tilt the flask to prevent incompletely burned material from falling into the liquid. Immediately after combustion is completed, shake the flask vigorously for 10 minutes to dissolve the combustion products. Place a small quantity of water around the rim of the flask, and carefully withdraw the stopper. Rinse the stopper, platinum wire, platinum gauze and sides of the flask with water. Transfer the liquid and liquids used for rinsing to a 50-ml volumetric flask and dilute to volume with water.

Combustion of phosphorus-containing residues
Dip the sample holder made from filter paper into methanolic sodium hydroxide TS, and then suspend it in a current of heated air. Immediately transfer about 0.2 ml of an aliquot of the extract as prepared above to the sample holder with the aid of 0.2-ml portions of chloroform R using a micropipette. Allow the solvent to evaporate from the paper, fold it to form a small packet about 1 cm² in area and place it in the centre of the platinum gauze. Insert a strip of filter paper, about 1 × 3 cm, as a fuse into the top of the holder, between the folds of the packet. Add 10 ml of sulfuric acid (~37 g/l) TS to the combustion flask and continue with the combustion as described above. Transfer the solution and the liquid used for rinsing to a 25-ml volumetric flask and dilute to volume with water.

2. Determination of chlorides

Apparatus
The determination is made with a spectrophotometer capable of measuring absorbance at 460 nm using absorption cells with path-lengths of 2 cm and 10 cm.

Method
Place 15 ml of the solution obtained after combustion in a 50-ml conical flask together with 1 ml of ferric ammonium sulfate (0.25 mol/l) VS and 3 ml of mercuric thiocyanate TS. Swirl the contents of the flask and allow to stand for 10 minutes. Transfer a portion of the solution to a 2-cm cell and measure the absorbance at 460 nm using water in the reference cell. The reading should be made promptly to minimize absorption of chloride from the air.

Prepare a standard solution of sodium chloride R containing 5 μg of chloride per ml. Transfer aliquots of this solution (0 ml, 2 ml, 4 ml, 6 ml, 8 ml and 10 ml) into a series of 50-ml conical flasks and dilute to 15 ml with water. Develop the colour and measure the absorbances as described above. Plot the absorbances against the chloride content of the dilutions in μg per ml and interpolate the chloride content of the solutions of the material tested.

3. Determination of phosphates

The phosphomolybdate method is based on the reaction of phosphate ions with ammonium molybdate to form a molybdophosphate complex, which is subsequently reduced to form a strongly blue-coloured molybdenum complex.
The intensity of the blue colour is measured spectrophotometrically. This method is applicable for the determination of any phosphates that have undergone a prior separation procedure.

Naturally occurring phosphates are present in most samples, and are often not removed during the clean-up procedure. In order to obtain background values, therefore, it is necessary to proceed with the determination for all samples, even those with no phosphate-containing pesticides. These background values should be subtracted from the results obtained on testing pesticide residues. Extracts of most uncontaminated materials contain about 0.05–0.1 mg/kg of phosphorus. Therefore, no contamination with organophosphate pesticides can be assumed for results in this range.

**Apparatus**
The determination is made with a spectrophotometer capable of measuring absorbance at 820 nm using an absorption cell with a path-length of 1 cm.

**Method**
Place 7 ml of the solution obtained after combustion in a calibrated 10-ml test-tube. Add 2.2 ml of sulfuric acid (300 g/l) TS and mix the solution well. Add 0.4 ml of ammonium molybdate (40 g/l) TS and swirl the mixture. Then add 0.4 ml of aminonaphtholsulfonic acid TS and swirl again. Heat the solution to 100 °C for 12 minutes (±2 minutes), cool, and transfer a portion to a 1-cm cell. Measure the absorbance at 820 nm using water in the reference cell.

Prepare standard dilutions with a known content of phosphate and measure the absorbance as described above. Plot the absorbances against the phosphate content of the dilutions in μg per ml and interpolate the phosphate content of the solutions of the material tested.

4. **Qualitative and quantitative determination of organochlorine pesticides**

**Preparation of sample**
Place 20 g of powdered plant material (sieve no. 180), accurately weighed, in a 500-ml beaker (tall form), mix with 98 ml of water and allow to macerate for at least 30 minutes. Add 200 ml of acetone R; the resulting volume of extraction solvent will be 295 ml. Extract for 5 minutes, while cooling, using a high-speed mixer. Filter the homogenized mixture through a porcelain filter (Büchner funnel, diameter 70 mm) fitted with a filter paper, using a slight vacuum, into a 250-ml graduated cylinder, allowing the process to last no longer than 1 minute, and then measure the volume (V) of the filtrate in ml.

**Method**
Transfer the filtrate prepared as above to a 500-ml separating funnel. Add a quantity of sodium chloride R equivalent in grams to one-tenth of the volume of the filtrate, then add 100 ml of dichloromethane R. Shake vigorously for 5 minutes, allow the phases to separate and discard the lower (aqueous) layer. Dry the acetone-dichloromethane phase, transfer it to a 500-ml conical flask, add 25 g of anhydrous sodium sulfate R and swirl occasionally. Next, filter the solution into a 500-ml flask with a ground-glass
stopper using a glass funnel (diameter 100 mm) containing purified glass-wool and anhydrous sodium sulfate R. Rinse the separating funnel, the conical flask and the glass funnel twice with 10 ml of ethyl acetate R. Add 5 ml of 2,2,4-trimethylpentane R, and concentrate the crude extract to about 2 ml in a rotary vacuum evaporator in a water-bath at 30–40 °C. Expel the remaining solvent in a gentle stream of air.

To purify by gel chromatography, macerate 50 g of suitable beads (e.g. S-X3 bio-beads) in an elution mixture of cyclohexane R and ethyl acetate R (1:1) and pour them into a chromatographic column (length 600 mm, diameter 25 mm) adapted for use with a vacuum pump. Rinse the gel bed with the elution mixture under air-free conditions. Dissolve the extract in the flask with 5 ml of ethyl acetate R. Add 2 g of anhydrous sodium sulfate R, swirl gently and add 5 ml of cyclohexane R. Filter the completely dissolved crude extract through a rapid filter into a 10-ml test-tube with a ground-glass stopper and close the tube immediately. Then transfer 5 ml of the filtrate on to the gel column. Elute with the elution mixture at an average rate of 5 ml/minute. Plant material components leave the gel column first, followed by the active ingredients of pesticides. Fractionation must be determined for each column, using appropriate reference substances.

Discard the first fraction (about 100 ml) containing the impurities. Collect the organochlorine pesticides appearing in the next eluate (about 70 ml) in a flask with a ground-glass stopper. Add 10 ml of 2,2,4-trimethylpentane R and concentrate the solution to about 5 ml in a rotary vacuum evaporator and a water-bath at 30–40 °C. Pipette another 5 ml of 2,2,4-trimethylpentane R into the flask and carefully evaporate the solution to about 1 ml (do not allow to become completely dry).

Calculate the amount of plant material, in grams, in the purified extract using the following formula:

\[
\frac{V}{590} \times \text{sample weight in g}
\]

where \( V \) = volume of filtrate.

To purify further, transfer 1 g of previously deactivated silica gel for column chromatography (70-230 mesh) containing 1.5% water, to a chromatographic column (length 25 cm, internal diameter 7 mm). Put 10 mm of anhydrous sodium sulfate R on top of the content of the column and cover with purified glass wool. Before use, rinse the column with 5 ml of hexane R. Allow the solvent to reach the surface of the column filling, then transfer quantitatively, by means of a pipette, the purified extract obtained by gel chromatography from the flask to the prepared silica gel column and rinse with 1 ml of hexane R. Set the flask aside for subsequent elutions.

Using a 10-ml volumetric flask as the receiver, elute any residues of polychlorinated biphenyls from the column with 10 ml of hexane R (eluate 0). Add 2 ml of an elution mixture composed of toluene R/hexane R (35:65) to the flask and swirl. Quantitatively transfer the solution to the column. Using another 10-ml volumetric flask as the receiver, elute the majority of the organochlorine pesticides from the silica gel column using 6 ml of the same elution mixture. Dilute the contents of the flasks to volume with the elution mixture (eluate 1).
Rinse the flask with 2 ml of toluene \textit{R} and transfer it quantitatively to the column. Collect the eluate in a third 10-ml volumetric flask. Add 8 ml of toluene \textit{R} to the flask, swirl and transfer the solution to the silica gel column; elute the remaining organochlorine pesticides using the same receiver. Dilute the contents of the flask to volume with toluene \textit{R} (eluate 2).

Evaluate the test solutions by capillary gas chromatography using an electron capture detector (ECD). Confirm the findings obtained for the main column (first separation system) with a second capillary column of different polarity (second separation system).

### Determination by gas chromatography

A capillary gas chromatograph with an ECD is used for the measurement. \textit{Helium R} is used as the carrier gas and a mixture of \textit{argon R} and \textit{methane R} (95:5) as an auxiliary gas for the detection.

#### First separation system

Use a vitreous silica column, 30 m long with an internal diameter of 0.25 mm, packed with a chemically bound phase of 5% phenyl, 95% methyl-polysiloxane. Use the following temperature programme:

- heat at 60 °C for 0.5 minutes;
- increase the temperature at a rate of 30 °C per minute to 160 °C and maintain this temperature for 2 minutes;
- increase the temperature at a rate of 2 °C per minute to 250 °C and maintain this temperature for 5 minutes.

Use a “split/split-free” injector to inject the sample solution and maintain the injection port at a temperature of 240 °C. Inject a volume of 1 μl at a rate of 30 seconds (“split-free”). The detector temperature should be 300 °C.

#### Second separation system

Use a vitreous silica column, 15 m long with an internal diameter of 0.25 mm, packed with a chemically bound phase of 7% cyanopropyl, 7% phenyl, 86% methyl-polysiloxane. Use the following temperature programme:

- heat at 60 °C for 12 seconds;
- increase the temperature at a rate of 30 °C per minute to 180 °C and maintain this temperature for 1 minute;
- increase the temperature at a rate of 2 °C per minute to 250 °C and maintain this temperature for 5 minutes.

Use an on-column injector to inject a volume of 1 μl of the sample solution. The detector temperature should be 300 °C.

