Importance of Chromatography Techniques in Phytomedicine Research

14

Aditi Gujrati, Sourabh Jain, Veenu Joshi, Shiv Shankar Shukla, Amber Vyas, and Vikas Jain

Abstract

Chromatography is a technique including thin-layer chromatography, column chromatography, high-performance liquid chromatography, and gas-liquid chromatography. These isolation techniques play a significant role in authentification, identification, isolation, and enrichment of phyto-molecules belonging to specific aromatic and medicinal plants. In thin-layer chromatography, the standard compound is used for authentification of the plant material after small-scale extraction. The large-scale extraction method was then carried accordingly with suitable solvent. Thin-layer chromatography also gives the basic idea for isolation because it requires proper combination of solvents for optimum Rf value. Column chromatography is the most basic technique for isolation of phyto-molecules. During isolation process both thin-layer chromatography and column chromatography are used simultaneously for identification of a compound in various fractions at

A. Gujrati · V. Jain (✉)
Mahakal Institute of Pharmaceutical Studies, Ujjain, Madhya Pradesh, India
e-mail: aditigujrati6107@gmail.com; vikasjain111180@gmail.com

S. Jain
Sagar Institute of Pharmaceutical Sciences, Sagar, Madhya Pradesh, India
e-mail: sourabhj47@gmail.com

V. Joshi
Center for Basic Science, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh, India
e-mail: vinu.jsh@gmail.com

S. S. Shukla
Columbia Institute of Pharmacy, Tekari, Raipur, Chhattisgarh, India
e-mail: shivpharma007@gmail.com

A. Vyas
University Institute of Pharmacy, Pt. Ravishankar Shukla University, Raipur, Chhattisgarh, India
e-mail: ambervyas@gmail.com

S. C. Mandal et al. (eds.), *Evidence Based Validation of Traditional Medicines*,
https://doi.org/10.1007/978-981-15-8127-4_14

different combinations of solvent used for isolation. For increasing the polarity of a particular solvent combination, thin-layer chromatography gives the basic information. The quantitative assessment of phyto-constituents is through high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) for nonvolatile components, whereas gas-liquid chromatography is used for volatile components in general context. High-performance thin-layer liquid chromatography is effective in quantification of biomarkers in extracts and fractions. High-performance liquid chromatography (HPLC) and gas chromatography (GC) are coupled with mass spectrophotometer (MS) to give a unique combination of LC-MS and GC-MS respectively, which are used for quantification of biomarkers along with their molecular weight. Recently, the work is carried out on enrichment of phyto-molecules using chromatographic techniques. In this chapter, we have focused on the recent advances in using chromatographic techniques at different levels of phytochemical investigation.

Keywords

Phyto-molecules · Thin-layer chromatography · Column chromatography · High-performance liquid chromatography · Gas chromatography · High-performance thin-layer chromatography

Abbreviations

APCI	Atmospheric pressure chemical ionization
CI	Chemical ionization
EI	Electron impact
ESI	Electrospray ionization
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectroscopy
GLC	Gas-liquid chromatography
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
LC-MS	Liquid chromatography-mass spectroscopy
MALDI	Matrix-assisted laser desorption/ionization
PC	Paper chromatography
TLC	Thin-layer chromatography

14.1 Introduction

The discovery of new drugs from plant sources involves a systematic scientific investigation using a multifaceted approach. It requires the knowledge of ethno-pharmacological uses, chemistry, biology, and toxicology of the plants leading to better understanding of concept correlated to drugs. It is also necessary to carry out community surveys, previous literature survey, and analysis of the outcomes of community interventions to evaluate the ethno-pharmacological uses of plants

against the various diseases. Discovery of drugs from plant sources is based on the constituents and their properties, which can be used for the treatment of any disease. Selection of appropriate isolation method involves the acquisition of knowledge related to ethno-use, which can help to work on the isolation of active drug substance from the complex framework of plant materials using the concepts of chemistry for the drug development. Chromatography represents the most flexible and readily available separation technique. It refers to a physical method for the isolation and detection of components or compounds from a mixture into separate components using stationary phase (consist of solid phase or layer of liquid adsorbed onto the surface of solid support) and a mobile phase (composed of liquid or a gaseous component). Thus, phytoconstituents can be easily separated and purified by the help of various chromatographic techniques (Archana Khale and Anubha 2011; Coskun 2016). However, the quality evaluation of drugs like physical and chemical stability, bioavailability, and identity characterization requires the proper isolation and drug development procedure.

