1.1 HERBAL MEDICINES

1.1.1 Introduction

Herbal drugs, the natural alternatives for chemical drugs are defined as “the art and science of restoring a sufferer to health by the use of plant remedies”. According to European Union definitions, herbal medicinal products (medicines) are “medicinal products containing as active ingredients exclusively plant material and/or vegetable drug preparations.” Herbal drug technology includes all the steps that are involved in converting botanical materials into medicines, where standardization and quality control with proper integration of modern scientific techniques and traditional knowledge will remain important.

All countries where medicinal plants and traditional medicines used are aware of the need for regulating the use of these medicinal substances. Some countries like India and China where the traditional systems continue to be used as a form of medicine have a heritage in the use of these substances long before the modern systems are used. In other countries the main system of medicine is the well developed modern system of medicine but traditional folk medicine, without teaching our textbooks has always been, to some extent, practiced in the countries for examples United Kingdom and Germany. Finally, countries like Canada and Australia where herbal medicine are not used, to any appreciable extent, in the past and the system of medicine always in use has been the modern allopathic system of medicine. However, there is a need for these countries also to regulate the use of medicinal plants because of migrant population who would like to continue to use the herbal
remedies they have been used to and because there is a growing interest in herbal medicines in the population of these countries\textsuperscript{2}.

Herbal drugs are finished labelled products that contain active ingredients such as aerial or underground parts of plant or other plant material or combinations thereof, whether in the crude state or as plant preparations. They are not the medicines that containing plant material combined with chemically defined active substances including isolated chemical constituents of plants.

When medicinal and aromatic plants and products derived from them is discussed by the general public, the notion about the exact meaning of these terms is more or less vague, unfortunately sometimes even in the academically trained profession of pharmacists. Everything comes to mind, from sliced greenery and salads recommended in grandma’s diary to grandpa’s home distilled spice brandy which he used to wash away their bad taste. In our context the discussion will solely focus on the medicinal use of herbal drugs and medicinal products derived thereof. Thus, in order to have a common base of understanding, one needs to have a common understanding about the definitions for Herbal drug, Herbal drug preparation and Herbal medicinal product.

**Herbal medicinal products** are medicinal products containing as active substances exclusively herbal drugs or herbal drug preparations.

**Herbal drugs** are plants or part of plants in an unprocessed state, which are used for a medicinal or pharmaceutical purpose. A herbal drug or a preparation thereof is regarded as one active substance in its entirely whether or not the constituents with therapeutic activity are known.
Herbal drug preparations are comminute or powdered herbal drugs, extracts, tinctures, fatty or essential oils, expressed juices, processed resins or gums, etc prepared from herbal drugs, and preparations whose production involves a fractionation, purification or concentration process. Other components such as solvents, diluents, preservatives may form part of herbal drug preparations. These terms used are the official definitions of the European pharmacopeia. In addition to these or variants of these definitions, still in use in different parts of the world, are:

Medicinal plant, Crude plant material, Vegetable drug, Botanicals, Article of botanical origin, Herbal substance for Herbal drugs, Plant preparations, Phytomedicines for Herbal drug preparations or Herbal medicinal Products.

1.1.2 Classification of Herbal Medicines

Herbal medicines are classified in various ways as mentioned below:

1. For practical purposes, herbal medicines can be classified into four categories, based on their origin, evolution and the forms of current usage. While these are not always mutually exclusive, these categories have sufficient distinguishing features for a constructive examination of the ways in which safety, efficacy and quality can be determined and improved.

Category 1: Indigenous herbal medicines

This category of herbal medicines is historically used in a local community or region and is very well known through long usage by the local population in terms of its composition, treatment and dosage. Detailed information on this category of traditional medicine, which also includes folk medicines, may or may not be available. It can be used freely by the local community or in the local region. However, if the medicines in this category enter the market or go
beyond the local community or region in the country, they have to meet the requirements of safety and efficacy laid down in the national regulations for herbal medicines.

Category 2: Herbal medicines in systems

Medicines in this category have been used for a long time. These are documented with their special theories and concepts, and accepted by the countries. For example, Ayurveda, Unani and Siddha would fall into this category of TM.

Category 3: Modified herbal medicines

These are herbal medicines as described above in categories 1 and 2, except that they have been modified in some way—either shape, or form including dose, dosage form, mode of administration, herbal medicinal ingredients, methods of preparation and medical indications. They have to meet the national regulatory requirements of safety and efficacy of herbal medicines.

Category 4: Imported products with a herbal medicine base

This category covers all imported herbal medicines including raw materials and products. Imported herbal medicines must be registered and marketed in the countries of origin. The safety and efficacy data have to be submitted to the national authority of the importing country and need to meet the requirements of safety and efficacy of regulation of herbal medicines in the recipient country⁴.
2. The second type of classification is as follows:

**Single or crude drug**:  
They are mainly whole, fragmented or cut plants, plant parts usually dried form, but sometimes fresh. They also include algae, fungi, lichens and certain exudates that have not been subjected to a specific treatment.

**Multi herbal formulation**:  
They are the formulations which are obtained by subjecting the herbal ingredients to various manufacturing processes such as extraction, distillation, expression, fractionation, purification, concentration, fermentation.

3. The third type of classification:

**Phytotherapeutics** or **Phytopharmaceuticals** sold as Over The Counter (OTC) products in modern dosage forms such as Tablets, Capsules & Liquids for oral use.

**Dietary Supplements** containing Herbal Products, also called Nutraceuticals available in modern dosage forms.

**Herbal Medicines** consisting of either Crude, Semi – Processed or Processed Medicinal Plants.

### 1.1.3 Requirements for Assessment of Safety of Herbal Medicines

A drug is defined as being safe if it causes no known or potential harm to users. There are three categories of safety those need to be considered, as these would dictate the nature of the safety requirements that would have to be ensured.

**Category 1:** safety established by use over long time

**Category 2:** safe under specific conditions of use (such herbal medicines should preferably be covered by well-established documentation)
Category 3: herbal medicines of uncertain safety (the safety data required for this class of drugs will be identical to that of any new substance)

Data will be required on the following:

• Acute toxicity
• Long-term toxicity

Data may also be necessary on the following:

• Organ-targeted toxicity
• Immunotoxicity
• Embryo/fetal and prenatal toxicity
• Mutagenicity/genotoxicity
• Carcinogenicity

General considerations for assessment of safety of herbal medicines

Any assessment of herbal medicines must be based on unambiguous identification and characterization of the constituents. A literature search must be performed. This should include the general literature such as handbooks specific to the individual form of therapy, modern handbooks on phytotherapy, phytochemistry and pharmacognosy, articles published in scientific journals, official monographs such as WHO monographs, national monographs and other authoritative data related to herbal medicines and, if available, database searches in online or offline databases, e.g. WHO adverse drug reaction database, National Library of Medicine’s Medline, etc. The searches should not only focus on the specific herbal medicinal preparation, but should include different parts of the plant, related plant species and information originating from chemotaxy. Toxicological information on single ingredients should be assessed for its relevance to the herbal medicines.
Specific requirements for assessment of safety of four categories of herbal medicines

Before any category of herbal medicine listed above is introduced into the market, the relevant safety category needs to be reviewed and the required safety data obtained, based on that particular safety category.

Category 1: Indigenous herbal medicines

These can be used freely by the local community or region, and no safety data would be required. However, if the medicines in this category are introduced into the market or moved beyond the local community or region, their safety has to be reviewed by the established national drug control agency. If the medicines belong to safety category 1, safety data are not needed.

Category 2: Herbal medicines in systems

The medicines in this category have been used for a long time and have been officially documented. Review of the safety category is necessary. If the medicines are in safety categories 1 or 2, safety data would not be needed. If the medicines belong to safety category 3, they have to meet the requirements for safety of ‘herbal medicines of uncertain safety.

Category 3: Modified herbal medicines

The medicines in this category can be modified in any way including dose, dosage form, mode of administration, herbal medicinal ingredients, methods of preparation, or medical indications based on categories 1 and 2. The medicines have to meet the requirements of safety of herbal medicines or requirements for the safety of ‘herbal medicines of uncertain safety’, depending on the modification.
Category 4: Imported/exported products with a herbal medicine base

Exported products shall require safety data, which have to meet the requirements for safety of herbal medicines or requirements for safety of ‘herbal medicines of uncertain safety’, depending on the safety requirement of the importing/recipient countries.

Regulatory Requirements

It is a known fact that millions of people around the world will always use herbal medicines because they believe in them. They also regard it as “their” system of medicine. These people only deal with practitioners whom they have always known and with whom they are comfortable. Most of the government bodies in third world countries, and increasingly in the western world, treat this faith of the population in traditional medicine and herbal remedies as an asset. We should also remember that many people in Europe, USA, and Japan are turning towards alternative medicine, due largely to the fact that there are frequent side effects to be faced by taking powerful synthetic allopathic drugs. Indeed, most of the people in these countries refer to the herbal medicines systems as Alternative Medicine System.

It is essential to know what regulatory and legislative controls on the manufacture and sale of such herbal medicines exist or required to be implemented in various places around the world. Linked to this area, of course the issues of quality control, both of the raw material and the finished product, and of standardization of herbal medicines.
1.1.4 WHO on Botanicals

World health organization (WHO) has tried to establish internationally recognizable regulatory guidelines to define basic criteria for the evaluation of quality, safety and efficacy of botanical medicines. WHO assists national regulatory authorities, scientific organization and manufactures to undertake an assessment of the documentation/submissions /dossiers in respect of such products. Guidelines for assessing the quality of such products. Guidelines for assessing the quality of botanical materials mainly emphasize the need to ensure the quality of medicinal plant products by using the modern techniques and applying suitable standards. A series of tests for assessing the quality of medicinal plant material have been described. For physical evaluation, parameters like ash value, extraction matter, volatile matter etc. have been suggested. Pharmacological evaluation has been recommended for certain norms like bitterness value and haemolytic activity. Detection of pesticidal residue, arsenic and heavy metal content, microbial load and radioactive contaminants has been suggested for safety of the botanical materials.

In 1997, WHO developed draft guidelines for methodology on research and evaluation of traditional medicine(TM). It mainly focuses on current major debates on safety and efficacy of traditional medicine. It also tries to provide answer for some of the challenging questions concerning evidence base of the evaluation of botanical medicine, and also recommend new approaches for carrying out clinical research. Specific objectives of these guidelines are to harmonize the use of certain accepted and important terms in TM.

Under the overall context of quality of botanical medicines, WHO developed the Guidelines on Good Agricultural and collection Practices (GACP) for
medicinal plants. GACP provides general technical guidance on obtaining medicinal plant materials of good quality for the sustainable production of herbal products classified as medicines. The main objectives of these guidelines are to guide the formulation of national and/or regional GACP guidelines and GACP monographs for medicinal plants and related standard operating procedures and to encourage and support the sustainable cultivation of medicinal plants of good quality.

WHO also has published monographs for selected medicinal plants. It will provide models to assist member states in developing their own monographs or formularies for these and other herbal medicines and facilitate information exchange among Member States. However, these are not pharmacopoeial monographs, rather they are comprehensive scientific references for drug regulatory authorities, physicians, traditional health practitioners, pharmacists, manufacturers, research scientists and the general public.

### 1.1.5 Herbal Drug Regulations in India

Recognizing the global demand, Government of India has realized Good Manufacturing Practices (GMPs) for the pharmacies manufacturing Ayurvedic, Siddha and Unani medicines to improve the quality and standard of drugs. The new rules came into force from June 2000 as an amendment to the Drugs and Cosmetics Act, 1940. These rules give details regarding essential infrastructure, personnel and quality control requirements for herbal drug manufacturing. Implementation of GMP requirements is mandatory to the industry. Qualifying units can get the GMP certificate immediately. Exemption has been given to the registered practitioners and teaching institutions that prepare medicines for their patients. Department of Indian
Systems of Medicine and Homeopathy (ISM&H) is trying to frame safety and efficacy regulations for licensing new patent and proprietary botanical medicines. Indian Pharmacopoeia covers few Ayurvedic medicines. Monographs have been given for some ayurvedic drugs like clove, guggul, opium, menthe, senna, the ayurvedic pharmacopoeia of India gives monographs for 258 different Ayurvedic drugs. The standards mentioned are quite inadequate to build quality of the botanical materials. Indian Drug Manufacturers Association (IDMA) has published Indian Herbal Pharmacopoeia (2002) with 52 monographs of widely used medicinal plants found in India. The latest available scientific data has been incorporated in these monographs.

Provision relating to the manufacture and control of Ayurvedic, Siddha and Unani drugs has been prescribed in the Drugs and Cosmetics Act. The individual section being described here is based on the regulation as prescribed in the Drugs and Cosmetics Act 1940, for the Ayurvedic, Siddha and Unani drugs. In the following section, the same relating to these systems is being described as mentioned in different sections of Drugs Act.