Use the “external standard” method for the qualitative and quantitative evaluation of the organochlorine pesticides in the test solutions with reference solutions of the following pesticides: \(\alpha\)-, \(\beta\)-, \(\gamma\)- and \(\delta\)-hexachlorocyclohexane (HCH); hexachlorobenzene; quintozene; aldrin; dieldrin; endrin; \(\alpha\)- and \(\beta\)-endosulfan; endosulfan sulfate; heptachlor, heptachlorepoxide; camphechlor; TDE, DDE and DDT (both \(o,p'\)- and \(p,p'\)-isomers); methoxychlor.
Measure the peak height of the pesticides obtained in the chromatograms and calculate the concentration of the residues in mg/kg using the following formula:

\[ \frac{h_t \times 10}{w} \times \frac{w_r}{h_r} \]

where:
- \( h_t \) = peak height obtained for the test solution in mm
- \( w \) = quantity of sample in the purified extract (g)
- \( w_r \) = quantity of pesticide in ng in the reference solution injected
- \( h_r \) = peak height obtained for the reference solution in mm.

5. **Analysis of esters of organophosphorus compounds**

Although most organophosphorus compounds undergo rapid decomposition, Member States may elect to test for them because of their harmful nature if present in significant concentrations. Testing may be more relevant in the case of herbal medicines used at high concentrations and frequency.

The extraction and the clean-up procedures can be performed as described above, but the detection requires a phosphorus flame ionization detector (P-FID).

6. **Determination of specific pesticide residues in medicinal plant material**

**General recommendations**

For the total determination, mix thoroughly 1 kg of plant material.

In order to obtain reliable chromatographic results, do one or more of the following:

- repeat the separation using another column;
- use a different separation system;
- use a different detector system;
- apply a coupling technique;
- prepare a derivative;
- perform chromatography with a mixture of the sample and a reference substance;
- change the sample preparation;
- use a fractionated elution during the column-chromatography clean-up procedure of the plant extract and test every fraction by chromatography; and
- compare the distribution coefficient of the material with that of a reference substance.

Prior to the quantitative determination of the material to be tested, check whether there is a linear relationship between the values obtained for the reference substance and its concentration over the range 0.1–2 times the standard concentration. Otherwise, prepare another concentration range or evaluate the results using a reference curve. Use any suitable mechanical or manual technique for the chromatographic determination.
Store the reference solutions protected from light to prevent decomposition. Use glass vessels closed with glass stoppers and keep them in a container saturated with the solvent employed to avoid any increase in concentration due to evaporation. Check the loss by evaporation by interim weighing of the vessels.

**Rate of recovery**

The rate of recovery (R) is the percentage of the reference material originally added to the plant material that is determined using the method described below.

### 7. Determination of desmetryn, prometryn and simazine residues

**Preparation of the plant material extract**

Place 10 g of powdered plant material in a 500-ml conical flask and add 125 ml of chloroform R.\(^1\) Shake the mixture for 60 minutes and filter under reduced pressure through a filter paper (medium grade) into a round-bottomed flask. Wash the residue with 3 successive volumes each of 25 ml of chloroform R.

**Method**

Concentrate the combined filtrates to a volume of 3–5 ml using a rotary vacuum evaporator and a water-bath at 40 °C. Transfer the extract to a chromatographic column as prepared below, rinsing the round-bottomed flask twice with 5 ml of chloroform R.

**Preparation of chromatographic column**

Use a glass tube (internal diameter 20–22 mm) with a restricted orifice and protected with a sintered-glass plate (e.g. P10 or P16, glass filter G4; or P40, glass filter G3). Fill the column with chloroform R, and then pour purified aluminium oxide R into it to form a 100-mm thick layer. The support material should remain covered with chloroform R. After transferring the extract and the rinsing liquids to the column, elute with 150 ml of chloroform R, at a rate of 1–2 drops per second, collecting the eluate in a round-bottomed flask. The first purifying process is completed when no further eluate drips from the column.

Evaporate the eluate to dryness using a rotary vacuum evaporator and a water-bath at 40 °C. To the residue add 10 ml of light petroleum R and transfer the mixture to a chromatographic column containing a layer of purified aluminium oxide R, 50 mm thick, in light petroleum R. Elute the mixture with 90 ml of light petroleum R, using this to rinse the round-bottomed flask, at a rate of 1–2 drops per second. Discard the eluate. Dissolve any remaining residue, which has not dissolved in light petroleum R in 10 ml of a mixture composed of 60 volumes of chloroform R, and 40 volumes of light petroleum R and transfer the solution to the column. Rinse the round-bottomed flask twice more with 10 ml of the solvent mixture. Transfer the liquid used for rinsing to the column. Elute with 120 ml of the same solvent mixture, at a rate of 1–2 drops per second and collect the eluate in a round-bottomed flask. The second purifying process is completed when no further eluate drips from the column.

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\(^1\) In future development of this method, replacement of chloroform by other appropriate solvents is recommended.
Evaporate the eluate to dryness using a rotary vacuum evaporator and a water-bath at 40 °C. To prepare a purified extract for the determination by gas chromatography, dissolve the residue in sufficient acetone R to produce a volume of 10 ml. If an especially purified extract is required, proceed as described below.

To the residue add 10 ml of light petroleum R and 10 ml of dimethyl sulfoxide R. Shake the mixture and transfer it to a separating funnel. Extract the dimethyl sulfoxide layer twice with 10 ml of light petroleum R. Discard the petroleum ether extract. Then add 100 ml of water to the dimethyl sulfoxide layer and extract 3 times, each with 20 ml of chloroform R. Extract the combined chloroform extracts twice with 20 ml of water and evaporate them to dryness using a rotary vacuum evaporator and a water-bath at 40 °C. Transfer the residue together with a mixture of 10 ml of light petroleum R and 10 ml of hydrochloric acid (1 mol/l) VS to a separating funnel and extract the mixture first with 10 ml and then with 5 ml of hydrochloric acid (1 mol/l) VS. Discard the petroleum ether layer and adjust the pH of the combined aqueous solutions to a value between 7 and 8 using sodium hydroxide (1 mol/l) VS. Extract the solution 3 times, each with 20 ml of chloroform R. Dry the combined chloroform extracts with anhydrous sodium sulfate R and filter into a round-bottomed flask, rinsing the funnel 3 times with 10.0-ml portions of chloroform R. Evaporate the filtrate to dryness using a rotary vacuum evaporator and a water-bath at 40 °C. Dissolve the residue in sufficient acetone R to produce 10 ml of especially purified extract to be used for the determination by gas chromatography.

Use the extracts as indicated in Table A6.1 for the following plant materials.

Table A6.1. Extract to be used for specific herbal materials

<table>
<thead>
<tr>
<th>No.</th>
<th>Material</th>
<th>No.</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flores Calendulae</td>
<td>10</td>
<td>Fructus Foeniculi</td>
</tr>
<tr>
<td>2</td>
<td>Flores Chamomillae</td>
<td>11</td>
<td>Herba Millefolii</td>
</tr>
<tr>
<td>3</td>
<td>Folia Melissae</td>
<td>12</td>
<td>Herba Plantaginis lanceolatae</td>
</tr>
<tr>
<td>4</td>
<td>Folia Menthae piperitae</td>
<td>13</td>
<td>Radix Althaeae</td>
</tr>
<tr>
<td>5</td>
<td>Folia Salviae</td>
<td>14</td>
<td>Radix Angelicae</td>
</tr>
<tr>
<td>6</td>
<td>Folia Thymi</td>
<td>15</td>
<td>Radix Levistici</td>
</tr>
<tr>
<td>7</td>
<td>Fructus Carvi</td>
<td>16</td>
<td>Radix Petroselini</td>
</tr>
<tr>
<td>8</td>
<td>Fructus Coriandri</td>
<td>17</td>
<td>Radix Valerianae</td>
</tr>
<tr>
<td>9</td>
<td>Fructus Cynobasti</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For materials no. 1 and 2, use an especially purified extract (see above); for materials no. 3–17, use a purified extract (see above).

**Determination of the rate of recovery**

Prepare five individual samples using each of the following procedures:

1. To prepare solution S2, first dissolve separately 0.04 g of each of the reference substances, desmetryn R, prometryn R and simazine R, in sufficient acetone R to produce 100 ml. Then place 5 ml of each solution in a 100-ml volumetric flask and dilute the mixture to volume with acetone R (S2). Place 10 g of powdered plant material in a 500-ml conical flask and add 1 ml of solution S2. Shake this mixture mechanically for 60 minutes; if necessary, repeat the operation...
manually and then proceed as described under “Preparation of the plant material extract”. Use either the purified or especially purified extract for the determination by gas chromatography, as specified in the test procedure for the plant material concerned.

2. Treat 10 g of powdered plant material as described under “Preparation of the plant material extract” (see above). Use either the purified extract or the especially purified extract for the determination by gas chromatography, as specified in the test procedure for each individual plant material.

Calculate the rate of recovery (R) as a percentage using the following formula:

\[
\frac{2(a-b)}{c}
\]

where

\[ a = \text{average quantity in mg/kg of the 5 residues obtained using procedure 1} \]
\[ b = \text{average quantity in mg/kg of the 5 residues obtained using procedure 2} \]
\[ c = \text{quantity of reference substances in mg contained in solution S} \_2 \text{during procedure 1}. \]

The rate should be within the range 70–120%. It is specific for each drug.

**Determination by gas chromatography**

Perform the determination as described in *the International Pharmacopoeia*.1

**Apparatus**

The equipment consists of:

- a glass column 1.2 m long, internal diameter 2 mm
- a suitable stationary liquid phase
- a suitable diatomaceous support.

Use nitrogen R as the carrier gas with a flow rate of 30 ml/min. The sample injection block should be maintained at 230 °C, the column at 190 °C and the detector, which should be nitrogen-selective, at 300 °C. In addition:

- volume of sample solution to be injected: 2.0 μl;
- separation characteristics: \[ h \leq 1.2 \times 10^{-3} \] for desmetryn R; \[ RS \geq 1.2 \] for prometryn R and simazine R;
- relative standard deviation (precision of chromatographic system): \[ sr. \leq 0.05 \] for desmetryn R, prometryn R and simazine R.

**Method**

- Chromatogram T. To determine the separation characteristics, inject solution \( S_2 \) (for the preparation of solution \( S_2 \) see “Determination of the rate of recovery” above). Chromatograms \( A_1-A_5 \). To determine the relative standard deviation inject solution \( S_2 \) and repeat the determination 5 times.

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• Chromatogram S₂. Inject 1 ml of solution S₂ for the determination of the rate of recovery. Dilute 1 ml of solution S₂ to 10 ml with acetone R and inject it for the chromatographic determination. The peaks on the chromatogram occur in the following sequence: prometryn, simazine, desmetryn.

• Chromatogram P₂. Inject the purified extract or the especially purified extract. Determine using an external standard: \( a = 0.0005 \). To convert the values obtained to percentage by weight, multiply the concentration in mg/kg by 10⁴.