There are several techniques currently applied to separate and identify the phytoconstituents. The extraction process is followed by separation of the constituents and isolation of the compounds using different chromatographic techniques. Further, application of spectroscopic techniques leads to identification and confirmation of the pure compounds. On the basis of the nature of the functional groups of compounds, if already known, they can be extracted at high, low, or room temperatures. Moreover, the extraction process is based on mass transfer, which is a unit operation, transferring soluble matter from solid to liquid phase. When a crude particle is immersed in a solvent, it is surrounded by boundary layer of the solvent, which starts penetrating inside the particle and subsequently forms solution of the constituents within the cells. Escape of this dissolved constituent takes place through the cell wall throughout the boundary layer, and the process continues till equilibrium is set up between both the sides.

14.1.1 Extraction Methods

Extraction methods involve the separation of the soluble plant constituents from the insoluble cellular residue. In small-scale extraction processes like maceration and percolation are generally slow, time-consuming and give insufficient extraction of the crude drugs. Whereas in large scale extraction process like modified soxhlet extraction large batches of crude drugs are extracted more easily and quickly.

14.1.1.1 Maceration

Maceration is the process of extracting medicinally active components of the plant material. It involves immersing of coarse or powdered crude drugs in a stoppered vessel containing suitable solvent. The above mixture is allowed to stand for at least 3–7 days in a warm place with intermittent shaking and finally the solvent containing dissolved constituents is collected for further processing. Maceration method is modified to multiple-stage extraction to increase the yield of the constituents in the extracts. The crude plant material is taken in the extractor, which is joined with a

circulatory pump and spray distributor along with number of connected tanks to collect the extraction solution. This is called as multiple-stage extraction. The solvent is added and circulated in the extractor containing plant material and is removed and stored as extracted solution in the receiver tanks. This operation is repeated thrice. When the fresh plant material is charged in the extractor, the stored solution is once again circulated and then removed as an extract. Likewise after three extractions, the plant material is removed from the extractor, again recharged with fresh drug, and the whole cycle is repeated. At the end of the process, the solvent moves out, and the crude extract is separated from the plant residue by centrifuging or pressing. Maceration is an old method in which active ingredients cannot be completely extracted (Silva et al. 2017; Raaman 2006).

14.1.1.2 Percolation

Percolation involves the constant flow of the solvent through the crude plant material, which continuously replaces the saturated solvent by fresh solvent. It involves a number of steps. In the first step of the process, powdered material is treated with sufficient amount of the solvent to make it uniformly wet and allowed to stand for 15–20 min. After which the above mixture is transferred into percolator which is generally a "V"-shaped vessel open at both ends. Again sufficient amount of the solvent is added for the saturation of the plant material, and the liquid starts to fall out from the outlet of the percolator (Fig. 14.1). Thereafter, the plant material is allowed to macerate in the vessel for 24 h, and percolation is continued gradually using sufficient solvent.

This process is based upon the flow of the solvent which is used until its point of saturation through the powdered material (Raaman 2006). This process yields products of greater concentration than the macerated products.

14.1.1.3 Modified Percolation

The conventional percolation process is modified by including evaporation, for getting more concentrated extract, especially when alcohol is used as solvent. In this method, the first portion of the percolate is collected and kept aside, and then subsequent portions are collected, concentrated, and added to the first portion. This method is known as reserve percolate method. By this method, the first portion which contains a large amount of the constituents is not subjected to heat, and also higher concentration of the extract is obtained.

In the modified process of percolation techniques, continuous or semi-extraction devices are used in some industries for handling the batches of varying size. The extraction battery which consists of a number of vessels in series is interconnected through pipelines and arranged so that the solvent can be added directly and the product can be removed from the vessel. Such type of extraction battery gives maximum efficiency of extraction with minimum use of the solvent; thus the extract obtained is more concentrated.