1.1.6 Regulatory Aspects and Approval of Herbal Drugs in Different Countries

The legal process of regulation and legislation of herbal medicines changes from country to country. The reason for this involves mainly cultural aspects and also the fact that herbal medicines are rarely studied scientifically. Thus, few herbal preparations have been tested for safety and efficacy. The WHO has published guidelines in order to define basic criteria for evaluating the quality, safety, and efficacy of herbal medicines aimed at assisting national
regulatory authorities, scientific organizations and manufacturers in this particular area. Furthermore, the WHO has prepared pharmacopoeic monographs on herbal medicines and the basis of guidelines for the assessment of herbal drugs. Several regulatory models for herbal medicines currently exist, including prescription drugs, over-the-counter drugs, traditional medicines and dietary supplements. Thus, the need to establish global and/or regional regulatory mechanisms for regulating herbal drugs seems obvious. A summary of the regulatory processes related to herbal drugs in some selected countries is presented below.

1.1.6.1 United States of America

1.1.6.1.1 Drug regulation 1906-1962

In USA many act and amendments were made to campaign against the unscrupulous practices of food and drug industries. But this all acts were centered of allopathic drug and herbal medicines were ignored. The different acts focused on particular single issue and did not cover the herbal medicines concept.

- Food and Drugs Act (1906): Required only that drug meet standards of strength and purity.
- Federal Food, Drug, and Cosmetic Act (1938): Required the manufacturer to prove the safety of a drug before it could be marketed.
- Durham-Humphrey Amendment (1951): defined prescription drugs as those unsafe for self-medication and which, therefore, should be used only under a doctor’s supervision.
- Kefauver-Harris Drug Amendments (1962): Before marketing a drug, manufacturer must prove not only safety, but also effectiveness for the
product’s intended use. Herbal medicines were grandfathered as drug but the FDA put them in regulatory limbo be sold as foods.

- Nutrition Labeling and Education Act (1990): required consistent, scientifically based labeling for almost all processed foods. Herbal medicines left in limbo.

- Dietary Supplement Health and Education Act (1994): Includes herbal medicines in the definition of a dietary supplement, assures consumers access to all supplements on the market as long as they are not unsafe, and allows for structure and function claims on the label. Since 1994, herbal medicines have been regulated under the Dietary Supplement Health and Education Act of 1994. On the basis of this law, herbal medicines are not evaluated by the Food and Drug Administration and, most important, these products are not intended to diagnose, treat, cure, or prevent diseases.

In USA, herbal remedies are referred to as homeopathic remedies. All such remedies, because these are offered for treatment of disease, are regarded as drugs. This means that if a herbal remedy is included in United States Pharmacopoeia, the official Homeopathic Pharmacopoeia or the National formulary, it will be recognized officially as a drug.

1.1.6.1.2 Some of the general guidelines used in USA are:

- Traditional herbal medicines or currently marketed botanical products, because of their extensive though uncontrolled use in humans, may require less preclinical information to support initial clinical trials than would be expected for synthetic or highly purified drugs.

- Requirements for Investigational New Drug (IND) applications of botanicals legally marketed in the United States as dietary supplements or
cosmetics. Very little new chemistry manufacturing and controls (CMC) or toxicologic data are needed to initiate early clinical, if there are no known safety issues associated with the product and it is used at approximately the same doses as those currently or traditionally used or recommended.

As the product is marketed and the dose thought to be appropriate and well tolerated is known, there should be little need for pilot or typical Phase 1 studies. Sponsors are allowed to initiate more definitive efficacy trials early in the development program. If there is doubt about the best dose of the product tested, a randomized, parallel, dose-response study may be particularly useful as an initial trial.

- Requirements for botanical product that has not been previously marketed in the United States or anywhere in the world

Certain additional information (CMC, toxicology, human use) is required to assist FDA in determining the safety of the product for use in initial clinical studies.

If the product is prepared, processed and used according to methodologies for which there is prior human experience, sufficient information may be available to support such studies without standard preclinical testing.

- Clinical trials of botanical products

There may be special problems associated with the incorporation of traditional methodologies, such as selection of doses and addition of new botanical ingredients based on response, which will need to be resolved.

The credible design for clinical trials studies will be randomized, double blind, and placebo-controlled (or dose-response). For most conditions
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potentially treated by botanical drugs (generally mildly symptomatic), active
control equivalence designs would not be credible.

For expanded i.e., Phase 3 clinical studies on a botanical drug product, more
detailed information on CMC and preclinical safety is necessary as compared
to the information required for a Phase 1 or Phase 2 study. This additional
information should be provided regardless of whether the product is currently
lawfully marketed in the United States or elsewhere as a dietary supplement.

All study data should conform to standard ethical guidelines of good clinical
practice (informed consent, approval from ethics committee) for all clinical
trials.

• Documentation for early trials (IND)
  Description of Product and Documentation of Human Use
  Description of Botanicals Used
  History of Use
  Current Investigational Use
  Chemistry, Manufacturing, and Controls Botanical Raw Material
  Botanical Drug Substance
  Botanical Drug Product
  Placebo
  Labelling
  Environmental Assessment or Claim of Categorical Exclusion
  Pharmacology/Toxicology Information
  • Exclusive marketing rights
1.1.6.2 Australia

Complementary medicine, including botanical medicines in Australia is regulated under therapeutic goods legislation. For managing the risk associated with therapeutic goods, it undergoes processes of manufacturers, pre-market assessment of product and post-market regulatory activity. Based on risk, Australia has developed two approaches for regulation of these therapeutic goods. Listed medicines are considered to be of lower risk than registered medicines. Most, but not all, complementary medicines are Listed medicines, which are individually assessed by the Therapeutic Goods Administration for compliance with legislation. They are not evaluated before release. They may only be formulated from ingredients that have undergone pre-market evaluation for safety and quality and are considered at low risk. Listed complementary medicines may only carry indications and claims for the symptomatic relief of non serious conditions, health maintenance, health enhancement and risk reduction. Registered medicines are individually evaluated for safety, quality and efficacy before they are released onto the market. An important feature of risk management in Australia is that early market access for low risk complementary medicines is supported by appropriate post-market regulatory activity.

1.1.6.3 China

In China, it is quite legal to sell medicinal plants and herbal in the free market, both in rural and urban areas. This would account for a large volume of the use of herbal remedies in the country. However, if a new medicinal plant product or a crude drug is to be imported from abroad to be sold in the Chinese market, then the approval of the provincial department of public
health is required. The Pharmacopoeia People’s Republic of China has got a section on “Standard for Processing of Chinese Materia Medica”. This new plant material or crude material being imported will have to be assessed according to the standards in the Pharmacopoeia and approval either given or not given. The origin of crude drug or of herbal product must always be clearly marked.

1.1.6.4 Brazil

Despite its immense flora, cultural aspect and the widespread use of herbal medicine, so far few efforts have been made in Brazil to establish the quality, safety and efficacy of these products. In 1994, the Ministry of Health created a commission to evaluate the situation of phytotherapeutic agents in Brazil. The commission proposed a directive based mainly on German and French regulations and on WHO guidelines for herbal drugs. In 1995, Directive Number 6 established the legal requirement for the registration of herbal drugs and defined the phytopharmaceutical product as a processed drug containing as active ingredients exclusively plant material and/or plant drug preparations. They are intended to treat, cure, alleviate, prevent and diagnose diseases. The legal requirements for registration of herbal medicines in Brazil demand complete documentation of efficacy, safety and well defined quality control.

1.1.6.5 Canada

In 1986, the Canadian Health Protection Branch (HPB) constituted a special committee (3 pharmacists, 2 herbalists, 1 nutritionist and 1 physician) and classified herbal drugs as Folk Medicine. The regulation is based on traditional uses, as long as the claim is validated by scientific studies. In 1990,
the HPB listed 64 herbs that were considered to be unsafe. In 1992, the HPB submitted a regulatory proposal to the Canadian Parliament and listed another 64 herbs that were considered to be adulterants. The Canadian regulatory system is consistent with WHO guidelines for the assessment of herbal medicines.

1.1.6.6 Germany

Germany’s Commission E (phytotherapy and herbal substances) was established in 1978. It is an independent division of the German Federal Health Agency that collects information on herbal medicines and evaluates them for safety and efficacy. The following methods and criteria are followed by Commission E: 1) traditional use; 2) chemical data; 3) experimental, pharmacological and toxicological studies; 4) clinical studies; 5) field and epidemiological studies; 6) patient case records submitted from physician’s files, and 7) additional studies, including unpublished proprietary data submitted by manufacturers. Two kinds of monographs are prepared: monopreparations and fixed combinations. The composition of Commission E is as follows: physicians, pharmacists, pharmacologists, toxicologists, industry representatives and laypersons, for a total of 24 members. Three possibilities for marketing herbal drugs exist: 1) temporary marking authorization for old herbal drugs until they are evaluated for safety and efficacy; 2) monographs of standardized marketing authorization, and 3) individual marketing authorization. Evaluations are published in the form of monographs that approve or disapprove the herbal drugs for over-the-counter use. Herbal medicines are sold in pharmacies, drugstores and health food stores. Some herbal medicines are controlled by a physician’s prescription.
Commission E has published about 300 monographs: 200 positives and 100 negatives. About 600-700 plants are sold in Germany. Approximately 70% of physicians prescribe registered herbal drugs. Part of annual sales is paid for by government health insurance.  

1.2 GLOBAL TRENDS & MARKET DRIVERS OF HERBAL PRODUCTS

1.2.1 Introduction

The herbs and botanicals market, as it applies to the dietary supplement, self-medication and functional food segments, is driven by consumer demographics and health concerns. Broadly speaking, these trends include anti-aging, weight control, joint and bone health, digestion/immunity, cardiovascular health/diabetes, cognition/memory, female/male health and the growing wellness and beauty trends. Another trend benefiting the herbs and botanicals market is the natural and exotic ingredients trend, which is taking off in functional foods, as well as medicinal products.

With the continued sedentary and hectic lifestyles of industrialized regions of the world and the relative increase of the senior segment of the world population, these trends are expected to grow.

At the same time, consumer education about the functional benefits of herbs and botanicals is increasing. Together with increased confidence due to solid science behind the products, market entry for new ingredients is becoming easier. On the other hand, bad press continues to affect the herbs and botanicals market, most notably whenever an herb, such as St. John's Wort, ginkgo or black cohosh, is reaching a certain market size and beginning to infringe upon the profits of synthetic competitors.
1.2.2 Market Size & Growth

The global market for herbal remedies across all segments (excluding soy, algae and fiber) currently brings in about $83 billion. Depending on the segment, growth is steady, ranging between 3% and 12%. Herbal dietary supplements ($11 billion) and herbal functional foods ($14 billion) make up over a third of the market. The global herbal pharmaceutical industry (including drugs from herbal precursors and registered herbal medicine) contributes $44 billion. Herbal beauty products make up the remaining $14 billion of the market.

In the global cosmetics market, herbal ingredients are estimated to have a 6% share of the market, and are exhibiting the strongest growth, between 8% and 12%.

In terms of geography, the global herbal medicines/ supplement market is divided among Germany (28%), Asia (19%), Japan (17%), France (13%), Rest of Europe (12%) and North America (11%).

In 2006, the top three herbs featured globally in medicines, supplements and functional foods were ginseng, ginkgo and noni. Table 1 shows the sales for these and other popular herbs worldwide.

This list is not expected to change much through 2008, though it is possible that plants currently under attack by the synthetic pharmaceuticals industry may decline in sales going forward. To offset this, other ingredients such as Coleus forskohlii and the so-called "superfruits" are soaring. However, there is currently no obvious single herbal "blockbuster" or rising star among the botanicals.
Among preferred botanicals used in cosmeceuticals are grape seed, bilberry, acerola, baobab, turmeric, ginkgo biloba, white and green tea, red clover, soy, tomato, comfrey, papaya, rosemary, wheat, evening primrose oil, sweet potatoes, carrots, olives, flax, aloe vera, coffee plant, centella asiatica, avocado and passion fruit.

### 1.2.3 Around the Globe

#### 1.2.3.1 Asia

In 2003, the Asian market for herbal supplements and herbal medicines (excluding Japan) brought in $2.4 billion in sales, which rose to $5.8 billion in 2004 and $6 billion in 2005. Today, the Asian market (excluding Japan) is estimated to be worth about $6.4 billion. This increase is expected to continue. In Japan alone, the market is worth well over $2.6 billion today.