The total maximum permissible amount of residues due to desmetryn, prometryn and simazine is 2 mg/kg of plant material.

8. Determination of specific organochlorine, organophosphorus and pyrethroid insecticide residues

Depending on the substance being examined, it may be necessary to modify, sometimes extensively, the procedure described below. In any case, it may be necessary to use, in addition, another column with a different polarity or another detection method (e.g. mass spectrometry) or a different method (e.g. immunochemical method) to confirm the results obtained.

This procedure is valid only for the analysis of samples of medicinal plant materials containing less than 15% water. Samples with a higher content of water may be dried, provided it has been shown that the drying procedure does not significantly affect the pesticide content.

**Preparation of sample**

**Extraction**

To 10 g of the substance being examined, coarsely powdered, add 100 ml of acetone R and allow to stand for 20 min. Add 1 ml of a solution containing 1.8 μg/ml of carbophenothion R in toluene R. Homogenize using a high-speed blender for 3 min. Filter and wash the filter cake with two quantities, each of 25 ml, of acetone R. Combine the filtrate and the washings and heat using a rotary evaporator at a temperature not exceeding 40 °C until the solvent has almost completely evaporated. To the residue add a few millilitres of toluene R and heat again until the acetone is completely removed. Dissolve the residue in 8 ml of toluene R. Filter through a membrane filter (45 μm), rinse the flask and the filter with toluene R and dilute to 10.0 ml with the same solvent (solution A).

**Purification**

• **Organochlorine, organophosphorus and pyrethroid insecticides**

Examine by size-exclusion chromatography.

The chromatographic procedure may be carried out using:

• a stainless steel column 0.30 m long and 7.8 mm in internal diameter packed with styrene-divinylbenzene copolymer R (5 μm);

• as mobile phase toluene R at a flow rate of 1 ml/min.

**Performance of the column.** Inject 100 μl of a solution containing 0.5 g/l of methyl red R and 0.5 g/l of oracet blue 2R in toluene R and proceed with the chromatography.
The column is not suitable unless the colour of the eluate changes from orange to blue at an elution volume of about 10.3 ml. If necessary calibrate the column using a solution containing, in *toluene R*, at a suitable concentration, the insecticide to be analysed with the lowest molecular mass (for example, dichlorvos) and that with the highest molecular mass (for example, deltamethrin). Determine which fraction of the eluate contains both insecticides.

**Purification of the test solution.** Inject a suitable volume of solution A (100 μl to 500 μl) and proceed with the chromatography. Collect the fraction as determined above (*solution B*). Organophosphorus insecticides are usually eluted between 8.8 ml and 10.9 ml. Organochlorine and pyrethroid insecticides are usually eluted between 8.5 ml and 10.3 ml.

- **Organochlorine and pyrethroid insecticides**

In a chromatography column, 0.10 m long and 5 mm in internal diameter, introduce a piece of defatted cotton and 0.5 g of silica gel treated as follows: heat *silica gel for chromatography R* in an oven at 150 °C for at least 4 h. Allow to cool and add dropwise a quantity of *water R* corresponding to 1.5% of the mass of silica gel used; shake vigorously until agglomerates have disappeared and continue shaking for 2 h using a mechanical shaker. Condition the column using 1.5 ml of *hexane R*. Prepacked columns containing about 0.5 g of a suitable silica gel may also be used provided they have been validated beforehand.

Concentrate solution B in a current of *helium for chromatography R* or *oxygen-free nitrogen R* almost to dryness and dilute to a suitable volume with *toluene R* (200 μl to 1 ml according to the volume injected in the preparation of solution B). Transfer quantitatively on to the column and proceed with the chromatography using 1.8 ml of *toluene R* as the mobile phase. Collect the eluate (*solution C*).

**Quantitative analysis**

*Organophosphorus insecticides* Examine by gas chromatography, using *carbophenothion R* as internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbophenothion.

*Test solution.* Concentrate solution B in a current of *helium for chromatography R* almost to dryness and dilute to 100 μl with *toluene R*.

*Reference solution.* Prepare at least 3 solutions in *toluene R* containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve.

The chromatographic procedure may be carried out using:

- a fused-silica column 30 m long and 0.32 mm in internal diameter, the internal wall of which is covered with a layer 0.25 μm thick of *poly(dimethyl)siloxane R*;
- *hydrogen for chromatography R* as the carrier gas. Other gases such as *helium for chromatography R* or *nitrogen for chromatography R* may also be used provided the chromatography is suitably validated;
- a phosphorus-nitrogen flame-ionization detector or an atomic emission spectrometry detector,
maintaining the temperature of the column at 80 °C for 1 min, then raising it at a rate of 30 °C/min to 150 °C, maintaining it at 150 °C for 3 min, then raising the temperature at a rate of 4 °C/min to 280 °C and maintaining at this temperature for 1 min, and maintaining the temperature of the injector port at 250 °C and that of the detector at 275 °C. Inject the chosen volume of each solution. When the chromatograms are recorded in the prescribed conditions, the relative retention times are approximately those listed in Table A6.2. Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

**Table A6.2. Relative retention times of organophosphorus insecticides**

<table>
<thead>
<tr>
<th>Substances</th>
<th>Relative retention times</th>
</tr>
</thead>
<tbody>
<tr>
<td>dichlorvos</td>
<td>0.20</td>
</tr>
<tr>
<td>fonofos</td>
<td>0.50</td>
</tr>
<tr>
<td>diazinon</td>
<td>0.52</td>
</tr>
<tr>
<td>parathion-methyl</td>
<td>0.59</td>
</tr>
<tr>
<td>chlorpyrifos-methyl</td>
<td>0.60</td>
</tr>
<tr>
<td>pirimiphos-methyl</td>
<td>0.66</td>
</tr>
<tr>
<td>malathion</td>
<td>0.67</td>
</tr>
<tr>
<td>parathion</td>
<td>0.69</td>
</tr>
<tr>
<td>chlorpyrifos</td>
<td>0.70</td>
</tr>
<tr>
<td>methidathion</td>
<td>0.78</td>
</tr>
<tr>
<td>ethion</td>
<td>0.96</td>
</tr>
<tr>
<td>carbophenothion</td>
<td>1.00</td>
</tr>
<tr>
<td>azinphos-methyl</td>
<td>1.17</td>
</tr>
<tr>
<td>phosalon</td>
<td>1.18</td>
</tr>
</tbody>
</table>

*Note:* The relative retention times are very close. If it is necessary to distinguish between two relative retention times that are very close, further development of a suitable method will be required.

**Organochlorine and pyrethroid insecticides**

Examine by gas chromatography, using carbophenothion as the internal standard. It may be necessary to use a second internal standard to identify possible interference with the peak corresponding to carbophenothion.

**Test solution.** Concentrate solution C in a current of helium for chromatography R or oxygen-free nitrogen R almost to dryness and dilute to 500 μl with toluene R.

**Reference solution.** Prepare at least three solutions in toluene R containing the insecticides to be determined and carbophenothion at concentrations suitable for plotting a calibration curve.

The chromatographic procedure may be carried out using:

- a fused silica column 30 m long and 0.32 mm in internal diameter the internal wall of which is covered with a 0.25 μm thick layer of poly(dimethyl)(diphenyl) siloxane R;
• hydrogen for chromatography R as the carrier gas. Other gases such as helium for chromatography R or nitrogen for chromatography R may also be used, provided the chromatography is suitably validated;
• an electron-capture detector;
• a device allowing direct cold on-column injection,

maintaining the temperature of the column at 80 °C for 1 min, then raising it at a rate of 30 °C/min to 150 °C, maintaining it at 150 °C for 3 min, then raising the temperature at a rate of 4 °C/min to 280 °C and maintaining at this temperature for 1 min, and maintaining the temperature of the injector port at 250 °C and that of the detector at 275 °C. Inject the chosen volume of each solution. When the chromatograms are recorded in the prescribed conditions, the relative retention times are approximately those listed in Table A6.3. Calculate the content of each insecticide from the peak areas and the concentrations of the solutions.

Table A6.3. Relative retention times of organochlorine and pyrethroid insecticides

<table>
<thead>
<tr>
<th>Substances</th>
<th>Relative retention times</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-hexachlorocyclohexane</td>
<td>0.44</td>
</tr>
<tr>
<td>hexachlorobenzene</td>
<td>0.45</td>
</tr>
<tr>
<td>β-hexachlorocyclohexane</td>
<td>0.49</td>
</tr>
<tr>
<td>lindane</td>
<td>0.49</td>
</tr>
<tr>
<td>δ-hexachlorocyclohexane</td>
<td>0.54</td>
</tr>
<tr>
<td>ε-hexachlorocyclohexane</td>
<td>0.56</td>
</tr>
<tr>
<td>heptachlor</td>
<td>0.61</td>
</tr>
<tr>
<td>aldrin</td>
<td>0.68</td>
</tr>
<tr>
<td>cis-heptachlor-epoxide</td>
<td>0.76</td>
</tr>
<tr>
<td>o,p'-DDE</td>
<td>0.81</td>
</tr>
<tr>
<td>α-endosulfan</td>
<td>0.82</td>
</tr>
<tr>
<td>dieldrin</td>
<td>0.87</td>
</tr>
<tr>
<td>p,p'-DDE</td>
<td>0.87</td>
</tr>
<tr>
<td>o,p'-DDD</td>
<td>0.89</td>
</tr>
<tr>
<td>endrin</td>
<td>0.91</td>
</tr>
<tr>
<td>β-endosulfan</td>
<td>0.92</td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>0.95</td>
</tr>
<tr>
<td>carbophenothion</td>
<td>1.00</td>
</tr>
<tr>
<td>p,p'-DDT</td>
<td>1.02</td>
</tr>
<tr>
<td>cis-permethrin</td>
<td>1.29</td>
</tr>
<tr>
<td>trans-permethrin</td>
<td>1.31</td>
</tr>
<tr>
<td>cypermethrin*</td>
<td>1.40</td>
</tr>
<tr>
<td>fenvalerate*</td>
<td>1.47 and 1.49</td>
</tr>
<tr>
<td>deltamethrin</td>
<td>1.54</td>
</tr>
</tbody>
</table>

* The substance shows several peaks.
Annex 7: List of culture media and strains used for microbiological analysis

Culture media

The following media are satisfactory, but other media may be used if they have similar nutritive and selective properties for the microorganisms to be tested. See Annex 8 for the list of reagents and solutions.