14.1.1.4 Continuous Extraction

Continuous extraction is used widely when the constituents and impurities both have partial solubility in the particular solvent (Silva et al. 2017). Soxhlet extraction is the

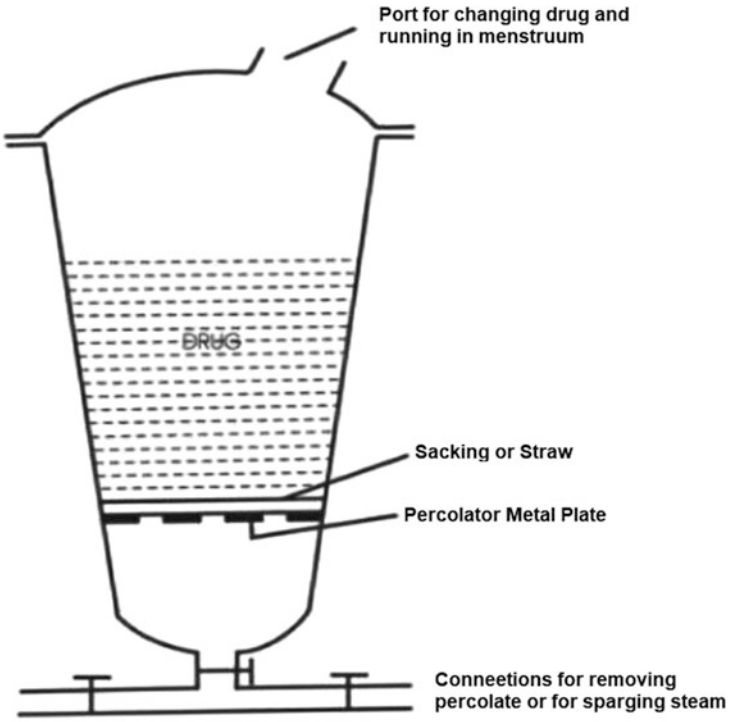

Fig. 14.1 Percolator

method of continuous extraction, in which the solvent is circulated through the extractor for a number of times. In this process, extraction is done by evaporating the solvent and collecting the vapors of the solvent into a condenser, and the condensed liquid is again returned for continuous extraction of the plant material.

Soxhlet apparatus (Fig. 14.2) designed for continuous extraction process includes the following:

- Extractor: Body of extractor is attached with siphon tube and side tube.
- Distillation flask: The extractor from the base is attached to the distillation tank.
- Condenser: Mouth of extractor is set to condenser with the help of standard joints.

In the soxhlet extraction process, first the crude powdered material is filled in the soxhlet apparatus directly or in a thimble of filter paper, and then the solvent is heated, evaporated, and siphoned back into the flask through the thimble. This sequence is repeated a number of times without changing the solvent to get sufficient extract. Fresh activated pieces of porcelain are added into the flask to avoid bumping of solvent.

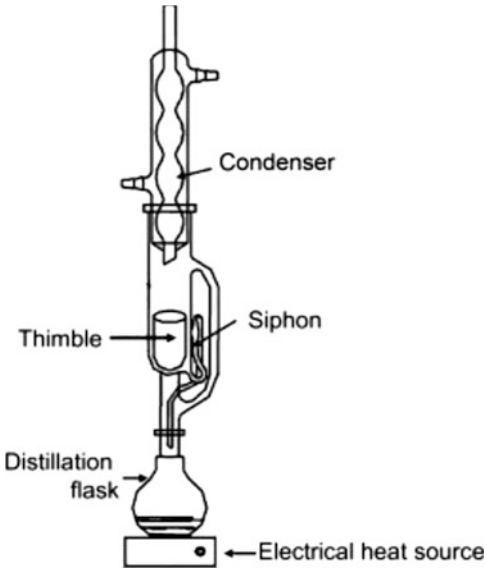

Fig. 14.2 Soxhlet extractor

14.1.1.5 Large-Scale Extraction

Large-scale extraction is meant for the extra-large batches of drug/plant material, in which various soxhlet extractor assemblies are modified (Fig. 14.3). The pilot plant extractor generally has a separate extractor and condenser unit. Separate inlet for loading the drug and outlet for drug discharge are also provided into it. The extractor body is divided into two parts: the upper one which contains plant material and lower one which is a distillation chamber. The distillation chamber is electrically heated, and the vapors of the solvent are passed to the condenser. The condensed liquid is sprayed on the bed of the powdered material with the help of a solvent distribution nozzle. Such large-scale extractors are provided with the outlet from the lower side of the extractor for removing the extract.