#### 1.2.3.2 U.S.

In 2005, U.S. herb and botanical sales were at $4.4 billion, with an estimated growth rate of nearly 4%. Retail sales of herbal/botanical products suffered a decline due to bad press and politics surrounding controversial ingredients in 2003, but the market seems to have recovered since, at least marginally. In 2006, sales grew to $4.6 billion, and they are currently estimated to be about $4.8 billion. This type of growth is expected to continue.

#### 1.2.3.3 Europe

The European market for herbal supplements and herbal medicines is currently worth $7.4 billion. An analysis based on data from IMS Health put the global European OTC market for herbal drugs at approximately $4.95 billion in 2003.
1.2.3.4 **Australia/New Zealand.** Herb and botanical sales are currently $0.45 billion in Australia and New Zealand. Sales development was $0.3 billion in 2003, rising to $0.4 billion in 2004 and increasingly slightly since then. The New Zealand supplement market has been growing 5% annually, rebounding following a downturn between 2000 and 2002.

1.2.3.5 **Latin America.** Sales of herbal and botanical products in Latin America are worth $0.9 billion. The sales development was flat, rising from $0.8 billion in 2003 to $0.9 billion in 2004 and holding steady.

1.2.3.6 **Rest of the World.** Estimates of herb and botanical sales in all other regions not mentioned previously, and excluding Australia/New Zealand and Latin America, are just below $1 billion. Generally, figures are hard to estimate, and the figures that do exist vary wildly from source to source. As we can see in recent years there is a spurt in the interest regarding survival of traditional systems of medication. In the global perspective, there is a shift towards the use of medicine of herbal origin, as the dangers and the shortcoming of modern medicine have started getting more apparent.

In almost all the traditional system of medicine, Rishis, Vaidyas and Hakims used to treat patients on individual basis, and prepare drug according to the requirement of the patient with consideration of the quality control aspect of individual medicines. But the scenario has changed now; herbal medicines are being manufactured on large scale in Pharmaceutical units, where manufacturers come across many problems such as availability of good quality and authentication raw material, availability of standards, proper standardization methodology of formulation, quality control parameters.
In modern concept it require necessary changes in their approach by that way concrete method of quality control in terms development of modern methodologies. Thus today quality assurance is thrust area for the evaluation of traditional used medicinal plants and herbal formulation\textsuperscript{14,15,16}. Thus standardization has become a key strategy for the acceptance of herbal medicines at global level.
1.3 STANDARDIZATION

1.3.1 Need of Standardizations

It is the cardinal responsibility of the regulatory authorities to ensure that the consumers get the medication, which guarantee purity, safety, potency and efficacy. This duty is discharged by the regulatory authorities by rigidly following various standards of quality prescribed for raw materials and finished products in pharmacopoeias controlling manufacturing formulate through the use of formularies and manufacturing operation through statutory imposed “Good manufacturing practices”. All these procedures logically applied to all types of medication whether included in modern system of medicine or one of the traditional system such as Ayurvedic system of medicine.

Herbal products have been enjoying renaissance among the customers throughout the world. However, one of the impediments in the acceptance of the plant based formulations is the lack of standard quality control profile. The quality of herbal medicine i.e. the profile of the constituents in the final product has implication in efficacy and safety. Due to complex nature and inherent variability of the constituents of plant based drugs, it is difficult to establish quality control parameters and modern analytical techniques are expected to help in circumventing this problem.\(^\text{17}\).

WHO estimates that about 80% of world population presently uses herbal medicine for some aspect of primary health care. WHO notes that of 119 plant-derived pharmaceutical medicines, about 74% are use in modern medicine in ways that are correlated directly with their traditional uses as plant medicines by native cultures.
According to WHO global survey on the national policy and regulation of traditional medicine, there are three common difficulties and challenges: lack of information sharing; lack of safety monitoring of herbal medicines; and lack of methods to evaluate the safety and efficacy. Correct identification and quality assurance of the starting material is, therefore, an essential prerequisite to ensure reproducible quality of herbal medicine, which contributes to its safety and efficacy. Reproducible safety and efficacy of phytopharmaceuticals is based on reproducible quality. Therefore a phytopharmaceutical want to be regarded as rational drugs, they need to be standardized and pharmaceutical quality must be approved. Also, in pharmacological, toxicological and clinical studies with herbal drugs, their composition needs to be well documented in order to obtain reproducible results. The WHO has recognized this problem and published guidelines to ensure the reliability and repeatability of research on herbal medicines. This concept should be followed not only in research, but also in the production and therapeutic applications of phytopharmaceuticals.¹

The quality control of crude drugs and herbal formulations is of paramount importance in justifying their acceptability in modern system of medicine. But one of the major problems faced by the herbal drug industry is nonavailability of rigid quality control profile for herbal material and their formulations.

Plant material and herbal remedies derived from them represent substantial portion of global market and in this respect internationally recognized guidelines for their quality assessment and quality control are necessary. WHO has emphasized the need to ensure quality control of medicinal plant
products by using modern technique and by applying suitable parameters and standards. In order to overcome certain inevitable shortcoming of the Pharmacopoeial monograph other quality control measures must be explored. Quality control has wide connotation and covers; many aspects of drug manufacture, distribution and sale is not restricted to final product analysis either regulatory or otherwise, while engaging in this task, it must be realized that some of the Quality control practices that work excellently either modern drug may not be appropriate with traditional drug \(^\text{17}\).

**1.3.2 Current Regulations for Standardization of Crude Drugs**

In India a great deal of bulk knowledge exists among ordinary people about the traditional use of herbal medicine. It is difficult to quantify the market size of the traditional Indian system, since most practitioners formulate and dispense their own recipes. The present annual turnover of product manufactured by large companies is estimated at approximately US $ 300 million compared to a turnover of approximately US $ 2.5 billions for modern drugs. According to the study on the attitude of modern medicine practitioners are relatively unfamiliar with Ayurvedic product even though some are practiced. They are willing to try an Ayurvedic product if it efficiency is scientifically proven and would try aliment such as cough, cold, diarrhea, stomach problem, reproductive disease, liver and skin disease\(^\text{18}\).

Patent proprietary Ayurvedic medicines are sold over the counter in pharmacies. These products appear to represent a major share of branded traditional medicine in India. Nevertheless systems like Ayurveda still need to gain an empirical support of modern medical sciences to make them credible and acceptable for all. An innovative research effort to define the advantage
of traditional system of medicine with respect to their safety and efficacy could result in a better utilization of these complementary systems of medicine. Internationally several pharmacopoeias have provided monographs stating parameter and standard of many herbs and some product made out of these herbs. Several pharmacopoeias like

- Chinese Herbal Pharmacopoeia
- United States Herbal Pharmacopoeia
- British Herbal Pharmacopoeia
- British Herbal Compendium
- Japanese Standards for Herbal Medicine
- The Ayurvedic Pharmacopoeia of India (API)

Lay down monograph for herbs and herbal products to maintain their quality in their respective nations. Government of India too has brought out Ayurvedic Pharmacopoeia India, which recommends basic quality parameters for eighty common Ayurvedic herbal drugs.  

1.3.3 Quality control

Quality control for efficacy and safety of herbal products is of paramount importance. Quality can be defined as the status of a drug that is determined by identity, purity, content, and other chemical, physical, or biological properties, or by the manufacturing processes. Quality control is a term that refers to processes involved in maintaining the quality and validity of a manufactured product.
1.3.3.1 In general, all medicines, whether they are of synthetic or of plant origin, should fulfill the basic requirements of being efficacious and safe, and this can be achieved by suitable clinical trials.

The term “herbal drugs” denotes plants or plant parts that have been converted into phytopharmaceuticals by means of simple processes involving harvesting, drying, and storage. A practical addition to the definition is also to include other crude products derived from plants, which no longer show any organic structure, such as essential oils, fatty oils, resins, and gums. Derived or isolated compounds in the processed state such as extracts or even isolated purified compounds (e.g. strychnine from *Strychnos nux-vomica*) or mixtures of compounds (e.g. abrin from *Abrus precatorius*) are, as a rule, not included in the definition.

1.3.3.2 In general, quality control is based on three important pharmacopoeial definitions:

1. Identity: Is the herb the one it should be?
2. Purity: Are there contaminants, e.g., in the form of other herbs which should not be there?
3. Content or assay: Is the content of active constituents within the defined limits?

It is obvious that the content is the most difficult one to assess, since in most herbal drugs the active constituents are unknown. Sometimes markers can be used which are, by definition, chemically defined constituents that are of interest for control purposes, independent of whether they have any therapeutic activity or not.
To prove identity and purity, criteria such as type of preparation, physical constants, adulteration, contaminants, moisture, ash content and solvent residues have to be checked. The correct identity of the crude herbal material, or the botanical quality, is of prime importance in establishing the quality control of herbal drugs.

**Identity** can be achieved by macro- and microscopical examinations. Voucher specimens are reliable reference sources. Outbreaks of diseases among plants may result in changes to the physical appearance of the plant and lead to incorrect identification. At times an incorrect botanical quality with respect to the labeling can be a problem. For example, in the 1990s, a South American product labeled as “Paraguay Tea” was associated with an outbreak of anticholinergic poisoning in New York. Subsequent chemical analysis revealed the presence of a class of constituents that was different from the metabolites normally found in the plant from which Paraguay tea is made.

**Purity** is closely linked with the safe use of drugs and deals with factors such as ash values, contaminants (e.g. foreign matter in the form of other herbs), and heavy metals. However, due to the application of improved analytical methods, modern purity evaluation also includes microbial contamination, aflatoxins, radioactivity, and pesticide residues. Analytical methods such as photometric analysis, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography (GC) can be employed in order to establish the constant composition of herbal preparations.

**Content or assay** is the most difficult area of quality control to perform, since in most herbal drugs the active constituents are not known. Sometimes
markers can be used. In all other cases, where no active constituent or marker can be defined for the herbal drug, the percentage extractable matter with a solvent may be used as a form of assay, an approach often seen in pharmacopeias. For example, when a herbal drug is used to make a tea, the hot water extractable matter, expressed as milligrams per gram of air-dried material, may serve this purpose. A special form of assay is the determination of essential oils by steam distillation. When the active constituents (e.g. sennosides in Senna) or markers (e.g. alkylamides in Echinacea) are known, a vast array of modern chemical analytical methods such as ultraviolet/visible spectroscopy (UV/VIS), TLC, HPLC, GC, mass spectrometry (MS), or a combination of GC and MS (GC/MS), can be employed.

1.3.3.3 Several problems not applicable to synthetic drugs influence the quality of herbal drugs:

1. Herbal drugs are usually mixtures of many constituents.
2. The active principle(s) is (are), in most cases unknown.
3. Selective analytical methods or reference compounds may not be available commercially.
4. Plant materials are chemically and naturally variable.
5. The source and quality of the raw material are variable.
6. The methods of harvesting, drying, storage, transportation, and processing (for example, mode of extraction and polarity of the extracting solvent, instability of constituents, etc.) have an effect.
Strict guidelines have to be followed for the successful production of a quality herbal drug. Among them are proper botanical identification, phytochemical screening, and standardization.

Standardization involves adjusting the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity by adding excipients or by mixing herbal drugs or herbal drug preparations. Botanical extracts made directly from crude plant material show substantial variation in composition, quality, and therapeutic effects.

Standardized extracts are high-quality extracts containing consistent levels of specified compounds, and they are subjected to rigorous quality controls during all phases of the growing, harvesting, and manufacturing processes.

No regulatory definition exists for standardization of dietary supplements. As a result, the term “standardization” may mean many different things. Some manufacturers use the term standardization incorrectly to refer to uniform manufacturing practices; following a recipe is not sufficient for a product to be called standardized. Therefore, the presence of the word “standardized” on a supplement label does not necessarily indicate product quality. When the active principles are unknown, marker substance(s) should be established for analytical purposes and standardization.

1.3.4 Quality evaluation:

Quality evaluation is a systematic examination of the extent to which an entity (part or product) is capable of meeting specified requirements. The result of quality evaluation may be used for qualification, approval and registration or accreditation purposes. A quality evaluation may be used to
determine manufacturing quality capability. One of the method for assessing quality evaluation is standardization.

Standardization is defined as adjusting the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity respectively by adding excipients or by mixing herbal drug extracts.

“Standardization” expression is used to describe all measures, which are taken during the manufacturing process and quality control leading to a reproducible quality. It also encompasses the entire field of study from birth of a plant to its clinical application. It also means adjusting the herbal drug preparation to a defined content of a constituent or a group of substances with known therapeutic activity respectively by adding excipients or by mixing herbal drugs or herbal drug preparations.

There are two types of standardization

In the first category, “true” standardization, a definite phytochemical or group of constituents is known to have activity. Ginkgo with its 26% ginkgo flavones and 6% terpenes is a classic example. These products are highly concentrated and no longer represent the whole herb, and are now considered as phytopharmaceuticals. In many cases they are vastly more effective than the whole herb.