Baird–Parker agar
Procedure. Dissolve 10 g of pancreatic digest of casein R, 5 g of beef extract R, 1 g of water-soluble yeast extract R, 5 g of lithium chloride R, 20 g of agar R, 12 g of glycine R and 10 g of sodium pyruvate R in sufficient water to produce 950 ml. Heat to boiling for 1 minute, shaking frequently and adjust the pH to 6.6–7.0 using sodium hydroxide (0.5 mol/l) VS. Sterilize in an autoclave at 121 °C for 15 minutes, cool to 45–50 °C and add 10 ml of a sterile 0.01 g/ml solution of potassium tellurite R and 50 ml of egg-yolk emulsion.

Brilliant green agar
Procedure. Dissolve 10 g of dried peptone R (meat and casein), 3 g of water-soluble yeast extract R, 5 g of sodium chloride R, 10 g of lactose R, 10 g of sucrose R, 20 g of agar R, 0.08 g of phenol red R and 12.5 mg of brilliant green R in sufficient water to produce 1000 ml. Heat to boiling for 1 minute. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 6.7–7.1. Immediately before use, sterilize in an autoclave at 121 °C for 15 minutes, cool to 50 °C and pour into Petri dishes.

Buffered sodium chloride–peptone solution pH 7.0
Procedure. Dissolve 3.56 g of potassium dihydrogen phosphate R, 7.23 g of disodium hydrogen phosphate R, 4.30 g of sodium chloride R and 1 g of dried peptone R (meat and casein) in sufficient water to produce 1000 ml. Polysorbate 20 R or polysorbate 80 R may be added, 0.001–0.01 g /ml. Sterilize in an autoclave at 121 °C for 15 minutes.

Casein–soybean digest agar
Procedure. Dissolve 15 g of pancreatic digest of casein R, 3 g of papaic digest of soybean meal R, 5 g of sodium chloride R and 15 g of agar R in sufficient water to produce 1000 ml. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 7.1–7.5. Sterilize in an autoclave at 121 °C for 15 minutes.

Cetrimide agar
Procedure. Dissolve 20 g of pancreatic digest of gelatin R, 1.4 g of magnesium chloride R, 10 g of potassium sulfate R, 0.3 g of cetrimide R, 13.6 g of agar R and 10 ml of glycerol R in sufficient water to produce 1000 ml. Heat to boiling for 1 minute with shaking. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 7.0–7.4. Sterilize in an autoclave at 121 °C for 15 minutes.
Cooked-meat medium

Procedure
Part I: Mix 454.0 g of ground beef heart (fat free) with 1000 ml of water (use purified water) and add 25 ml of 1 M sodium hydroxide. Heat to boiling and simmer for 20 minutes with frequent stirring. Cool and check the pH, which should be about 7.2; adjust it if necessary. Filter through several layers of gauze; squeeze out the excess liquid. Spread the meat particles to partially dry and place in suitable vessels.

Part II: Filter the fluid obtained from part I through three pieces of coarse filter paper to clarify. Then filter through one piece of finer filter paper (Whatman No. 1 or equivalent is suitable). Dissolve the following ingredients in the filtrate: peptic digest of animal tissues (20.0 g), dextrose monohydrate (2.0 g), and sodium chloride (5.0 g), and adjust the volume to 1000 ml with water (use purified water). Add the fluid to the vessels, using about four to five parts of the fluid to one part of the meat. pH after sterilization: 7.2 +/- 0.1.

Defibrinated sheep blood agar medium (five per cent) (blood agar medium)

Procedure. Heat casein-soybean digest agar medium and cool to 45–50 °C in a water-bath. Add sufficient amount of defibrinated sheep blood to make 5 per cent and mix.

Deoxycholate citrate agar

Procedure. Dissolve 10 g of beef extract R, 10 g of dried peptone R (meat), 10 g of lactose R, 20 g of sodium citrate R, 1 g of iron (III) citrate R, 5 g of sodium deoxycholate, 13.5 g of agar R and 20 mg of neutral red R in sufficient water to produce 1000 ml. Heat gently to boiling for 1 minute, cool to 50 °C and adjust the pH to 7.1–7.5 using sodium hydroxide (0.05 mol/l) VS. Pour into Petri dishes. Do not heat in an autoclave.

Enterobacteriaceae enrichment broth (Mossel)

Procedure. Dissolve 10 g of pancreatic digest of gelatin R, 5 g of glucose hydrate R, 20 g of dehydrated ox bile R, 2 g of potassium dihydrogen phosphate R, 8 g of disodium hydrogen phosphate R and 15 mg of brilliant green R in sufficient water to produce 1000 ml. Heat to boiling for 30 minutes and cool immediately. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 7.0–7.4.

Kligler’s iron agar (KIA)

Procedure. Dissolve the agar in the meat infusion broth, or alternatively in meat extract broth, by heating in a boiling water-bath or in steam at 100° C. Bring the molten nutrient agar to 80 °C in a water-bath. Add and dissolve the lactose, peptone, proteose peptone, NaCl, glucose, ferrous sulfate, and sodium thiosulfate and mix well. Adjust the pH to 7.4. Add 6 ml of 0.5% solution of phenol red and mix well. Distribute in screw-cap tubes (15 × 150 or 16 × 160 mm) in 5–6 ml amounts and sterilize by autoclaving at 121 °C for 15 minutes. Allow the medium to cool and set with a slant of 2.5 cm and a butt 2.5 cm deep. Record batch number and date on the label and then store at room temperature not exceeding 25 °C.

An additional 10 g of peptone, 3 g of beef extract, 3 g of yeast extract, and one litre of distilled water may be used in place of meat infusion broth.
Lactose broth
Procedure. Dissolve 3 g of beef extract R, 5 g of pancreatic digest of gelatin R and 5 g of lactose R in sufficient water to produce 1000 ml. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 6.7–7.1. Sterilize in an autoclave at 121 °C for 15 minutes.

MacConkey agar
Procedure. Dissolve 17 g of pancreatic digest of gelatin R, 3 g of dried peptone R (meat and casein), 10 g of lactose R, 5 g of sodium chloride R, 1.5 g of bile salts R, 30 mg of neutral red R and 1 mg of crystal violet R in sufficient water to produce 1000 ml. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 6.9–7.3. Heat to boiling for 1 minute with constant shaking then sterilize in an autoclave at 121 °C for 15 minutes.

MacConkey broth
Procedure. Dissolve 20 g of pancreatic digest of gelatin R, 10 g of lactose R, 5 g of dehydrated ox bile R and 10 mg of bromocresol purple R in sufficient water to produce 1000 ml. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 7.1–7.5. Sterilize in an autoclave at 121 °C for 15 minutes.

Soybean-casein-digest medium
Procedure. Dissolve 17 g of pancreatic digest of casein R, 3 g of papaic digest of soybean meal R, 5 g of sodium chloride R, 2.5 g of dipotassium hydrogen phosphate R and 2.5 g of glucose hydrate R in sufficient water to produce 1000 ml. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 7.1–7.5. Sterilize in an autoclave at 121 °C for 15 minutes.

Soybeancasein-digest medium
Procedure. Dissolve 10 g of dried peptone R (meat and casein), 40 g of glucose hydrate R and 15 g of agar R in sufficient water to produce 1000 ml. Using acetic acid (~60 g/l) TS adjust the pH to 5.4–5.8. Sterilize in an autoclave at 121 °C for 15 minutes. Immediately before use, add sterile solutions of 0.10 g of benzylpenicillin sodium R and 0.1 g of tetracycline R per litre of medium or alternatively, before sterilization, add 0.05 g of chloramphenicol R per litre of medium.

Tetrathionate bile brilliant green broth
Procedure. Dissolve 8.6 g of dried peptone R, 8 g of dehydrated ox bile R, 6.4 g of sodium chloride R, 20 g of calcium carbonate R, 20 g of potassium tetrathionate R and 0.07 g of brilliant green R in sufficient water to produce 1000 ml. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 6.8–7.2. Heat just to boiling; do not reheat.

Triple sugar iron agar
Procedure. Dissolve 3 g of beef extract R, 3 g of water-soluble yeast extract, 20 g of dried peptone R (casein and beef), 5 g of sodium chloride R, 10 g of lactose R, 10 g of sucrose R, 1 g of glucose hydrate R, 0.3 g of brown ammonium iron(III) citrate R, 0.3 g of sodium thiosulfate R, 25 mg of phenol red R and 12 g of agar R in sufficient water to produce 1000 ml. Heat to boiling for 1 minute with shaking. Using sodium hydroxide (0.05 mol/l) VS adjust the pH to 7.2–7.6. Distribute in tubes and sterilize in an autoclave at 121 °C for 15 minutes. Allow to set in an inclined position covered with a butt.

Violet-red bile agar with glucose and lactose
Procedure. Dissolve 3.0 g of water-soluble yeast extract R, 7.0 g of pancreatic digest of gelatin R, 1.5 g of bile salts R, 10.0 g of lactose R, 5.0 g of sodium chloride R, 10.0 g of
glucose hydrate R, 15.0 g of agar R, 30 mg of neutral red R and 2.0 mg of crystal violet R in sufficient water to produce 1000 ml. Heat to boiling and adjust the pH to 7.2–7.6 using sodium hydroxide (0.05 mol/l) VS. Do not heat in an autoclave.

**Xylose, lysine, deoxycholate agar**

 Procedure. Dissolve 3.5 g of xylose R, 5 g of L-lysine R, 7.5 g of lactose R, 7.5 g of sucrose R, 5 g of sodium chloride R, 3 g of water-soluble yeast extract R, 0.08 g of phenol red R, 13.5 g of agar R, 2.5 g of sodium deoxycholate R, 6.8 g of sodium thiosulfate R and 0.8 g of brown ammonium iron(III) citrate R in sufficient water to produce 1000 ml. Using sodium hydroxide (0.05 mol/l) VS, adjust the pH to 7.2–7.6. Heat just to boiling, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

**Strains of microorganisms**

The strains of microorganism referred to throughout the text are suitable, but others may be used if they have similar properties. The designations of the strains and the addresses from which they may be obtained are listed in Table A7.1.