The other type of standardization is based on manufacturers guaranteeing the presence of a certain percentage of marker compounds; these are not indicators of therapeutic activity or quality of the herb.
1.3.5 WHO Guidelines for Quality Standardized Herbal Formulations

- Quality control of crude drugs material, plant preparations and finished products.
- Stability assessment and shelf life.
- Safety assessment; documentation of safety based on experience or toxicological studies.
- Assessment of efficacy by ethnomedical informations and biological activity evaluations.
1.3.5.1 The standardization of crude drug materials include the following steps:

1. Authentication (Stage of collection, parts of the plant collected, regional status, botanical identity like phytomorphology, microscopical and histological analysis, taxonomical identity, etc.)

2. Foreign matter (herbs collected should be free from soil, insect parts or animal excreta, etc.)

3. Organoleptic evaluation (sensory characters – taste, appearance, odor, feel of the drug, etc.)

4. Tissues of diagnostic importance present in the drug powder.

5. Ash values and extractive values.

6. Volatile matter

7. Moisture content determination

8. Chromatographic and spectroscopic evaluation: TLC, HPTLC, HPLC methods will provide qualitative and semi quantitative information about the main active constituents present in the crude drug as chemical markers in the TLC fingerprint evaluation of herbals (FEH). The quality of the drug can also be assessed on the basis of the chromatographic fingerprint.

9. Determination of heavy metals – e.g. cadmium, lead, arsenic, etc.

10. Pesticide residue – WHO and FAO (Food and Agricultural Organization) set limits of pesticides, which are usually present in the herbs. These pesticides are mixed with the herbs during the time of cultivation. Mainly pesticides like DDT, BHC, toxaphene, and aldrin cause serious side-effects in human beings if the crude drugs are mixed with these agents.
11. Microbial contamination – usually medicinal plants containing bacteria and molds are coming from soil and atmosphere. Analysis of the limits of *E. coli* and molds clearly throws light towards the harvesting and production practices. The substance known as afflatoxins will produce serious side-effects if consumed along with the crude drugs. Afflatoxins should be completely removed or should not be present.

**Table No 1.1 Limits for Microbial Contamination**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Finished product (CFU/g)</th>
<th>Raw materials(CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>$10^1$</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Salmonella</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total aerobic bacteria</td>
<td>$10^5$</td>
<td>-</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>$10^3$</td>
<td>-</td>
</tr>
</tbody>
</table>

12. Radioactive contamination – Microbial growth in herbals are usually avoided by irradiation. This process may sterilize the plant material but the radioactivity hazard should be taken into account. The radioactivity of the plant samples should be checked accordingly to the guidelines of International Atomic Energy (IAE) in Vienna and that of WHO.

In order to obtain quality oriented herbal products care should be taken right from the proper identification of plants; season and area of collection, extraction, isolation and verification process.
1.3.5.2 The stability parameters for the herbal formulations which includes physical parameters, chemical parameters, and microbiological parameters.

1. Physical parameters include color, appearance, odor, clarity, viscosity, moisture content, pH, disintegration time, friability, hardness, flowability, flocculation, sedimentation, settling rate and ash values.

2. Chemical parameters includes limit tests, extractive values, chemical assays, etc.

Chromatographic analysis of herbals can be done using TLC, HPLC, HPTLC and GC, UV, Fluorimetry, GC-MS, etc.

3. Microbiological parameters include total viable content, total mold count, total enterobacterial and their count. Limiters can be utilized as a quantitative or semiquantitative tool to ascertain and control the amount of impurities like the reagents used during abstraction of various herbs, impurities coming directly from the manufacturing vessels, impurities from the solvents, etc.

4. Chemical decomposition of substances present in the formulation also produces several toxic or impure compounds during storage in undesirable conditions. Contaminants may come directly from the atmosphere also. This include mainly dust, sulfur dioxide, H₂S, CO₂, Arsenic, moisture, etc.
1.3.5.3 The brief description of WHO guidelines are as follows: 20,23-28

1. Organoleptic evaluation

Organoleptic evaluation of drugs refers to the evaluation of a drug by colour, odour, size, shape, taste and special features including touch, texture etc. Since the majority of information on the identity, purity and quality of the material can be drawn from these observations, they are of primary importance before any further testing can be carried out.

For this purpose authentic specimen of the material under study and samples of pharmacopoeial quality should be available to serve as a reference.

This evaluation procedure provides the simplest and quickest means to establish the identity and purity and thereby ensure quality of a particular sample.

If it is found to be devoid of or significantly different from the specified sensory characters like colour, consistency, odour, etc., it is considered as not fulfilling the requirements.

However judgment based on the sensory characteristics like odour, taste etc., may vary from person to person and time to time based on individual's nature. So the description of this features are very difficult so that often the characteristic like odour and taste can only described as 'characteristic' and reference made to the analyst's memory.

No preliminary treatment is necessary for evaluating the sample in this manner excepting the softening and stretching of the wrinkled and contracted leaves and flowers etc.
2. Botanical Evaluation

Quality control of herbal drugs has traditionally been based on appearance and today microscopic evaluation is indispensable in the initial identification of herbs, as well as in identifying small fragments of crude or powdered herbs, and detection of foreign matter and adulterants. A primary visual evaluation, which seldom needs more than a simple magnifying lens, can be used to ensure that the plant is of the required species, and that the right part of the plant is being used. At other times, microscopic analysis is needed to determine the correct species and/or that the correct part of the species is present. For instance, pollen morphology may be used in the case of flowers to identify the species, and the presence of certain microscopic structures such as leaf stomata can be used to identify the plant part used. Although this may seem obvious, it is of prime importance, especially when different parts of the same plant are to be used for different treatments. Stinging nettle (Urtica urens) is a classic example where the aerial parts are used to treat rheumatism, while the roots are applied for benign prostate hyperplasia.

3. Physical Evaluation

a. Determination of Foreign Matter

Herbal drugs should be made from the stated part of the plant and be devoid of other parts of the same plant or other plants. They should be entirely free from moulds or insects, including excreta and visible contaminant such as sand and stones, poisonous and harmful foreign matter and chemical residues. Animal matter such as insects and “invisible” microbial contaminants, which can produce toxins, are also among the potential contaminants of herbal medicines. Macroscopic examination can easily be employed to determine the
presence of foreign matter, although microscopy is indispensable in certain special cases (for example, starch deliberately added to “dilute” the plant material). Furthermore, when foreign matter consists, for example, of a chemical residue, TLC is often needed to detect the contaminants.

b. Determination of Ash

To determine ash content the plant material is burnt and the residual ash is measured as total and acid-insoluble ash. Total ash is the measure of the total amount of material left after burning and includes ash derived from the part of the plant itself and acid-insoluble ash. The latter is the residue obtained after boiling the total ash with dilute hydrochloric acid, and burning the remaining insoluble matter. The second procedure measures the amount of silica present, especially in the form of sand and siliceous earth.

c. Determination of Heavy Metals

Contamination by toxic metals can either be accidental or intentional. Contamination by heavy metals such as mercury, lead, copper, cadmium, and arsenic in herbal remedies can be attributed to many causes, including environmental pollution, and can pose clinically relevant dangers for the health of the user and should therefore be limited.

A simple, straightforward determination of heavy metals can be found in many pharmacopeias and is based on color reactions with special reagents such as thioacetamide or diethylthiocarbamate, and the amount present is estimated by comparison with a standard.

Instrumental analyses have to be employed when the metals are present in trace quantities, in admixture, or when the analyses have to be quantitative. The main methods commonly used are atomic absorption spectrophotometry
Standardization of Some Plant-Based Formulations By Modern Analytical Techniques

4. Determination of Microbial Contaminants and Aflatoxins

Medicinal plants may be associated with a broad variety of microbial contaminants, represented by bacteria, fungi, and viruses. Inevitably, this microbiological background depends on several environmental factors and exerts an important impact on the overall quality of herbal products and preparations.

Herbal drugs normally carry a number of bacteria and molds, often originating in the soil. Poor methods of harvesting, cleaning, drying, handling, and storage may also cause additional contamination, as may be the case with *Escherichia coli* or *Salmonella* spp. While a large range of bacteria and fungi are from naturally occurring microflora, aerobic spore-forming bacteria frequently predominate.

Laboratory procedures investigating microbial contaminations are laid down in the well-known pharmacopeias, as well as in the WHO guidelines. In general, a complete procedure consists of determining the total aerobic microbial count, the total fungal count, and the total Enterobacteriaceae count, together with tests for the presence of *Escherichia coli*, *Staphylococcus aureus*, *Shigella*, and *Pseudomonas aeruginosa* and *Salmonella* spp. The European Pharmacopoeia also specifies that *E. coli* and *Salmonella* spp. should be absent from herbal preparations. However it is not always these two pathogenic bacteria that cause clinical problems. For example, a fatal case of listeriosis was caused by contamination of alfalfa tablets with the Gram positive bacillus *Listeria monocytogenes*. 

(AAS), inductively coupled plasma (ICP) and neutron activation analysis (NAA).
Materials of vegetable origin tend to show much higher levels of microbial contamination than synthetic products and the requirements for microbial contamination in the European Pharmacopoeia allow higher levels of microbial contamination in herbal remedies than in synthetic pharmaceuticals. The allowed contamination level may also depend on the method of processing of the drug.

The presence of fungi should be carefully investigated and/or monitored, since some common species produce toxins, especially aflatoxins. Aflatoxins in herbal drugs can be dangerous to health even if they are absorbed in minute amounts. Aflatoxin-producing fungi sometimes build up during storage. Procedures for the determination of aflatoxin contamination in herbal drugs are published by the WHO. After a thorough clean-up procedure, TLC is used for confirmation.

5. Determination of Pesticide Residues

Even though there are no serious reports of toxicity due to the presence of pesticides and fumigants, it is important that herbs and herbal products are free of these chemicals or at least are controlled for the absence of unsafe levels. Herbal drugs are liable to contain pesticide residues, which accumulate from agricultural practices, such as spraying, treatment of soils during cultivation, and administering of fumigants during storage. However, it may be desirable to test herbal drugs for broad groups in general, rather than for individual pesticides. Many pesticides contain chlorine in the molecule, which, for example, can be measured by analysis of total organic chlorine. In an analogous way, insecticides containing phosphate can be detected by measuring total organic phosphorus.
Samples of herbal material are extracted by a standard procedure, impurities are removed by partition and/or adsorption, and individual pesticides are measured by GC, MS, or GC/MS. Some simple procedures have been published by the WHO and the European Pharmacopoeia has laid down general limits for pesticide residues in medicine.

6. Determination of Radioactive Contamination

There are many sources of ionization radiation, including radionuclides, occurring in the environment. Hence a certain degree of exposure is inevitable. Dangerous contamination, however, may be the consequence of a nuclear accident. The WHO, in close cooperation with several other international organizations, has developed guidelines in the event of a widespread contamination by radionuclides resulting from major nuclear accidents. These publications emphasize that the health risk, in general, due to radioactive contamination from naturally occurring radionuclides is not a real concern, but those arising from major nuclear accidents such as the nuclear accident in Chernobyl, may be serious and depend on the specific radionuclide, the level of contamination, and the quantity of the contaminant consumed. Taking into account the quantity of herbal medicine normally consumed by an individual, they are unlikely to be a health risk. Therefore, at present, no limits are proposed for radioactive contamination.

7. Biological evaluation

Pharmacological activity of certain drugs has been applied to evaluate and standardize them. The assays on living animal and on their intact or isolated organs can indicate the strength of the drug or their preparations. All living organism are used, these assays are known as Biological assays or Bioassay.
Analytical Methods

The quantitative determination of constituents has been made easy by recent developments in analytical instrumentation. Recent advances in the isolation, purification, and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the determination and analysis of quality and the process of standardization of herbal preparations. Classification of plants and organisms by their chemical constituents is referred to as chemotaxonomy. TLC, HPLC, GC, quantitative TLC (QTLC), and high-performance TLC (HPTLC) can determine the homogeneity of a plant extract. Over-pressured layer chromatography (OPLC), infrared and UV-VIS spectrometry, MS, GC, liquid chromatography (LC) used alone, or in combinations such as GC/MS, LC/MS, and MS/MS, and nuclear magnetic resonance (NMR), are powerful tools, often used for standardization and to control the quality of both the raw material and the finished product. The results from these sophisticated techniques provide a chemical fingerprint as to the nature of chemicals or impurities present in the plant or extract.\(^7\)

1.3.5.4 Modern Herbal Ayurvedic Monographs

In the modern herbal ayurvedic monographs the standardization parameters are discussed in a comprehensive way. According to the modern ayurvedic monograph the quality control protocols include the following:

1. **Title, synonyms**, publications related to that plant, constituents present, analytical methods.