**Table A7.1. Addresses for obtaining strains of microorganism**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection, 12301 Park Lawn Drive, Rockville, MD 20852, USA</td>
</tr>
<tr>
<td>CIP</td>
<td>Collection de l’Institut Pasteur, Service de la Collection Nationale de Cultures de Microorganismes (CNCM), 25 rue du Docteur Roux, F 75015 Paris, France</td>
</tr>
<tr>
<td>IFO</td>
<td>Institute for Fermentation, Osaka (IFO) microorganism strains, The Culture Collection Division, The NITE Biological Resource Center (NBRC), Department of Biotechnology, National Institute of Technology and Evaluation (NITE), 2-5-8, Kazusa-kamatari, Kisarazu City, Chiba 292-0818, Japan</td>
</tr>
<tr>
<td>NCIMB</td>
<td>National Collection of Industrial and Marine Bacteria, 23 St Machar Drive, Aberdeen AB24 3RY, Scotland</td>
</tr>
<tr>
<td>NCPF</td>
<td>National Collection of Pathogenic Fungi, PHLS Mycology Reference Laboratory, Public Health Laboratory, Kingsdown, Bristol BS2 8EL, England</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT, England</td>
</tr>
</tbody>
</table>
Annex 8: List of reagents and solutions

The reagents, test solutions and volumetric solutions mentioned in this publication are described below. Reagents are denoted by the abbreviation R, test solutions by the abbreviation TS, and volumetric solutions by the abbreviations VS. The concentration of the reagent solutions is expressed in g/l, that is, grams of anhydrous substance per litre of water or solvent, as indicated. Where no solvent is indicated, demineralized water should be used. The procedures for the preparation of test solutions that require special attention are given in detail. The designation of \(d\) denotes the relative density \(d_{20}^\circ\), i.e. measured in air at 20 °C in relation to water at 20 °C. Colour index (CI) numbers are provided for strains.

**Acetic acid, glacial**, R. C\(_2\)H\(_4\)O\(_2\); \(d\sim1.048\).
A suitable commercially available reagent.

**Acetic acid (~300 g/l) TS.** A solution of glacial acetic acid R containing about 300 g of C\(_2\)H\(_4\)O\(_2\) per litre (approximately 5 mol/l); \(d\sim1.037\).

**Acetic acid (~60 g/l) TS.** Acetic acid (~300 g/l) TS, diluted to contain about 60 g of C\(_2\)H\(_4\)O\(_2\) per litre (approximately 1 mol/l); \(d\sim1.008\).

**Acetic acid, dilute.**
Dilute 6 g of glacial acetic acid with water to make 100 ml (1 mol/l).

**Acetone R.** C\(_3\)H\(_6\)O.
A suitable commercially available reagent.

**Acetonitrile R.** Methyl cyanide, C\(_2\)H\(_3\)N.
*Description.* A clear, colourless liquid.
*Miscibility.* Freely soluble with water.
A suitable commercially available reagent.

**Acidic tin (II) chloride TS.**
Dissolve 8 g of tin (II) chloride dihydrate in 500 ml of hydrochloric acid. Preserve in glass-stoppered bottles. Use within 3 months.

**Aflatoxin mixture TS.**
*Procedure.* Prepare a mixed working standard in a mixture of 98 volumes of chloroform R and 2 volumes of acetonitrile R, containing 0.5 μg of each of aflatoxins B\(_1\) and G\(_1\) per ml, and 0.1 μg of each of aflatoxins B\(_2\) and G\(_2\) per ml.
*Note.* Aflatoxins are highly toxic and should be handled with care. National legal requirements should be followed.
Suitable commercially available working standards.

**Agar R.**
A suitable commercially available reagent.

**Aluminium chloride R.** AlCl\(_3\)-6H\(_2\)O.
A suitable commercially available reagent.
Aluminium oxide, purified, R. Al₂O₃.  
A suitable commercially available reagent for column chromatography.

1,2,4-Aminonaphtholsulfonic acid R. C₁₀H₉NO₄S.  
**Description.** A white to slightly brownish pink powder.  
**Solubility.** Sparingly soluble in water.

**Aminonaphtholsulfonic acid TS.**  
**Procedure.** Add 0.25 g of 1,2,4-aminonaphtholsulfonic acid R to 100 ml of freshly prepared sodium metabisulfite (150 g/l) TS with mechanical stirring. After stirring for 15 minutes, add 0.5 g of anhydrous sodium sulfite R. After stirring for an additional 5 minutes, filter the mixture.  
**Storage.** Keep in a brown bottle.  
**Note.** This reagent should be prepared freshly every week.

Ammonia solution NH₄OH (R or TS)  
Ammonia water. A suitable commercially available reagent: specific gravity: about 0.90; density: 0.908 g/ml; content: 28–30%.

Ammonia TS.  
To 400 ml of ammonia solution add water to make 1000 ml (10%).

Ammonia (~100 g/l) TS. Ammonia (~260 g/l) TS, diluted to contain about 100 g of NH₃ per litre (approximately 6 mol/l); d 0.956.

Ammonium dihydrogen phosphate R. (NH₄)H₂PO₄.  
Monobasic ammonium phosphate. A white, crystalline powder or colourless crystals, freely soluble in water.

Ammonium iron(III) citrate, brown, R. Ferric ammonium citrate, brown; soluble ferric citrate.  
Contains about 9% of NH₃, 16.5–18.5% of Fe, and about 65% of hydrated citric acid.  
**Description.** Reddish brown granules, garnet-red transparent scales, or brownish yellow powder; odourless or slight odour of NH₃. Very deliquescent.  
**Solubility.** Very soluble in water; practically insoluble in ethanol (~750 g/l) TS.  
**Storage.** Store in a well closed container, protected from light.  
A suitable commercially available reagent.

Ammonium molybdate R. (NH₄)₆Mo₇O₂₄·4H₂O.  
A suitable commercially available reagent.

Ammonium molybdate (40 g/l) TS. A solution of ammonium molybdate R containing about 40 g of (NH₄)₆Mo₇O₂₄·4H₂O per litre.

Ammonium oxalate R. C₂H₆N₂O₄·H₂O.  
A suitable commercially available reagent.

Ammonium oxalate (25 g/l) TS. A solution of ammonium oxalate R containing about 27 g of C₂H₆N₂O₄ per litre.

Ammonium thiocyanate R. CH₄N₂S.  
A suitable commercially available reagent.
Ammonium thiocyanate (75 g/l) TS.
A solution of ammonium thiocyanate R containing about 75 g of CH₄N₂S per litre (approximately 1 mol/l).

Argon R. Ar.
Contains not less than 999.95 ml of Ar per litre.
A suitable commercially available reagent.

Arsenic, dilute, AsTS.
One millilitre contains 10 μg of arsenic.
Procedure. Dilute 1 ml of strong arsenic AsTS with sufficient water to produce 100 ml.
Note. Dilute arsenic AsTS must be freshly prepared.

Arsenic, strong, AsTS.
Procedure. Dissolve 0.132 g of arsenic trioxide R in 6 ml of sodium hydroxide (~80 g/l) TS, by gentle heating. Dilute the cooled solution with 20 ml of water, and add 50 ml of hydrochloric acid (~250 g/l) TS and sufficient water to produce 100 ml.

Arsenic trioxide R. As₂O₃.
A suitable commercially available reagent.

Beef extract R. A residue from beef broth obtained by extracting fresh, sound, lean beef by cooking with water and evaporating the resulting broth at a low temperature, usually under reduced pressure until a thick pasty residue is obtained.
A suitable commercially available reagent.

Benzylpenicillin sodium R. C₁₆H₁₇N₂NaO₄S.

Bile salts R.
Description. A concentrate of beef bile, the principal constituent of which is sodium deoxycholate, determined as cholic acid.
Solubility. Soluble in water and in ethanol (~750 g/l) TS.
Acidity. pH of a 0.02 g/ml solution 5.8–6.2.
A suitable commercially available reagent.

Brilliant green R. Malachite green G; basic green 1; C.I. 42040; C₂₇H₃₄N₂O₄S.
Description. Small, glistening golden crystals.
Solubility. Soluble in water and ethanol (~750 g/l) TS.
A suitable commercially available reagent.

Bromine AsTS.
Procedure. Dissolve 30 g of potassium bromide R in 40 ml of water, add 30 g of bromine R and dilute with sufficient water to produce 100 ml. The solution complies with the following test. Evaporate 10 ml nearly to dryness on a water-bath, add 50 ml of water, 10 ml of hydrochloric acid (~250 g/l) AsTS, and sufficient stannous chloride AsTS to reduce the remaining bromine, and apply the general test for arsenic. The colour of the stain produced is not more intense than that produced from a 1-ml standard stain, showing that the amount of arsenic does not exceed 1 μg/ml.
Bromocresol purple R. C_{21}H_{16}Br_{2}O_{5}S.
A suitable commercially available reagent.

Calcium carbonate R1. CaCO_{3}.
A suitable commercially available reagent.

Carbophenothion. C_{11}H_{16}ClO_{2}PS_{3}. \( O,O\)-Diethyl S-[(4-chlorophenyl)thio]methyl-phosphorodithionate.
Yellowish liquid, practically insoluble in water, miscible with organic solvents. \( \rho_{20} \): about 1.27.
A suitable commercially available reagent.

Cetrimide R. Contains not less than 96.0% and not more than 101.0% of alkyltrimethylammonium bromide, calculated as C_{17}H_{38}BrN with reference to the dried substance.
Description. A white or almost white, voluminous, free-flowing powder; slight characteristic odour.
Solubility. Soluble in two parts of water; freely soluble in ethanol (~750 g/l) TS.
A suitable commercially available reagent.

Chloramphenicol R. C_{11}H_{12}Cl_{2}N_{2}O_{3}. Quality conforms to the monograph in The international pharmacopoeia, Vol. 2, p. 64.

Chloroform R. CHCl_{3}.
A suitable commercially available reagent.

Crystal violet R. C_{25}H_{30}ClN_{3}.
A suitable commercially available reagent.

Cyclohexane R. C_{6}H_{12}.
A suitable commercially available reagent.

Desmetryn R. C_{9}H_{17}N_{5}S. 2-methylmercapto-4-methylamino-6-isopropylamino-S-triazine.
A commercially available reagent suitable for use as a reference material.

Dichloromethane R. Methylene chloride, CH_{2}Cl_{2}.
Description. A clear colourless, mobile liquid.
Miscibility. Freely miscible with ethanol (~750 g/l) TS and ether R.
Boiling range. Not less than 95% distils between 39 and 41 °C.
Residue on evaporation. After evaporation on a water-bath and drying at 105 °C, leaves not more than 0.5 mg/ml.
A suitable commercially available reagent.

Dimethyl sulfoxide R. C_{2}H_{6}OS.
Description. A colourless liquid; odourless or with a slight unpleasant odour.
Mass density \( (\rho_{20}) \). 1.10 kg/l.
A suitable commercially available reagent.

Dimethyl yellow R. C.I. 11020; 4-dimethylaminoazobenzene; C_{14}H_{15}N_{3}.
Caution. Dimethyl yellow R is carcinogenic.
Description. Produces a red colour in moderately acidic alcoholic solutions and yellow colour in weakly acidic and alkaline solutions.

Homogeneity. Carry out the method for thin-layer chromatography, using silica gel G as the coating substance and dichloromethane R as the mobile phase. Apply 10 μl of a 0.1 mg/ml solution in dichloromethane R. After removing the plate from the chromatographic chamber allow it to dry in air. Only one spot appears on the chromatogram.

A suitable commercially available reagent.

Dipotassium hydrogen phosphate R. K₂HPO₄.
A suitable commercially available reagent.

Disodium edetate R. C₁₀H₁₄N₂Na₂O₈.2H₂O.
A suitable commercially available reagent.

Disodium hydrogen phosphate R. Na₂HPO₄.12H₂O.
A suitable commercially available reagent.

Ethanol R.
A suitable commercially available reagent.

Ethanol (~750 g/l) TS.
A suitable commercially available reagent.

Ether R. C₄H₁₀O.
A suitable commercially available reagent.

Ether/light petroleum TS1.
Procedure. Dilute 60 ml of ether R with sufficient distilled light petroleum R to produce 1000 ml.

Ether/light petroleum TS2.
Procedure. Dilute 150 ml of ether R with sufficient distilled light petroleum R to produce 1000 ml.

Ether/light petroleum TS3.
Procedure. Dilute 500 ml of ether R with sufficient distilled light petroleum R to produce 1000 ml.

Ethyl acetate R. C₄H₈O₂.
A suitable commercially available reagent.

Ferric ammonium sulfate R. FeH₄NO₃S₂·12H₂O.
This reagent should be free of chlorides.
A suitable commercially available reagent.

Ferric ammonium sulfate (0.25 mol/l) VS. Ferric ammonium sulfate R, dissolved in nitric acid (~750 g/l) TS to contain 120.5 g of FeH₄NO₃S₂·12H₂O in 1000 ml.
Procedure. Dissolve 120.5 g of ferric ammonium sulfate R in a sufficient quantity of nitric acid (~750 g/l) TS to produce 1000 ml. The reagent should be free of chlorides.
Ferrous sulfate. FeSO₄·7H₂O
A suitable commercially available reagent.

Florisil R.
A suitable commercially available material for column chromatography.

Glucose hydrate R. Monohydrate of α-D-glucopyranose, C₆H₁₂O₆·H₂O. Contains not less than 99.0% and not more than 101.5% of C₆H₁₂O₆, calculated with reference to the dried substance.
Description. Colourless crystals or a white crystalline or granular powder; odourless.
Solubility. Soluble in about 1 part of water and in about 60 parts of ethanol (~750 g/l) TS; more soluble in boiling water and in boiling ethanol (~750 g/l) TS.
Acidity. Dissolve 5 g in 50 ml of carbon-dioxide-free water R. Neutralization requires not more than 0.5 ml of carbonate-free sodium hydroxide (0.02 mol/l) VS, phenolphthalein/ethanol TS being used as indicator.
Specific optical rotation. Dissolve 100 mg, previously dried to constant weight, in 1 ml of water, and add a few drops of ammonia (~100 g/l) TS; [α]D²⁰ = +52 to +53 °.
Soluble starch or sulfites. Dissolve 1 g in 10 ml of water and add 1 drop of iodine TS; the liquid is coloured yellow.
Loss on drying. Dry to constant weight at 105 °C; loses not less than 80 mg/g and not more than 100 mg/g.
Sulfated ash. Not more than 1.0 mg/g.
Assay. Dissolve about 0.1 g, accurately weighed, in 50 ml of water, add 30 ml of iodine (0.1 mol/l) VS and 10 ml of sodium carbonate (50 g/l) TS, and allow to stand for 20 minutes. Add 15 ml of hydrochloric acid (~70 g/l) TS and titrate the excess of iodine with sodium thiosulfate (0.1 mol/l) VS, using starch TS as indicator. Perform a blank determination and make any necessary corrections. Each ml of iodine (0.1 mol/l) VS is equivalent to 9.008 mg of C₆H₁₂O₆.

Glycerol R. Propane-1,2,3-triol with small amounts of water, C₃H₈O₃. Contains not less than 970 g/kg of C₃H₈O₃.
Description. A clear, almost colourless, syrupy and hygroscopic liquid; odourless.
Miscibility. Miscible with water and ethanol (~750 g/l) TS; practically immiscible with ether R and chloroform R.
Mass density (ρ₂₀). Not less than 1.256 kg/l.
Refractive index (nD). Not less than 1.469.
Acrolein and other reducing substances. Mix 1 ml with 1 ml of ammonia (~100 g/l) TS and heat in a water-bath at 60 °C for 5 minutes; the liquid is not coloured yellow. Remove from the water-bath and add 3 drops of silver nitrate (40 g/l) TS; the liquid does not become coloured within 5 minutes.
Sulfated ash. Not more than 0.5 mg/ml.
A suitable commercially available reagent.

Glycine R. Aminoacetic acid, C₂H₅NO₂.
A suitable commercially available reagent.

Helium R. He. Contains not less than 999.95 ml of He per litre.
A suitable commercially available reagent.

Helium for chromatography. He.
Contains not less than 99.995 per cent V/V of He.
A suitable commercially available reagent.
Hexane. C\textsubscript{6}H\textsubscript{14}.
A colourless, flammable liquid, practically insoluble in water, miscible with ethanol and with ether.

\begin{align*}
\rho_{20}^\circ &: 0.659 \text{ to } 0.663. \\
n_D^{20} &: 1.375 \text{ to } 1.376
\end{align*}

Distillation range. Not less than 95 per cent distils between 67 °C and 69 °C.
A suitable commercially available reagent.

Hexane R. n-Hexane, C\textsubscript{6}H\textsubscript{14}.
Description. A colourless, mobile, highly inflammable liquid.
Boiling range. Distils completely over a range of 1 °C between 67.5 and 69.5 °C.

Mass density (\(\rho_{20}\)). 0.658–0.659 kg/l.
Refractive index (\(n_D^{20}\)). 1.374–1.375.
A suitable commercially available reagent.

Hydrochloric acid R. HCl
A suitable commercially available reagent.

Hydrochloric acid (1 mol/l) VS. Hydrochloric acid (~250 g/l) TS, diluted with water to contain 36.47 g of HCl in 1000 ml.

Method of standardization. Ascertain the exact concentration of the 1 mol/l solution in the following manner. Dissolve about 1.5 g, accurately weighed, of anhydrous sodium carbonate R (previously dried at 270 °C for 1 hour) in 50 ml of water and titrate with the hydrochloric acid solution, using methyl orange/ethanol TS as indicator. Each 52.99 mg of anhydrous sodium carbonate R is equivalent to 1 ml of hydrochloric acid (1 mol/l) VS.

Hydrochloric acid (~420 g/l) TS. \(d \sim 1.18\).
A suitable commercially available reagent.

Hydrochloric acid (~250 g/l) AsTS. Hydrochloric acid (~250 g/l) TS that complies with the following tests A and B.

A. Dilute 10 ml with sufficient water to produce 50 ml, add 5 ml of ammonium thiocyanate (75 g/l) TS and stir immediately; no colour is produced.

B. To 50 ml add 0.2 ml of bromine AsTS, evaporate on a water-bath until reduced to 16 ml, adding more bromine AsTS if necessary to ensure that an excess, as indicated by the colour, is present throughout the evaporation. Add 50 ml of water and 5 drops of stannous chloride AsTS and apply the general test for arsenic. The colour of the stain produced is not more intense than that produced from a 0.2-ml standard stain, showing that the amount of arsenic does not exceed 0.05 μg/ml.

Hydrochloric acid (~250 g/l) TS. A solution of hydrochloric acid (~420 g/l) TS in water, containing approximately 250 g of HCl per litre; \(d \sim 1.12\).

Hydrochloric acid (~250 g/l), stannated, AsTS.
Procedure. Dilute 1 ml of stannous chloride AsTS with sufficient hydrochloric acid (~250 g/l) AsTS to produce 100 ml.

Hydrochloric acid (~70 g/l) TS.
Procedure. Dilute 260 ml of hydrochloric acid (~250 g/l) TS with sufficient water to produce 1000 ml (approximately 2 mol/l); \(d \sim 1.035\).
Hydrochloric acid, dilute, R
Dilute 23.6 ml of hydrochloric acid with water to make 100 ml (10%).

Hydrogen for chromatography. H₂.
Contains not less than 99.95 per cent V/V of H₂.
A suitable commercially available reagent.

Indophenol blue R. C.I. 49700; C₁₈H₂₆N₂O₃.
Description. A violet-black powder.
Solubility. Practically insoluble in water; soluble in chloroform R.
Homogeneity. Carry out the method for thin-layer chromatography, using silica gel G as the coating substance and dichloromethane R as the mobile phase.
Apply 10 μl of a 0.1 mg/ml solution in dichloromethane R and develop. After removing the plate from the chromatographic chamber allow it to dry in air.
Only one spot appears on the chromatogram.
A suitable commercially available reagent.

Iodine R. I₂.
A suitable commercially available reagent.

Iodine TS.
Procedure. Dissolve 2.6 g of iodine R and 3 g of potassium iodide R in sufficient water to produce 100 ml (approximately 0.1 mol/l).

Iodine (0.1 mol/l) VS. Iodine R and potassium iodide R, dissolved in water to contain 25.38 g of I₂ and 36.0 g of KI in 1000 ml.
Method of standardization. Ascertain the exact concentration of the 0.1 mol/l solution by titrating 25.0 ml with sodium thiosulfate (0.1 mol/l) VS, using starch TS as indicator.

Iron(III) citrate R. Ferric citrate, C₆H₅FeO₇.H₂O.
A suitable commercially available reagent.

Lactose R. C₁₂H₂₂O₁₁.
A suitable commercially available reagent.

Lead acetate R. C₄H₆O₄Pb·3H₂O.
A suitable commercially available reagent.

Lead acetate (80 g/l) TS. A solution of lead acetate R in freshly boiled water containing about 80 g/l of C₄H₆O₄Pb (approximately 0.25 mol/l).

Lead(II) acetate TS.
To 9.5 g of lead(II) acetate trihydrate add freshly boiled and cooled water to make 100 ml. Preserve in tightly stoppered bottles (0.25 mol/l).

Lithium chloride R. LiCl.
Description. White, deliquescent crystals or granules.
Solubility. Freely soluble in water; soluble in acetone R and ethanol (~ 750 g/l) TS.
Storage. Store in a tightly closed container.
A suitable commercially available reagent.
**L-Lysine R.** C$_6$H$_{14}$N$_2$O$_2$.

*Description.* Crystalline needles or hexagonal plates.