   Descriptive evaluation: Description of the drug, phytomorphological, microscopical, organoleptic evaluations, foreign matter, foreign minerals, etc.
2. **Physicochemical parameters**

Identity: Physical and chemical identity, chromatographic finger prints, ash values, extractive values, moisture content.

Strength: Ethanol and water extractive values, volatile oil and alkaloidal assays, quantitative estimation protocols, etc.

3. **Biological Activity Evaluation**

Bitterness values, astringency, swelling factor, form index, hemolytic index, etc.

4. **Toxicological evaluation**

- Pesticide residues, heavy metals, microbial contamination
- Aflatoxins
- Radioactive Contaminants

5. **Therapeutic Evaluation**

Bioassay is well established that the biological potency of the herbal constituents is due to not one but a mixture of bioactive plant constituents and the relative properties of a single bioactive compound can vary from batch to batch while the biological activity remains within the desirable limits
1.4 MARKER COMPOUND – AN ANALYTICAL TOOL

1.4.1 Markers

Markers are chemically defined constituents or groups of constituents of a herbal substance, herbal preparation or a herbal medicinal product which are of interest for control purposes independent of whether they have any therapeutic activity. Markers serve to calculate the quantity of herbal substance(s) or herbal preparation(s) in the Herbal Medicinal Product if the marker has been quantitatively determined in the herbal substance or herbal preparations.

1.4.2 Markers classification of eight categories of chemical markers, namely

(1) Therapeutic components,
(2) Bioactive components,
(3) Synergistic components,
(4)Characteristic components,
(5) Main components,
(6) Correlative components,
(7) Toxic components,
(8) General components used with fingerprint spectrum.

These eight categories are defined and discussed in the subsequent sections.
Therapeutic components

Therapeutic components possess direct therapeutic effects of a herbal medicine. They may be used as chemical markers for both qualitative and quantitative assessments.

Originated from the bulbs of *Fritillaria* species (family Liliaceae), *Bulbus fritillariae* (*Beimu*) is commonly prescribed as an antitussive and expectorant in Chinese medicine practice. Five different *Bulbus Fritillariae* derived from nine *Fritillaria* species are documented in the Chinese Pharmacopoeia. Isosteroidal alkaloids of *Bulbus fritillariae*, including verticine, verticinone and imperialine, were identified as the major therapeutic components that account for the antitussive effect. Therefore, isosteroidal alkaloids were selected as the chemical markers for the quality assessment of *Bulbus fritillariae* using a series of chromatographic techniques such as pre-column derivatizing gas chromatography – flame ionization detection (GC-FID), direct GC-FID, gas chromatography – mass spectrometry (GC-MS), pre-column derivatizing high-performance liquid chromatography – ultraviolet detection (HPLC-UV), high-performance liquid chromatography – evaporative light scattering detection (HPLC-ELSD) and high-performance liquid chromatography – mass spectrometry (HPLC-MS) methods. Artemisinin from *Herba Artemisiae Annuae* (*Qinghao*) is another example of therapeutic component. *Herba Artemisiae Annuae* is well known for its potent anti-malarial activity. Artemisinin inhibits *Plasmodium falciparum* and *Plasmodium vivax*, two pathogens that cause malaria. Artemisinin is now used as a chemical marker in HPLC-ELSD, GC-FID and GC-MS for...
assessing the quality of the plant (parts and whole) at various stages, including the green and dead leaves of the plant\textsuperscript{38,39}.

(2) \textbf{Bioactive components}

Bioactive components are structurally different chemicals within a herbal medicine; while individual components may not have direct therapeutic effects, the combination of their bioactivities does contribute to the therapeutic effects. Bioactive components may be used as chemical markers for qualitative and quantitative assessment.

According to Chinese medicine theories, \textit{Radix Astragali (Huangqi)}, derived from the roots of \textit{Astragalus membranaceus} (Fish.) Bge. or \textit{A. membranaceus} var. \textit{mongholicus} (Bge.) Hsiao, is used to reinforce \textit{qi}. Isoflavonoids, saponins and polysaccharides of \textit{Radix Astragali} showed pharmacological actions in immune and circulatory systems, which were consistent with the Chinese medicine indications\textsuperscript{40}. These bioactive components, including isoflavonoids and saponins, were used simultaneously in the evaluation of the quality of \textit{Radix Astragali}\textsuperscript{41-43}.

(3) \textbf{Synergistic components}

Synergistic components do not contribute to the therapeutic effects or related bioactivities directly. However, they act synergistically to reinforce the bioactivities of other components, thereby modulating the therapeutic effects of the herbal medicine. Synergistic components may be used as chemical markers for qualitative and quantitative assessment.

The products of St John's wort (\textit{Hypericum perforatum} L.) are popular for treating mild depression\textsuperscript{44}. Butterweck et al. reviewed the research progress on the phytochemistry and pharmacology of St John's wort\textsuperscript{45,46}.\par
Naphthodianthrone, hypericin, and hyperforin (a phloroglucinol derivative) were identified as the major components that contribute to the pharmacological activities of St John's wort. Rutin, a ubiquitous flavonoid of natural products, demonstrated synergistic antidepressant actions in St John's wort. In a forced swimming test on rats, extracts of St John's wort with various chemical profiles were tested, among which the extract containing about 3% of rutin showed positive effects, whereas the extracts containing less than 3% of rutin were inactive. The extracts became active when the level of rutin was increased to about 3%. However, rutin alone did not show any effects under the same conditions\textsuperscript{47}. These results suggest that chemicals in St John's wort work synergistically to achieve the antidepressant effects. Therefore, naphthadianthrones, phloroglucinols and flavonoids may be used as chemical markers for the quality control of St John's wort\textsuperscript{48-52}.

(4) **Characteristic components**

While characteristic components may contribute to the therapeutic effects, they must be specific and/or unique ingredients of a herbal medicine.

Terpene lactones in the leaves of *Ginkgo biloba* L. (*Yinxing*) exemplify characteristic components. EGb 761, a standardized leaf extract of *Ginkgo biloba* is a well defined product for the treatment of cardiovascular diseases, memory loss and cognitive disorders associated with age-related dementia\textsuperscript{53}. Flavonoids and terpene lactones are responsible for the medicinal effects of EGb 761. Flavonoids, terpene lactones including ginkgolides A, B and C, and bilobalide are chemical markers for the quality control of *Ginkgo biloba* leaf extracts\textsuperscript{54-57}. EGb 761 contains 6% of terpene lactones (2.8–3.4% of
ginkgolides A, B and C, and 2.6–3.2% of bilobalide) and 24% of flavone glycosides. Aglycons are primarily quercetin, kaempferol and isorhamnetin. Valerenic acids, the characteristic components of valerian derived from the roots of *Valeriana officinalis* L., have sedative effects and improve sleep quality \(^58,59\). Valerenic acids are used as chemical markers to evaluate the quality of valerian preparations although their sedative effects have not been fully elucidated \(^60\). These chemical markers are also used for studying stability test for valerian ground materials and extracts\(^61\).

(5) **Main components**

Main components are the most abundant in a herbal medicine (or significantly more abundant than other components). They are not characteristic components and their bioactivities may not be known. Main components may be used for both qualitative and quantitative analysis of herbal medicines especially for differentiation and stability evaluation.

Four well-known Chinese herbal medicines derived from the genus *Panax*, namely (1) *Radix et Rhizoma Ginseng* (*Renshen*), (2) *Radix et Rhizoma Ginseng Rubra* (*Hongshen*), (3) *Radix Panacis Quinquefolii* (*Xiyangshen*) and (4) *Radix et Rhizoma Notoginseng* (*Sanqi*) \(^62\), contain triterpenoid saponins including ginsenoside Rg1, Re, Rb1 and notoginsenoside R1 as their main components. Through qualitative and quantitative comparison of the saponin profiles, these four herbs can be differentiated from one another \(^63\)–\(^73\).

(6) **Correlative components**

Correlative components in herbal medicines have close relationship with one another. For example, these components may be the precursors, products or metabolites of a chemical or enzymatic reaction. Correlative components can
be used as chemical markers to evaluate the quality of herbal medicines originated from different geographical regions and stored for different periods of time\textsuperscript{74-76}.

(7) Toxic components

Traditional Chinese medicine literature and modern toxicological studies documented some toxic components of medicinal herbs. For instance, aristolochic acids (AAs) and pyrrolizidine alkaloids (PAs) may cause nephrotoxicity and heptotoxicity respectively\textsuperscript{77-99}.

(8) General components coupled with 'fingerprints'

General components are common to many species / genera and specific components present in a particular species, genus or family. These components may be used with 'fingerprints' for quality control purposes.

Lobetyolin, a polyacetylene compound, is used as a marker for \textit{Radix Codonopsis} (\textit{Dangshen}) in thin-layer chromatography (TLC). \textit{Radix Codonopsis} is derived from the roots of three \textit{Codonopsis} species, namely \textit{Codonopsis pilosula} (Franch.) Nannf., \textit{C. pilosula} Nannf. var. \textit{modesta} (Nannf.) L. T. Shen or \textit{C. tangshen} Oliv.\textsuperscript{68}. Study showed that other five \textit{Codonopsis} species that are common substitutes of \textit{Radix Codonopsis} also contain lobetyolin. They are \textit{C. tubulosa} Kom., \textit{C. subglobosa} W. W. Smith, \textit{C. clematidea} (Schynek) C. B. Cl., \textit{C. canescens} Nannf. and \textit{C. lanceolata} (Sieb. et Zucc.) Trautv. Moreover, the roots of \textit{Campanumoea javanica} Bl. and \textit{Platycodon grandiflorum} (Jacq.) A. DC. (family \textit{Campanulaceae}), which are easily confused with \textit{Radix Codonopsis}, also contained lobetyolin. Therefore, lobetyolin may be used as a general chemical marker coupled with HPLC-UV 'fingerprints' to differentiate \textit{Radix Codonopsis} from its substitutes and
adulterants. As a chemical component may have more than one attribute, a component may belong to multiple categories. For example, ginkgolides A, B and C, and bilobalide are not only characteristic components, but also bioactive components of *Ginkgo biloba*. Ginsenoside Rg1, Re and Rb1 are both main and bioactive components of *Panax ginseng*. 
1.4.3 Isolation of markers

The three steps are involved in marker generation:

1. Extraction of plant material
2. Isolation of specific marker/s or bioactive compounds
3. Characterization of the isolated compound

1.4.3.1 Extraction of plant material

1. CONVENTIONAL SOXHLET EXTRACTION

Principles and mechanisms

Classical techniques for the solvent extraction of nutraceuticals from plant matrices are based on the choice of solvent coupled with the use of heat and/or agitation. Existing classical techniques used to obtain nutraceuticals from plants include: Soxhlet, hydrostillation and maceration with an alcohol–water mixture or hot fat.

Soxhlet, which has been used for a long time, is a standard technique and the main reference for evaluating the performance of other solid–liquid extraction (or leaching) methods. Soxhlet extraction is a general and well-established technique, which surpasses in performance other conventional extraction techniques except for, in limited field of applications, the extraction of thermolabile compounds.

In a conventional Soxhlet system plant material is placed in a thimble-holder, and filled with condensed fresh solvent from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. In the solvent flask, solute is separated from the solvent using distillation. Solute is left in the flask and fresh solvent
passes back into the plant solid bed. The operation is repeated until complete extraction is achieved.

**Advantages and disadvantages of Soxhlet extraction**

The advantages of conventional Soxhlet extraction include (1) the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with the solid matrix (2) maintaining a relatively high extraction temperature with heat from the distillation flask, and (3) no filtration requirement after leaching. Also, the Soxhlet method is very simple and cheap.

The main disadvantages of conventional Soxhlet extraction include (1) the extraction time is long; (2) a large amount of solvent is used; (3) agitation cannot be provided in the Soxhlet device to accelerate the process; (4) the large amount of solvent used requires an evaporation/concentration procedure; and (5) the possibility of thermal decomposition of the target compounds cannot be ignored as the extraction usually occurs at the boiling point of the solvent for a long time. The long time requirement and the requirement of large amounts of solvent lead to wide criticism of the conventional Soxhlet extraction method.

**II. SONICATION-ASSISTED EXTRACTION**

**Principles and mechanisms**

Sound waves, which have frequencies higher than 20 kHz, are mechanical vibrations in a solid, liquid and gas. Unlike electromagnetic waves, sound waves must travel in a matter and they involve expansion and compression cycles during travel in the medium. Expansion pulls molecules apart and compression pushes them together. The expansion can create bubbles in a
liquid and produce negative pressure. The bubbles form, grow and finally collapse. Close to a solid boundary, cavity collapse is asymmetric and produces high-speed jets of liquid. The liquid jets have strong impact on the solid surface.

Two general designs of ultrasound-assisted extractors are ultrasonic baths or closed extractors fitted with an ultrasonic horn transducer. The mechanical effects of ultrasound induce a greater penetration of solvent into cellular materials and improve mass transfer. Ultrasound in extraction can also disrupt biological cell walls, facilitating the release of contents. Therefore, efficient cell disruption and effective mass transfer are cited as two major factors leading to the enhancement of extraction with ultrasonic power. Scanning electron micrographs (SEM) have provided evidence of the mechanical effects of ultrasound, mainly shown by the destruction of cell walls and release of cell contents. In contrast to conventional extractions, plant extracts diffuse across cell walls due to ultrasound, causing cell rupture over a shorter period.

Advantages and disadvantages of sonication-assisted extraction

Ultrasound-assisted extraction is an inexpensive, simple and efficient alternative to conventional extraction techniques. The main benefits of use of ultrasound in solid–liquid extraction include the increase of extraction yield and faster kinetics. Ultrasound can also reduce the operating temperature allowing the extraction of thermolabile compounds. Compared with other novel extraction techniques such as microwave-assisted extraction, the ultrasound apparatus is cheaper and its operation is easier.
Furthermore, the ultrasound-assisted extraction, like Soxhlet extraction, can be used with any solvent for extracting a wide variety of natural compounds. However, the effects of ultrasound on extraction yield and kinetics may be linked to the nature of the plant matrix. The presence of a dispersed phase contributes to the ultrasound wave attenuation and the active part of ultrasound inside the extractor is restricted to a zone located in the vicinity of the ultrasonic emitter. Therefore, those two factors must be considered carefully in the design of ultrasound-assisted extractors.

III. MICROWAVE-ASSISTED EXTRACTION

Principles and mechanisms

Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz. Domestic and industrial microwaves generally operate at 2.45 GHz, and occasionally at 0.915 GHz in the USA and at 0.896 GHz in Europe. Microwaves are transmitted as waves, which can penetrate biomaterials and interact with polar molecules such as water in the biomaterials to create heat. Consequently, microwaves can heat a whole material to penetration depth simultaneously.

Microwave-assisted extraction (MAE) offers a rapid delivery of energy to a total volume of solvent and solid plant matrix with subsequent heating of the solvent and solid matrix, efficiently and homogeneously. Because water within the plant matrix absorbs microwave energy, cell disruption is promoted by internal superheating, which facilitates desorption of chemicals from the matrix, improving the recovery of nutraceuticals. The effect of microwave energy is thus strongly dependent on the dielectric susceptibility of both the solvent and the solid plant matrix.
There are two types of commercially available MAE systems: closed extraction vessels under controlled pressure and temperature, and focused microwave ovens at atmospheric pressure\textsuperscript{107}. The closed MAE system is generally used for extraction under drastic conditions such as high extraction temperature. The pressure in the vessel essentially depends on the volume and the boiling point of the solvents. The focused MAE system can be operated at a maximum temperature determined by the boiling point of the solvents at atmospheric pressure. Ericsson and Colmsjo introduced a dynamic MAE system, which was demonstrated to yield extract equivalent to yield of extract from Soxhlet extraction, but in a much shorter time\textsuperscript{107}.

**Advantages and disadvantages of microwave-assisted extraction**

MAE has been considered as a potential alternative to traditional solid–liquid extraction for the extraction of metabolites from plants. It has been used to extract nutraceuticals for several reasons: (1) reduced extraction time (2) reduced solvent usage and (3) improved extraction yield. MAE is also comparable to other modern extraction techniques such as supercritical fluid extraction due to its process simplicity and low cost.

**IV. SUPERCRITICAL FLUID EXTRACTION**

**Principles and mechanisms**

Supercritical state is achieved when the temperature and the pressure of a substance is raised over its critical value. The supercritical fluid has characteristics of both gases and liquids. Compared with liquid solvents, supercritical fluids have several major advantages:
(1) the dissolving power of a supercritical fluid solvent depends on its density, which is highly adjustable by changing the pressure or/and temperature;

(2) the supercritical fluid has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer. During SFE, raw plant material is loaded into an extraction vessel, which is equipped with temperature controllers and pressure valves at both inlet and outlet to keep desired extraction conditions. The extraction vessel is pressurized with the fluid by a pump. The fluid and the dissolved compounds are transported to separators, where the salvation power of the fluid is decreased by decreasing the pressure or increasing the temperature of the fluid. The product is then collected via a valve located in the lower part of the separators. The fluid is further regenerated and cycled.

Advantages and disadvantages of supercritical fluid extraction

SFE offers unusual possibilities for selective extractions and fractionations because the solubility of a chemical in a supercritical fluid can be manipulated by changing the pressure and/or temperature of the fluid. Furthermore, supercritical fluids have a density of a liquid and can solubilize a solid like a liquid solvent. The solubility of a solid in a supercritical fluid increases with the density of the fluid, which can be achieved at high pressures.

Therefore, SFE can eliminate the concentration process, which usually is time-consuming. Furthermore, the solutes can be separated from a supercritical solvent without a loss of volatiles due to the extreme volatility of the supercritical fluid.
Supercritical CO$_2$ extraction uses a moderate extraction temperature as low as 30°C. The low supercritical temperature of CO$_2$ makes it attractive for the extraction of heat sensible compounds. SFE can be directly coupled with a chromatographic method for simultaneously extracting and quantifying highly volatile extracted compounds.

However, the economics and onerous operating conditions of the SFE processes has restricted the applications to some very specialized fields such as essential oil extraction, coffee decaffeination and to university research.

V. ACCELERATED SOLVENT EXTRACTION

Principles and mechanisms

Accelerated solvent extraction (ASE) is a solid–liquid extraction process performed at elevated temperatures, usually between 50 and 200°C and at pressures between 10 and 15 MPa. Therefore, accelerated solvent extraction is a form of pressurized solvent extraction that is quite similar to SFE. Extraction is carried out under pressure to maintain the solvent in its liquid state at high temperature. The solvent is still below its critical condition during ASE. Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state, thus achieving safe and rapid extraction. Also, pressure allows the extraction cell to be filled faster and helps to force liquid into the solid matrix. Elevated temperatures enhance diffusivity of the solvent resulting in increased extraction kinetics.$^{105,121,122}$

Although the solvent used in ASE is usually organic solvents. Pressurized hot water, or subcritical water can also be used in an ASE apparatus, which is usually called pressurized hot water extraction or subcritical water extraction.$^{123}$
Advantages and disadvantages of accelerated solvent extraction

Use of non-toxic extracting solvents such as carbon dioxide and water has economic and environmental benefits. Supercritical CO$_2$ extraction has been reported to be a valuable novel extraction technique for the extraction of nutraceuticals. However, a considerable quantity of polar modifier has to be added to carbon dioxide to extract polar compounds. Accelerated solvent extraction is considered as a potential alternative technique to SFE for the extraction of polar compounds$^{121}$. Compared with traditional Soxhlet extraction, there is a dramatic decrease in the amount of solvent and the extraction time for ASE$^{122}$. Particular attention should be paid to the accelerated solvent extraction performed with high extraction temperature, which may lead to degradation of thermolabile compounds.

1.4.3.2 Analytical methods for Isolation and Characterization of plant constituent/s.

Detection and isolation of phytoconstituents can be achieved by chromatographic techniques. Chromatographic technique is a separation process that depends upon the differential distribution of components of mixture between a mobile phase and stationary phase. Use of modern chromatographic techniques can achieve separation and detection of very low concentration of a compound in a complex mixture.

However, the chromatographic techniques that are commonly used for separation of constituent/s are Column Chromatography, Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC).
A. Column Chromatography

Column Chromatography is Liquid Chromatography in which a mobile phase in the form of a liquid passes over the stationary phase packed in a column. The column is either a glass or a metallic column. The column absorption chromatography is the oldest one and has been derivatized into other forms like gel permeation, ion exchange, affinity and column partition. In column absorption chromatography, fairly large number of adsorbents is used like starch, calcium carbonate, magnesia, lime, silica gel and alumina. To optimize the resolution, many mobile phases are used either in combination or alone like petroleum ether, chloroform, acetone, water etc., for better absorption it is essential to consider the polarity of the sample, adsorbent and the mobile phase.\(^{124}\)

B. Thin Layer Chromatography

In 1958, Stahl demonstrated application of TLC in analysis, a method based on absorption chromatography. It is at present an important analytical tool for qualitative and quantitative analysis of number of natural products.\(^{125}\)

TLC has become the workhouse of the drug industry for the all-important determination of product purity. It has also widespread use in clinical laboratories and is the backbone of many biochemical and biological studies. TLC is a type of planar chromatography. Typical TLC studies are performed on flat glass or plastic plates coated with thin layer of finely divided particles; this layer constitutes the stationary phase. The mobile phase moves through the stationary phase by capillary action. Among the various chromatography techniques, TLC has the special advantage of speed, versatility and sensitivity. The greater speed of TLC is due to the more compact nature
adsorbent, when it is spread on a plate. Versatility is due to the number of different adsorbent such as silica gel, cellulose, aluminum oxide etc. The sensitivity of TLC is such, that separation of less than µg amount of material can be achieved if necessary.\textsuperscript{126,127}

C. High Performance Thin Layer Chromatography (HPTLC)

Nowadays HPTLC is applied to obtain fingerprint of herbal formulations. This technique involves accurate and precise instrumental application of the sample on adsorbent layer. It is designed to achieve much faster and better separation. Development of the chromatogram in equipments ensures distortion free and direct quantitative evaluation by means of densitometer. The sample fractions are quantified by scanning the chromatogram with a light beam in the visible or ultraviolet range of the spectrum and measuring the absorbance or fluorescence by diffuse reflectance\textsuperscript{128}.

D. Preparative Thin Layer Chromatography

This technique is used when significant quantities of sample components are to be isolated and purified for subsequent analysis by IR, NMR, GC-MS, and LC-MS. The thickness of the adsorbent layer on preparative plates is much more as compared to normal TLC plates; as a result it can accommodate more amount of sample. After development, the adsorbent layer corresponding to each band is scraped off and component is extracted with a suitable solvent\textsuperscript{128}.
E. High performance liquid chromatography (HPLC)

It is the main analytical technique for quantitative measurements of active compounds since its development in late 1960’s and early 1970’s. It is widely accepted separation technique for both sample analysis and purification. In HPLC, the mobile phase is forced through the column under high pressure with isocratic or gradient elution. HPLC is the most widely used of all and the reason for the popularity of the method is its sensitivity, its ready adaptability to accurate quantitative determinations, its suitability for separately nonvolatile species or thermally fragile ones, and above all, its widespread applicability to substances that are of prime interest to industry, to many fields of science, and to the public.

Using all these modern and sophisticated methods of analysis, Ayurveda can certainly be at par with all the modern methods of health can thus acquiring a global status with ready acceptance and utility.

The spectral techniques that are commonly used for characterization of constituent/s are UV spectroscopy, IR spectroscopy, Nuclear Magnetic Resonance spectroscopy and Mass spectroscopy.

F. Ultra-Violet and Visible spectroscopy

Ultraviolet and Visible (UV-Vis) absorption spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Absorption measurements can be at a single wavelength or over an extended spectral range. Ultraviolet and visible light are energetic enough to promote outer electrons to higher energy levels, and UV-Vis spectroscopy is usually applied to molecules or inorganic complexes in solution. The UV-Vis spectra have broad features that are of limited use for...
sample identification but are very useful for quantitative measurements. The concentration of an analyte in solution can be determined by measuring the absorbance at some wavelength and applying the Beer-Lambert Law.\(^\text{130}\)

**G. Infrared spectroscopy**

Infrared (IR) spectroscopy is one of the most powerful analytical techniques which offer the possibility of chemical identification. One of the most important advantages of IR spectroscopy over the other methods of structural analysis is that it provides useful information about the structure of molecule quickly, without tiresome evaluation methods. The technique is based upon the simple fact that a chemical substance show marked selective absorption in the infrared region. After absorption of IR radiations, the molecules of a chemical substance vibrate at many rates of vibration, giving rise to close packed absorption bands, called an IR absorption spectrum which may extend over wide wavelength range.\(^\text{131}\)

**H. Nuclear Magnetic Resonance spectroscopy**

Nuclear Magnetic Resonance (NMR) is a branch of spectroscopy in which radio frequency waves induce transitions between magnetic energy levels of nuclei of a molecule. NMR is a powerful tool for investigating nuclear structure. NMR is a technique that enables us to study the shape and structure of molecules. In particular, it reveals the different chemical environments of various form of hydrogen present in a molecule, from which we can ascertain the structures of the molecules with which we are dealing.\(^\text{132}\)

**I. Mass Spectroscopy**

Mass spectrometry is an analytical technique that identifies the chemical composition of a compound or sample on the basis of the mass-to-charge
ratio of charged particles. The method employs chemical fragmentation of a sample into charged particles (ions) and measurements of two properties, charge and mass, of the resulting particles, the ratio of which is deduced by passing the particles through electric and magnetic fields in a mass spectrometer. The technique has both qualitative and quantitative uses, such as identifying unknown compounds, determining the isotopic composition of elements in a compound, determining the structure of a compound by observing its fragmentation, quantifying the amount of a compound in a sample using carefully designed methods (e.g., by comparison with known quantities of heavy isotopes) and determining other physical, chemical, or biological properties of compounds.