*Solubility.* Soluble in water; very slightly soluble in ethanol (~750 g/l) TS; insoluble in ether R.

*Melting point.* About 213 °C with decomposition.

*Specific optical rotation.* Dissolve 0.2 g in 10 ml of hydrochloric acid (~250 g/l) TS; $[\alpha]_{D}^{20}$ = about +21.5 °.

A suitable commercially available reagent.

**Magnesium chloride R.** MgCl$_2$·6H$_2$O.

A suitable commercially available reagent.

**Magnesium nitrate R.** Mg(NO$_3$)$_2$

*See* Magnesium nitrate hexahydrate.

**Magnesium nitrate hexahydrate R.** Mg(NO$_3$)$_2$·6H$_2$O

A suitable commercially available reagent.

**Mercuric bromide R.** HgBr$_2$.

A suitable commercially available reagent.

Mercuric bromide AsTS.

*Procedure.* Dissolve 5 g of mercuric bromide R in sufficient ethanol (~ 750 g/l) TS to produce 100 ml.

Mercuric bromide paper AsR.

*Procedure.* Use smooth, white filter-paper weighing 65–120 g/m$^2$. The thickness of the paper in mm should be approximately equal numerically to the weight expressed as above, divided by 400. Soak pieces of filter-paper, not less than 25 mm in width, in mercuric bromide AsTS, decant the superfluous liquid, suspend the paper over a non-metallic thread and allow it to dry, protected from light.

*Storage.* Store the mercuric bromide paper AsR in stoppered bottles in the dark.

*Note.* Paper that has been exposed to sunlight or to vapours of ammonia must not be used as it produces only a pale stain or no stain at all.

**Mercuric thiocyanate R.** C$_2$HgN$_2$S$_2$.

A suitable commercially available reagent.

**Mercuric thiocyanate TS.** A saturated solution of mercuric thiocyanate R in ethanol (~ 750 g/l) TS.

**Mercury R.** Hg.

A suitable commercially available reagent.

**Methane R.** CH$_4$.

A suitable commercially available reagent.

**Methanol R.** CH$_4$O.

A suitable commercially available reagent.
Methyl orange R. Sodium salt of 4’-dimethylaminoazobenzene-4-sulfonic acid, \(C_{14}H_{14}N_3NaO_3S\).
A suitable commercially available reagent.

**Methyl orange TS.**
Dissolve 0.1 g of methyl orange in 100 ml of water, and filter if necessary.

**Methyl orange/ethanol TS.**
*Procedure.* Dissolve 0.04 g of methyl orange R in sufficient ethanol (~ 150 g/l) TS to produce 100 ml.

**Methyl red.** C.I. 13020. 2-(4-Dimethylamino-phenylazo)benzoic acid. \(C_{15}H_{16}N_3O_2\).
A dark-red powder or violet crystals, practically insoluble in water, soluble in alcohol.
A suitable commercially available reagent.

**Neutral red R.** C.I. 50040; C.I. Basic red; \(C_{15}H_{14}ClN_4\).
A suitable commercially available reagent.

**Nitric acid (~ 750 g/l) TS.**
*Procedure.* Dilute 750 ml of nitric acid (~1000 g/l) TS with sufficient water to produce 1000 ml (approximately 12 mol/l).

**Nitric acid (~ 1000 g/l) TS.** \(d \sim 1.41\).
A suitable commercially available reagent.

**Nitric acid R.** HNO₃ (concentration: 69–70%, density: about 1.42 g/ml)
A suitable commercially available reagent.

**Nitrogen.** \(N_2\).
Nitrogen, washed and dried.
A suitable commercially available reagent.

**Nitrogen for chromatography.**
Contains not less than 99.95 per cent \(V/V\) of \(N_2\).
A suitable commercially available reagent.

**Nitrogen, oxygen-free.**
Nitrogen R which has been freed from oxygen by passing it through alkaline pyrogallol solution R.

**Nitrogen R.** \(N_2\).
A suitable commercially available reagent.

**Oracet blue 2R.** CI 61110. 1-amino-4-(phenylamino)anthracene-9,10-dione. \(C_{20}H_{14}N_2O_2\). mp: about 194 °C.
A suitable commercially available reagent.

**Ox bile, dehydrated, R.** Dehydrated, purified fresh bile.
A suitable commercially available reagent.

**Pancreatic digest of casein R.**
A suitable commercially available reagent.
Pancreatic digest of gelatin R.
A suitable commercially available reagent.

Papaic digest of soybean meal R.
A suitable commercially available reagent.

Peptone, dried, R. A variety of peptones are available from casein, meat, beef or a mixture of these.
A suitable commercially available reagent.

Perchloric acid (~ 1170 g/l) TS. d ~ 1.67.
A suitable commercially available reagent.

Petroleum, light, R.
A suitable commercially available reagent.

Phenolphthalein R. C_{20}H_{14}O_{4}.
A suitable commercially available reagent.

Phenolphthalein TS.
Dissolve 1 g of phenolphthalein in 100 ml of ethanol R.

Phenolphthalein/ethanol TS.
Procedure. Dissolve 1.0 g of phenolphthalein R in sufficient ethanol (~ 750 g/l) TS to produce 100 ml.

Phenol red R. Phenolsulfonphthalein, C_{19}H_{14}O_{5}S.
A suitable commercially available reagent.

Phosphate buffer solution, pH 7.2
Mix 50 ml of 0.2 mol/l potassium dihydrogen phosphate TS for buffer solution and 34.7 ml of 0.2 mol/l sodium hydroxide VS, and add water to make 200 ml.

Poly(dimethyl)(diphenyl)siloxane.
Contains 95 per cent of methyl groups and 5 per cent of phenyl groups. DB-5, SE52.
Stationary phase for gas chromatography.
A suitable commercially available material for column chromatography.

Poly(dimethyl)siloxane.
Silicone gum rubber (methyl). Organosilicon polymer with the appearance of a semi-liquid, colourless gum.
A suitable commercially available material for column chromatography.


Potassium bromide R. KBr.
A suitable commercially available reagent.
Potassium dihydrogen phosphate R. \( \text{KH}_2\text{PO}_4 \).
A suitable commercially available reagent.

Potassium dihydrogen phosphate TS (0.2 mol/l)
Dissolve 27.22 g of potassium dihydrogen phosphate in water to make 1000 ml.

Potassium hydrogen phthalate R. \( \text{C}_8\text{H}_5\text{KO}_4 \).
A suitable commercially available reagent.

Potassium hydroxide. KOH
A suitable commercially available reagent.

Potassium iodide R. KI.
A suitable commercially available reagent.

Potassium iodide TS.
Dissolve 16.5 g of potassium iodide in water to make 100 ml. Preserve in light-resistant containers. Prepare before use (1 mol/l).

Potassium iodide AsR.
Potassium iodide R that complies with the following test: Dissolve 10 g of potassium iodide R in 25 ml of hydrochloric acid (~ 250 g/l) as TS and 35 ml of water, add 2 drops of stannous chloride as TS and apply the general test for arsenic; no visible stain is produced.

Potassium sulfate R. \( \text{K}_2\text{SO}_4 \).
A suitable commercially available reagent.

Potassium tellurite R. \( \text{K}_2\text{TeO}_3 \) (approx.)
A suitable commercially available reagent.

Potassium tetrathionate R. \( \text{K}_2\text{S}_4\text{O}_6 \).
A suitable commercially available reagent.

Prometryn R. \( \text{C}_{10}\text{H}_{19}\text{N}_5\text{S} \).
A commercially available reagent suitable for use as a reference material.

Propylene glycol R. Propane diol, \( \text{C}_3\text{H}_8\text{O}_2 \).
A suitable commercially available reagent.

2-Propanol R. Isopropyl alcohol; \( \text{C}_3\text{H}_8\text{O} \).
A suitable commercially available reagent.

Pyrogallol R. \( \text{C}_6\text{H}_6\text{O}_3 \). Benzene-1,2,3-triol.
White crystals, becoming brownish on exposure to air and light, very soluble in water, in alcohol and in ether, slightly soluble in carbon disulfide. On exposure to air, aqueous solutions, and more rapidly alkaline solutions, become brown owing to the absorption of oxygen.
mp: about 131 °C.
Store protected from light.
A suitable commercially available reagent.
Pyrogallol solution, alkaline.
Dissolve 0.5 g pyrogallol R in 2 ml of carbon dioxide-free water R. Dissolve 12 g of potassium hydroxide R in 8 ml of carbon dioxide-free water R. Mix the two solutions immediately before use.

Silica gel G
Description. A fine, white, homogeneous powder with an average particle size of between 10 and 44 μm containing about 130 g of calcium sulfate, hemihydrate per kg.
Content of calcium sulfate. Place about 0.25 g, accurately weighed, in a flask with a ground-glass stopper, add 3 ml of hydrochloric acid (~70 g/l) TS and 100 ml of water and shake vigorously for 30 minutes. Filter through a sintered-glass filter and wash the residue. Using the combined filtrate and washings, carry out the assay for calcium by complexometry (The international pharmacopoeia, Vol. 1, page 128). Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 7.26 mg of CaSO₄·½H₂O (MW 145.1).

pH. Shake 1 g with 10 ml of carbon-dioxide-free water R for 5 minutes. Measure the pH potentiometrically (The international pharmacopoeia, Vol. 1, p. 96); pH is about 7.
Preparation. Suspend 30 g in 60 ml of water, shaking vigorously for 30 seconds. Carefully coat the cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.
Separating power. Apply separately to the adsorbent layer 10 μl of 0.10 mg/ml solutions containing respectively indophenol blue R, Sudan red G R and dimethyl yellow R in toluene R. Develop the plate with the same solvent over a distance of 10 cm. The chromatogram shows three clearly separated spots, the spot of indophenol blue near the points of application, that of dimethyl yellow in the middle of the chromatogram, and that of Sudan red G between the two.

Silica gel R.
A suitable commercially available material for column chromatography.

Silver nitrate R. AgNO₃.
A suitable commercially available reagent.

Silver nitrate (40 g/l) TS. A solution of silver nitrate R containing about 42.5 g of AgNO₃ per litre (approximately 0.25 mol/l).

Simazine R. C₇H₁₂CIN₅.
A commercially available reagent suitable for use as a reference material.

Soda lime R.
A suitable commercially available reagent.

Sodium carbonate R. Na₂CO₃·10H₂O.
A suitable commercially available reagent.

Sodium carbonate, anhydrous, R. Na₂CO₃.
A suitable commercially available reagent.

Sodium carbonate (50 g/l) TS. A solution of sodium carbonate R containing about 50 g of Na₂CO₃ per litre (approximately 0.5 mol/l).
Sodium chloride R. NaCl.
A suitable commercially available reagent.

Sodium chloride (400 g/l) TS. A solution of sodium chloride R containing about 400 g of NaCl per litre.

Sodium citrate R. C₆H₅Na₃O₇·2H₂O.
Quality of substance conforms to the monograph in The international pharmacopoeia, vol. 3, p. 192.

Sodium deoxycholate R. C₂₃H₃₉NaO₄. Containing not less than 90% of C₂₃H₃₉NaO₄.
A suitable commercially available reagent.

Sodium hydroxide R. NaOH.
A suitable commercially available reagent.

Sodium hydroxide (1 M).
Dissolve 162 g of sodium hydroxide in 150 ml of carbon dioxide-free water R, cool the solution to room temperature and filter through hardened filter paper. Dilute 54.5 ml of the clear filtrate with carbon dioxide-free water R to 1000 ml.
Standardization: weigh accurately about 5 g of potassium hydrogen phthalate, previously crushed lightly and dried at 120 °C for 2 hours, and dissolve in 75 ml of carbon dioxide-free water R. Add 0.1 ml of phenolphthalein TS, and titrate with the sodium hydroxide solution to the production of a permanent pink colour. Each 204.2 mg of potassium hydrogen phthalate is equivalent to 1 ml of 0.1 M sodium hydroxide VS.
Storage. Solutions of alkali hydroxides absorb carbon dioxide when exposed to air. They should therefore be stored in bottles with suitable non-glass, well-fitting stoppers, provided with a tube filled with the soda lime.
Note: prepare solutions at lower concentrations (e.g., 0.1 M, 0.01 M) by quantitatively diluting accurately measured volumes of the 1 M solution with sufficient carbon dioxide-free water R to yield the desired concentration. Restandardize the solution frequently.

Sodium hydroxide (1 mol/l) VS. Sodium hydroxide R dissolved in water to produce a solution containing 40.01 g of NaOH in 1000 ml.
Method of standardization. Ascertain the exact concentration of the 1 mol/l solution in the following manner: dry about 5 g of potassium hydrogen phthalate R at 105 °C for 3 hours and weigh accurately. If the potassium hydrogen phthalate is in the form of large crystals, they should be crushed before drying. Dissolve in 75 ml of carbon dioxide-free water R and titrate with the sodium hydroxide solution, using phenolphthalein/ethanol TS as indicator. Each 0.2042 g of potassium hydrogen phthalate is equivalent to 1 ml of sodium hydroxide (1 mol/l) VS.
Storage. Solutions of sodium hydroxide should be restandardized frequently.

Sodium hydroxide (0.5 mol/l) VS. Sodium hydroxide R dissolved in water to produce a solution containing 20.00 g of NaOH in 1000 ml.
Method of standardization. Ascertain the exact concentration of the solution following the method described under sodium hydroxide (1 mol/l) VS.
**Sodium hydroxide (0.05 mol/l) VS.** Sodium hydroxide R, dissolved in water to produce a solution containing 2.000 g of NaOH in 1000 ml. 
*Method of standardization.* Ascertain the exact concentration of the solution following the method described under sodium hydroxide (1 mol/l) VS.

**Sodium hydroxide (0.02 mol/l), carbonate-free, VS.** Sodium hydroxide R, dissolved in water to produce a solution containing 0.8001 g of NaOH in 1000 ml. 
*Method of standardization.* Ascertain the exact concentration of the solution following the method described under sodium hydroxide (1 mol/l) VS.

**Sodium hydroxide (~ 240 g/l) TS.** A solution of sodium hydroxide R containing about 240 g of NaOH per litre of carbon-dioxide-free water R.

Sodium hydroxide, methanolic TS.
*Procedure.* Dissolve 2.5 g of sodium hydroxide R in 10 ml of carbon-dioxide-free water R. Add 1 ml of propylene glycol R and dilute to 100 ml with methanol R.

**Sodium metabisulfite R.** Na$_2$O$_5$S$_2$.
A suitable commercially available reagent.

**Sodium metabisulfite (150 g/l) TS.**
A solution of sodium metabisulfite R containing about 150 g of Na$_2$O$_5$S$_2$ per litre.

**Sodium pyruvate R.** C$_3$H$_3$NaO$_2$.
*Description.* An almost white to white powder or a crystalline powder.
*Solubility.* Soluble in water.
A suitable commercially available reagent.

**Sodium sulfate, anhydrous, R.** Na$_2$SO$_3$.
A suitable commercially available reagent.

**Sodium sulfide TS.** Na$_2$S
Dissolve 5 g of sodium sulfide ennaehydrate in a mixture of 10 ml of water and 30 ml of glycerin. Or dissolve 5 g of sodium hydroxide in a mixture of 30 ml of water and 90 ml of glycerin, saturate half the volume of this solution with hydrogen sulfide, while cooling, and mix with the remaining half. Preserve in well-filled, light-resistant bottles. Use within 3 months.

**Sodium sulfide ennaehydrate R.** Na$_2$S$_9$H$_2$O
A suitable commercially available reagent.

**Sodium sulfite, anhydrous, R.** Na$_2$SO$_3$.
A suitable commercially available reagent.

**Sodium tetrahydroborate R.** NaBH$_4$.
Hygroscopic crystals, freely soluble in water, soluble in ethanol.

**Sodium thiosulfate R.** Na$_2$S$_2$O$_3$.5H$_2$O.
A suitable commercially available reagent.

**Sodium thiosulfate (0.1 mol/l) VS.** Sodium thiosulfate R, dissolved in water to produce a solution containing 15.82 g of Na$_2$S$_2$O$_3$ in 1000 ml.
Method of standardization. Ascertain the exact concentration of the 0.1 mol/l solution in the following manner: transfer 30.0 ml of potassium dichromate (0.0167 mol/l) VS to a glass-stoppered flask and dilute with 50 ml of water. Add 2 g of potassium iodide R and 5 ml of hydrochloric acid (~250 g/l) TS, stopper and allow to stand for 10 minutes. Dilute with 100 ml of water and titrate the liberated iodine with the sodium thiosulfate solution, using starch TS as indicator. Sodium thiosulfate solutions should be restandardized frequently.

Stannous chloride R. SnCl₂·2H₂O.
A suitable commercially available reagent.

Stannous chloride AsTS.
 Procedure. Prepare from stannous chloride TS by adding an equal volume of hydrochloric acid (~250 g/l) TS, boiling down to the original volume, and filtering through a fine-grained filter-paper.

Test for arsenic. To 10 ml add 6 ml of water and 10 ml of hydrochloric acid (~250 g/l) AsTS, and distil 16 ml. To the distillate add 50 ml of water and 2 drops of stannous chloride AsTS and apply the general test for arsenic. The colour of the stain produced is not more intense than that produced from a 1-ml standard stain, showing that the amount of arsenic does not exceed 1 μg/ml.

Starch R.
A suitable commercially available reagent.

Starch, soluble, R.
A suitable commercially available reagent.

Starch TS.
 Procedure. Mix 0.5 g of starch R or of soluble starch R with 5 ml of water, and add this solution, with constant stirring, to sufficient water to produce about 100 ml; boil for a few minutes, cool, and filter.

Note. Starch TS should be freshly prepared.

Styrene-divinylbenzene copolymer.
Porous, rigid, cross-linked polymer beads. Several grades are available with different sizes of beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.
A suitable commercially available material.

Sucrose R. C₁₂H₂₂O₁₁.
A suitable commercially available reagent.

Sudan red G R. 1-(4-Phenylazophenylazo)-2-naphthol; sudan III; solvent red 23; C.I. 26100; C₂₂H₁₆N₄O.
Description. A reddish brown powder.
Solubility. Practically insoluble in water; soluble in chloroform R.
A suitable commercially available reagent.

Sulfuric acid R. H₂SO₄
A suitable commercially available reagent.
Sulfuric acid (~1760 g/l) TS. \( d \approx 1.84. \)
A suitable commercially available reagent.

**Sulfuric acid (~300 g/l) TS.**

*Procedure.* Add 171 ml of sulfuric acid (~ 1760 g/l) TS to sufficient water to produce 1000 ml (approximately 3 mol/l).

**Sulfuric acid (~ 37 g/l) TS.**

*Procedure.* Add 21.5 ml of sulfuric acid (~ 1760 g/l) TS to sufficient water to produce 1000 ml (approximately 0.375 mol/l).

**Tetracycline R.** \( \text{C}_{22}\text{H}_{24}\text{N}_{2}\text{O}_{8}. \)
A suitable commercially available reagent.

**N,N,N’,N’-Tetramethyl-p-phenylenediamine dihydrochloride R.** \( \text{C}_{10}\text{H}_{16}\text{N}_{2}\text{H}_{2}\text{Cl}_{2}. \)

*Description.* Whitish grey crystals.
A suitable commercially available reagent.

**Toluene R.** \( \text{C}_7\text{H}_8. \) Methylbenzene.
A clear, colourless, flammable liquid, very slightly soluble in water, miscible with alcohol. \( d_{20}^0: 0.865 \text{ to } 0.870. \)
bp: about 110 °C.
A suitable commercially available reagent.

**Trifluoroacetic acid (TFA).** \( \text{CF}_3\text{COOH}. \)
A suitable commercially available reagent.

**2,2,4-Trimethylpentane R.** \( \text{C}_8\text{H}_{18}. \)
A suitable commercially available reagent.

**Water, carbon-dioxide-free, R.** Water that has been boiled vigorously for a few minutes and protected from the atmosphere during cooling and storage.

**Yeast extract, water-soluble, R.**
A suitable commercially available reagent.

**Zinc R, Zn; granulate, powder, or dust.**
A suitable commercially available reagent.

**Zinc, AsR, granulated.** Granulated zinc R that complies with the following tests:

*Limit of arsenic.* Add 10 ml of stannated hydrochloric acid (~ 250 g/l) AsTS to 50 ml of water, and apply the general test for arsenic; use 10 g of granulated zinc R and allow the reaction to continue for 1 hour; no visible stain is produced.

*Test for sensitivity.* Repeat the test for arsenic with the addition of 0.1 ml of dilute arsenic AsTS; a faint, but distinct yellow stain is produced.

**Zinc acetate R.** \( \text{C}_4\text{H}_6\text{O}_4\text{Zn},2\text{H}_2\text{O}. \)
A suitable commercially available reagent.

**Zinc acetate/aluminium chloride TS.**

*Procedure.* Dissolve 200 g of zinc acetate R and 5 g of aluminium chloride R in sufficient water to produce 1000 ml.