1.4.3.3 Applications of chemical markers

1. Identification of adulterants

Derived from the resin of *Garcinia hanburyi* Hook f. (family Guttiferae), gamboges (Tenghuang) has been used in China to treat scabies, tinea and malignant boil, and in Thailand to treat infected wounds, pain and oedema. Characteristic polyprenylated caged xanthones including gambogic acid, gambogenic acid were isolated as the main and bioactive components of gamboges. In one study, an adulterant of gamboges was differentiated from the authentic sample by an HPLC-UV method using eight caged xanthones as chemical markers.

2. Differentiation of herbal medicines with multiple sources

*Radix Stemonae* (Baibu) is a traditional antitussive and insecticidal herbal medicine derived from the roots of three *Stemonae* species, namely *Stemona tuberosa* Lour, *S. sessilifolia* (Mig.) Mig. and *S. japonica* Mig. The
Introduction

Stemona alkaloids were pharmacologically proven to be responsible for the antitussive and insecticidal effects of *Radix Stemonae* \(^{100-105}\). It is observed that the chemical profiles of these three Stemona species varied greatly. Croomine-type alkaloids such as croomine were detected in all three species, while protostemonine-type alkaloids such as protostemonine and maistemonine were detected in *S. japonica* and *S. sessilifolia*. Moreover, stichoneurine-type alkaloids such as stemoninine, neotuberostemonine and tuberostemonine were only found in *S. tuberosa*. Stemona alkaloids may be used as markers to discriminate the three Stemona species \(^{134,135}\).

3. **Determination of the best harvesting time**

*Rhizoma Chuanxiong* (*Chuanxiong*) is one of the traditional Chinese medicinal herbs frequently used to treat cerebro- and cardio-vascular diseases. Various chemical compounds have been isolated and identified from *Rhizoma Chuanxiong*, including ferulic acid, senkyunolide I, senkyunolide H, senkyunolide A, coniferyl ferulate, Z-ligustilide, 3-butylidenephthalide, riligustilide and levistolide A. These chemicals have multiple biological activities which may contribute to the therapeutic effects of the herb. Thus, major bioactive components senkyunolide A, coniferyl ferulate, Z-ligustilide, ferulic acid, 3-butylidenephthalide, riligustilide and levistolide A may be used as markers to select the best harvesting time. A study using these markers suggested that the best harvesting time for *Rhizoma Chuanxiong* is from mid April to late May \(^{136-140}\).

4. **Confirmation of collection sites**

The studies on the chemistry and antitussive activities of *Radix Stemonae*, four chemical profiles of *S. tuberosa* of different geographic sources were
characterised using croomine, stemoninine, neotuberostemonine or tuberostemonine as markers. Moreover, the total alkaloid of *S. tuberosa* exhibited various levels of antitussive activities in a citric acid-induced guinea pig cough model. Croomine, stemoninine, neotuberostemonine and tuberostemonine all possess significant antitussive activities, however, croomine (croomine type) act on the central nervous system pathway, whereas the other three alkaloids (stichoneurine type) acted on the peripheral pathway of cough reflex. In terms of safety, those containing stichoneurine-type alkaloids are more suitable *Radix Stemonae* sources than those containing croomine as the major component. Croomine, stemoninine, neotuberostemonine, and tuberostemonine may be used as markers to confirm the collection sites for *S. tuberosa* (e.g. Shizhu and Erbian in Sichuan province, Masupo and Baoshan in Yunnan province, Shanglin in Guangxi province or Yudu in Jiangxi province, China) which contains higher levels of stemoninine, neotuberostemonine or tuberostemonine, and a low level of croomine.\(^{141-143}\)

5. **Assessment of processing methods**

In general practice, most herbs must be processed to reduce toxicity. For example, *aconite* derived from the root of *Aconitum carmichaeli* Debx\(^ {144}\), is a well known toxic and potent herbal medicine. Cases of intoxication and even death were reported in China and Japan\(^ {145-147}\). The herb is processed by boiling in water for 4–6 hours or steaming for 6–8 hours\(^ {148}\). The toxic components of this herb are diester-diterpene *Aconitum* alkaloids, such as aconitine, mesaconitine and hypeaconitine. When processed, these alkaloids hydrolyse into their respective analogues collectively known as monoester
alkaloids. Monoester alkaloids are much less toxic than diester alkaloids. These six *Aconitum* alkaloids may be used to evaluate *Radix Aconiti*.

6. **Quality evaluation of herbal parts**

Traditionally, *Radix Astragali* is graded according to its diameter, length and physical appearance. Isoflavonoids and saponins were recognised as the major bioactive components attributed to the therapeutic effects of *Radix Astragali*. These two types of components were used to evaluate the quality of *Radix Astragali* in our study, in which 25 samples of *Radix Astragali* were collected from four cultivating regions in China. The contents of 11 main isoflavonoids and three major astragalosides were analysed. Contrary to the traditional notion, thin roots contained more astragalosides than thick ones. There was no difference in isoflavonoid content between the thin and thick roots, or the bark and the xylem. These results suggest that the thin root *Radix Astragali* is of better quality.

7. **Identification and quantitative determination of proprietary products**

*Qingfu Guanjie Shu* (QGS, also known as JCICM-6) capsule is a proprietary product to treat rheumatoid arthritis. QGS has significant suppressive effects on arthritic and acute inflammation in animal models. The formula of QGS is composed of five anti-inflammatory and anti-arthritic herbs, namely *Caulis Sinomenii*, *Radix Paeoniae Alba*, *Cortex Moutan*, *Rhizoma Curcumae Longae* and *Radix Aconiti Lateralis Preparata*. Sinomenine, paeoniflorin, paenonol, cucurmin and hyperaconitine are the major constituents of the five herbs respectively, all of which have significant *in vivo* and *in vitro* effects including anti-inflammation, analgesia, anti-arthritis and
immunosuppression\textsuperscript{159-163}. Thus, HPLC methods were developed with these five chemicals as markers in the manufacturing process of QGS\textsuperscript{164,165}.

8. **Stability test of proprietary products**

Stability test is used to evaluate product quality over time and determine recommended shelf life. The five markers mentioned above were used as indicators to evaluate the product stability of QGS. For example, the accelerated conditional stability test was carried out with four time points in a period of three months in chambers at 40 ± 2°C and 75 ± 5% of humidity\textsuperscript{166}.

9. **Diagnosis of herbal intoxication**

Toxic components may be used as chemical markers in screening methods, e.g. rapid diagnosis of acute hidden aconite poisoning in urine samples by HPLC-MS\textsuperscript{167}. Five pairs of aconite alkaloids (i.e. aconitine and benzoylaconitine, yunaconitine and deacetyl-yunaconitine, mesaconitine and benzoylmesaconitine, hyponaconitine and benzoylhypaconitine, and crasscauline A and deacetyl-crasscauline A) were chosen as markers to develop a LC-MS screening method. The screening method was applied to a clinical investigation of 15 cases of suspected herbal poisoning, of which 11 cases were tested by LC-MS\textsuperscript{168}.

10. **Quantitative Analysis**

Quantitative analysis is carried on with marker by chromatographic technique. This quantitative analysis of herbal products using marker compound is termed as “**Marker Based Standardization**” or “**Fingerprint Analysis**” by Chromatographic Techniques.

The primary goal of the method(s) is to provide validated methods to be used for the quantization of the compound(s) most correlated with pharmacological...
activity or qualitative markers as determined by the primary pharmacological
literature, constituent declaration in product labeling, and a survey of experts.
The method(s) will be selected from the primary analytical literature by a
Methods Selection Committee with priority given to compendial methods
when available.
Primary factors for considering a method as appropriate include accuracy of
the findings, speed, basic ruggedness, applicability to a large segment of the
manufacturing community, and avoidance of the use of toxic reagents and
solvents. When necessary, comparative tests shall be conducted to determine
which of the available method(s) is most appropriate. The validation process
minimally includes: standard precision, linearity, sample precision using
replicate samples, sample linearity, selectivity (co-elution, sensitivity to
analyte degradation), retention times, and limits of detection. The detailed
method validation is described in next chapter.
1.5 METHOD VALIDATION

1.5.1 Introduction

Method validation is the process by which it is established that performance characteristics of the method meet the requirements for the intended analytical applications. Methods need to be validated or revalidated before their introduction into routine use\textsuperscript{169}. The International Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use\textsuperscript{170} has developed a text on the validation of analytical procedures. The United States Food and Drug Administration (USFDA) have proposed guidelines on submitting samples and analytical data for methods validation\textsuperscript{171-173}. The United States Pharmacopoeia (USP) has published specific guidelines for method validation for compound evaluation\textsuperscript{174}. The document includes definitions for eight validation characteristics. An extension with more detailed methodology is in preparation and nearly completed\textsuperscript{175}. The United States Environmental Protection Agency (US EPA) prepared a guidance for methods development and validation for the Resource Conservation and Recovery Act (RCRA) \textsuperscript{176}. The pharmaceutical industry uses methodology published in the literature \textsuperscript{177,178}. The most comprehensive document was published as the ‘Conference Report of the Washington Conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies held in 1990 (sponsored by the American Association of Pharmaceutical Scientists, the AOAC and the US FDA, among others) \textsuperscript{179}. The report presents guiding principles for validation of studies in both human and animal subjects that may be referred to in developing future formal guidelines. Representatives of
the pharmaceutical and chemical industry have published papers on the validation of analytical methods. Hokanson applied the life cycle approach, developed for computerized systems, to the validation and revalidation of methods. Green gave a practical guide for analytical method validation with a description of a set of minimum requirements for a method. Renger and his colleagues described the validation of a specific analytical procedure for the analysis of theophylline in a tablet using high performance thin layer chromatography (HPTLC). The validation procedure in that article is based on requirements for European Union multistate registration. Wegscheider has published procedures for method validation with special focus on calibration, recovery experiments, method comparison and investigation of ruggedness. The Association of Official Analytical Chemists (AOAC) has developed a Peer-Verified Methods validation program with detailed guidelines on what parameters should be validated.

According to different guidelines, validation is defined as follows:

**FDA-guidelines:**

Validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes.

**EU-guidelines**

Action of proving, in accordance with GMP-principles that any procedure, process, equipment, material, activity or system actually leads to the expected results.
ICH-guidelines

Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use.\textsuperscript{185}

1.5.2 Purposes of Method Validation Studies\textsuperscript{186}:

- To quantifiably characterize system performance
- To assess potential for error
- To identify method-to-method differences
- To meet regulatory guidelines

1.5.3 Types of Analytical Procedures to be Validated

The discussion of the validation of analytical procedures is directed to the four most common types of analytical procedures:

- Identification tests.
- Quantitative tests for impurities' content.
- Limit tests for the control of impurities.
- Quantitative tests of the active moiety in samples of drug substance or drug product or other selected component(s) in the drug product. Although there are many other analytical procedures, such as dissolution testing for drug products or particle size determination for drug substance, these have not been addressed in the initial text on validation of analytical procedures. Validation of these additional analytical procedures are equally important to those listed herein and may be addressed in subsequent documents.\textsuperscript{170}

1.5.3.1. Strategy for Validation of Methods

The validity of a specific method should be demonstrated in laboratory experiments using samples or standards that are similar to the unknown samples analyzed in the routine. The preparation and execution should follow
a validation protocol, preferably written in a step by step instruction format.

Possible steps for a complete method validation are listed below.

**1.5.3.2 Steps in Method Validation**

1. Develop a validation protocol or operating procedure for the Validation
2. Define the application, purpose and scope of the method
3. Define the performance parameters and acceptance criteria
4. Define validation experiments
5. Verify relevant performance characteristics of equipment
6. Qualify materials, e.g. standards and reagents
7. Perform pre-validation experiments
8. Adjust method parameters or and acceptance criteria if necessary
9. Perform full internal (and external) validation experiments
10. Develop SOPs for executing the method in the routine
11. Define criteria for revalidation
12. Define type and frequency of system suitability tests and or analytical quality control (AQC) checks for the routine
13. Document validation experiments and results in the validation.

First the scope of the method and its validation criteria should be defined. These include: Compounds, matrices, type of information, qualitative or quantitative, detection and quantitation limits, linear range, precision and accuracy, type of equipment and location. The scope of the method should include the different types of equipment and the locations where the method will be run. The method’s performance characteristics should be based on the intended use of the method. For example, if the method will be used for qualitative trace level analysis, there is no need to test and validate the
method’s linearity over the full dynamic range of the equipment. Initial parameters should be chosen according to the analyst’s best judgment. Finally, parameters should be agreed between the lab generating the data and the client using the data. Instruments performance should be verified using generic standards, before an instrument is used to validate a method. These studies should include the approximate precision, working range and detection limits. If the preliminary validation data appear to be inappropriate, either the method itself or the equipment or the analysis technique or the acceptance limits should be changed. In this way method development and validation is an iterative process. For example, in liquid chromatography selectivity is achieved through selection of mobile phase composition. For quantitative measurements the resolution factor between two peaks should be 2.5 or higher. If this value is not achieved, the mobile phase composition needs further optimization. There are no official guidelines on the sequence of validation experiments and the optimal sequence can depend on the method itself.

1.5.3.3 A validation report should be prepared that includes:

- Objective and scope of the method (applicability, type)
- Type of compounds and matrix
- Detailed chemicals, reagents, reference standards and control sample preparations
- Procedures for quality checks of standards and chemicals used
- Safety considerations
- Method parameters
- Critical parameters indicated from robustness testing
• Listing of equipment and its functional and performance requirements, e.g. cell dimensions, baseline noise, column temperature range

• Detailed conditions on how the experiments were conducted, including sample preparation

• Statistical procedures and representative calculations

• Procedures for quality control in the routine (e.g., system suitability tests)

• Representative plots, e.g. chromatograms, spectra and calibration curves

• Method acceptance limit performance data

• The expected uncertainty of measurement results

• Criteria for revalidation

• Person who developed and initially validated the method

• Summary and conclusions

1.5.3.4 Validation of Standard Methods

A laboratory applying a specific method should ensure that they have documentary evidence that the method has been appropriately validated. “The responsibility is with the user to ensure that the validation documented in the method is sufficiently complete to meet his or her needs.” When standard methods are used, their scope should be in line with the scope of the laboratories, method requirements and the suitability of the entire analytical system in the specific laboratory’s environment should be verified for the method. The laboratory should demonstrate the validity of the method in the laboratories environment. Full validation of a standard method is recommended where no information on type and results of validation can be found in the standard method documentation.
1.5.3.4.1 Revalidation

A revalidation is necessary whenever a method is changed and the new parameter is outside the operating range. Operating ranges should be defined for each method based on experience with similar methods, or they should be investigated during method developments. These ranges should be verified during method validation in robustness studies and should be part of the method characteristics. Availability of such operating ranges makes it easier to decide when a method should be revalidated. If, for example, the operating range of the column temperature has been specified to be between 30 and 40°C, if, for whatever reason, the new operating parameter has been selected as 41°C, then the method should be revalidated. Revalidation is also required if the sample matrix changes and if the instrument type changes.

1.5.3.5 Parameters For Method Validation: 170, 171, 190, 191

The parameters as defined by the ICH and by other organizations and authors are specificity, selectivity, precision, repeatability, intermediate precision, reproducibility, accuracy, trueness, bias, linearity range, limit of detection, limit of quantitation, robustness and ruggedness.

1.5.3.5.1 SPECIFICITY

An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure. It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination). In this case a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination.
1.5.3.5.1.1 Identification

Suitable identification tests should be able to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (perhaps by comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sound scientific judgement with a consideration of the interferences that could occur.

1.5.3.5.1.2 Assay and Impurity Test(s)

For chromatographic procedures, representative chromatograms should be used to demonstrate specificity and individual components should be appropriately labelled. Similar considerations should be given to other separation techniques. Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be demonstrated by the resolution of the two components which elute closest to each other. In cases where a non-specific assay is used, other supporting analytical procedures should be used to demonstrate overall specificity. For example, where a titration is adopted to assay the active substance for release, the combination of the assay and a suitable test for impurities can be used. The approach is similar for both assay and impurity tests.
1.5.3.5.1.2.1. Discrimination of analytes where impurities are available

For the assay, this should involve demonstration of the discrimination of the analyte in the presence of impurities and/or excipients; practically, this can be done by spiking pure substances (active substance or product) with appropriate levels of impurities and/or excipients and demonstrating that the assay result is unaffected by the presence of these materials (by comparison with the assay result obtained on unspiked samples). For the impurity test, the discrimination may be established by spiking active substance or product with appropriate levels of impurities and demonstrating the separation of these impurities individually and/or from other components in the sample matrix.

1.5.3.5.1.2.2. Discrimination of the analyte where impurities are not available

If impurity or degradation product standards are unavailable, specificity may be demonstrated by comparing the test results of samples containing impurities or degradation products to a second well-characterised procedure e.g.: pharmacopoeial method or other validated analytical procedure (independent procedure). As appropriate, this should include samples stored under relevant stress conditions: light, heat, humidity, acid/base hydrolysis and oxidation.

- for the assay, the two results should be compared.
- for the impurity tests, the impurity profiles should be compared.

Peak purity tests may be useful to show that the analyte chromatographic peak is not attributable to more than one component (e.g., diode array, mass spectrometry).
1.5.3.5.2. LINEARITY

A linear relationship should be evaluated across the range (see section 3) of the analytical procedure. It may be demonstrated directly on the active substance (by dilution of a standard stock solution) and/or on separate weighings of synthetic mixtures of the product components, using the proposed procedure. The latter aspect can be studied during investigation of the range.

Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample. For the establishment of linearity, a minimum of 5 concentrations is recommended. Other approaches should be justified.
1.5.3.5.3. RANGE

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

The following minimum specified ranges should be considered:

- for the assay of an active substance or a finished product: normally from 80 to 120 percent of the test concentration;

- for content uniformity, covering a minimum of 70 to 130 percent of the test concentration, unless a wider more appropriate range, based on the nature of the dosage form (e.g., metered dose inhalers), is justified;

- for dissolution testing: +/-20% over the specified range; e.g., if the specifications for a controlled released product cover a region from 20%, after 1 hour, up to 90%, after 24 hours, the validated range would be 0-110% of the label claim.

- for the determination of an impurity: from the reporting level of an impurity 1 to 120% of the specification; for impurities known to be unusually potent or to produce toxic or unexpected pharmacological effects, the detection/quantitation limit should be commensurate with the level at which the impurities must be controlled.

Note: for validation of impurity test procedures carried out during development, it may be necessary to consider the range around a suggested (probable) limit;
if assay and purity are performed together as one test and only a 100% standard is used, linearity should cover the range from the reporting level of the impurities to 120% of the assay specification;

1.5.3.5.4. ACCURACY

Accuracy should be established across the specified range of the analytical procedure.

1.5.3.5.4.1 Assay

1.5.3.5.4.1.1 Active Substance

Several methods of determining accuracy are available:

a) application of an analytical procedure to an analyte of known purity (e.g. reference material);

b) comparison of the results of the proposed analytical procedure with those of a second well-characterised procedure, the accuracy of which is stated and/or defined

c) accuracy may be inferred once precision, linearity and specificity have been established.

1.5.3.5.4.1.2 Medicinal Product

Several methods for determining accuracy are available:

a) application of the analytical procedure to synthetic mixtures of the product components to which known quantities of the substance to be analysed have been added;

b) in cases where it is impossible to obtain samples of all product components, it may be acceptable either to add known quantities of the analyte to the product or to compare the results obtained from a second, well characterised procedure, the accuracy of which is stated and/or defined
c) accuracy may be inferred once precision, linearity and specificity have been established.

### 1.5.3.5.2 Impurities (Quantitation)

Accuracy should be assessed on samples (substance/product) spiked with known amounts of impurities.

In cases where it is impossible to obtain samples of certain impurities and/or degradation products, it is considered acceptable to compare results obtained by an independent procedure. The response factor of the drug substance can be used. It should be clear how the individual or total impurities are to be determined, e.g., weight/weight or area percent, in all cases with respect to the major analyte.

### 1.5.3.5.3 Recommended Data

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g., 3 concentrations/3 replicates each of the total analytical procedure).

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals.

### 1.5.3.5.5. PRECISION

Validation of tests for assay and for quantitative determination of impurities includes an investigation of precision.

#### 1.5.3.5.1.5.5.1 Repeatability

Repeatability should be assessed using:

- a) a minimum of 9 determinations covering the specified range for the procedure (e.g., 3 concentrations/3 replicates each) or
a) a minimum of 6 determinations at 100% of the test concentration.

1.5.3.5.2 Intermediate Precision

The extent to which intermediate precision should be established depends on the circumstances under which the procedure is intended to be used. The applicant should establish the effects of random events on the precision of the analytical procedure. Typical variations to be studied include days, analysts, equipment, etc. It is not considered necessary to study these effects individually. The use of an experimental design (matrix) is encouraged.

1.5.3.5.3 Reproducibility

Reproducibility is assessed by means of an inter-laboratory trial. Reproducibility should be considered in case of the standardisation of an analytical procedure, for instance, for inclusion of procedures in pharmacopoeias. This data is not part of the marketing authorisation dossier.

1.5.3.5.4 Recommended Data

The standard deviation, relative standard deviation (coefficient of variation) and confidence interval should be reported for each type of precision investigated.

1.5.3.5.6 Detection Limit

Several approaches for determining the detection limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

1.5.3.5.6.1 Based on Visual Evaluation

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods. The detection limit is determined by the
analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

1.5.3.5.6.2 Based on Signal-to-Noise

This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

1.5.3.5.6.3 Based on the Standard Deviation of the Response and the Slope

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3}{\sigma S}$$

where \(\sigma\) = the standard deviation of the response

\(S\) = the slope of the calibration curve

The slope \(S\) may be estimated from the calibration curve of the analyte. The estimate of \(\sigma\) may be carried out in a variety of ways, for example:

1.5.3.5.6.3.1 Based on the Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

1.5.3.5.6.3.2 Based on the Calibration Curve

A specific calibration curve should be studied using samples containing an analyte in the range of DL. The residual standard deviation of a regression
line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

**1.5.3.5.6.4 Recommended Data**

The detection limit and the method used for determining the detection limit should be presented. If DL is determined based on visual evaluation or based on signal to noise ratio, the presentation of the relevant chromatograms is considered acceptable for justification. In cases where an estimated value for the detection limit is obtained by calculation or extrapolation, this estimate may subsequently be validated by the independent analysis of a suitable number of samples known to be near or prepared at the detection limit.

**1.5.3.5.7. QUANTITATION LIMIT**

Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental. Approaches other than those listed below may be acceptable.

**1.5.3.5.7.1 Based on Visual Evaluation**

Visual evaluation may be used for non-instrumental methods but may also be used with instrumental methods.

The quantitation limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision.

**1.5.3.5.7.2 Based on Signal-to-Noise Approach**

This approach can only be applied to analytical procedures that exhibit baseline noise.

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte
with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

1.5.3.5.7.3 Based on the Standard Deviation of the Response and the Slope

The quantitation limit (QL) may be expressed as:

\[ QL = \frac{10}{\sigma S} \]

where \( \sigma \) = the standard deviation of the response, \( S \) = the slope of the calibration curve

The slope \( S \) may be estimated from the calibration curve of the analyte. The estimate of \( \sigma \) may be carried out in a variety of ways including:

1.5.3.5.7.3.1 Based on Standard Deviation of the Blank

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

1.5.3.5.7.3.2 Based on the Calibration Curve

A specific calibration curve should be studied using samples, containing an analyte in the range of QL. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

1.5.3.5.7.4 Recommended Data

The quantitation limit and the method used for determining the quantitation limit should be presented.

The limit should be subsequently validated by the analysis of a suitable number of samples known to be near or prepared at the quantitation limit.
1.5.3.5.8. ROBUSTNESS

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used.

Examples of typical variations are:

• Stability of analytical solutions,
• Extraction time

In the case of liquid chromatography, examples of typical variations are

• Influence of variations of pH in a mobile phase,
• Influence of variations in mobile phase composition,
• Different columns (different lots and/or suppliers),
• Temperature,
• Flow rate.

In the case of gas-chromatography, examples of typical variations are

• Different columns (different lots and/or suppliers),
• Temperature
• Flow rate.
1.5.3.5.9. SYSTEM SUITABILITY TESTING

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.