14.2 Method of Separation

The crude extract obtained by extraction process is subjected to separation and isolation of the pure compound, which requires specific and specialized techniques. The selection of appropriate method and/or technique depends on solubility and volatility of the compounds to be separated from the crude extract. Techniques which are used for separation and purification include the following:

(a) Paper chromatography (PC)
(b) Thin-layer chromatography (TLC)
(c) Gas-liquid chromatography (GLC)
(d) High-performance liquid chromatography (HPLC)

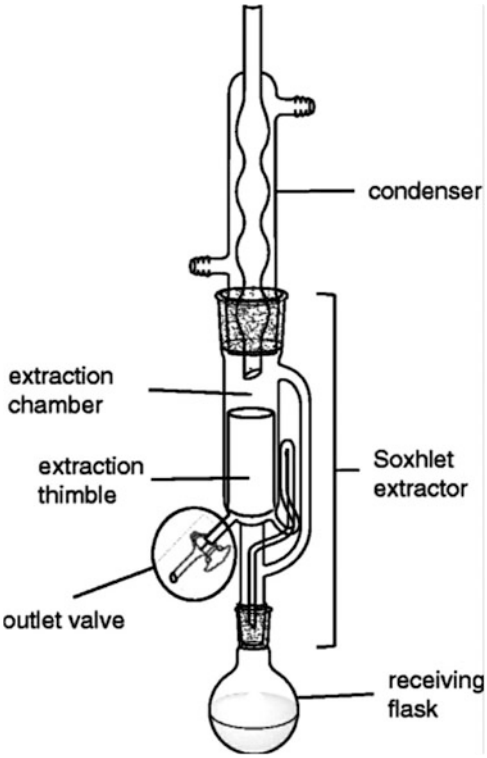

Fig. 14.3 Modified soxhlet extractor

(e) High-performance thin-layer liquid chromatography (HPTLC)

Paper chromatography is generally useful for hydrophilic components like amino acids, nucleic acid bases, carbohydrates, phenolic compounds, and organic acids. While TLC is preferred for the separation of lipophilic components like carotenoids, steroids, lipids, and chlorophyll, the third technique, i.e., GLC is mainly used for the separation of volatile components like fatty acids, mono as well as sesquiterpenes, sulfur compounds, and hydrocarbons. However, the volatility of the compounds can be enhanced by conversion to esters or ethers. In contrast, the less volatile substances can be separated by using HPLC, which has the benefit of column efficiency along with the high speed analysis. All of these methods are applied for the identification, purification, isolation, and/or separation of the compounds. For large-scale isolation column containing adsorbents (as used in PC and TLC) are used and the technique is known as column chromatography. For a large amount of crude material, these columns are coupled with automatic fraction collectors to get a high yield of purified compound (Fan et al. 2006; Hamburger and 193 Cordell 1987; Zygmunt and Namiesnik 2003). Generally, a combination of the aforementioned techniques is the best strategy to isolate a pure compound.

14.2.1 Paper Chromatography

Paper chromatography is a very simple method, in which a filter paper sheet is used as stationary phase for separation of components of a mixture. The Rf values obtained in this method are quite reproducible, which are important to authenticate the results, especially in the case of constituents like anthocyanins, which do not have defined physical properties.

Paper chromatography is of two types: partition chromatography and adsorption chromatography. In the former, compounds are partitioned between water-immiscible alcoholic solvent (e.g., n-butanol) and water. On the other hand, the latter involves the selective adsorption of compounds in the presence of aqueous solvent as in case of common purines and pyrimidines, phenolics, and plant glycosides. Generally PC is done by descending the Whatman filter paper in tanks which is suitable for two-dimensional separation of the compounds. A variety of "modified" filter papers are commercially available for separation of specific compounds. For instance, addition of alumina or salicylic acid onto cellulose paper helps in reducing its polarity, while soaking it in silicone or paraffin oil makes it suitable to carry out "reversed-phase" chromatography.

In this technique, detection of particular class of compounds is done by spraying or dipping the developed paper in specific chromatographic reagent and the compounds are eluted as colored or fluorescent spots under UV light. In few cases the paper may be heated in order to develop the colors. The Rf value can be calculated by measuring the distance from the origin to the center of the spot formed by the compound to the distance travelled by the solvent. This always appears as a fraction between the range of 0.001 and 0.99. Also, Rm value can be calculated for the comparison of structurally correlated compounds (Ye et al. 2007) by the following formula:

$$Rm = \log (1/Rf - 1)$$

14.2.2 Thin-Layer Chromatography (TLC)

TLC is a versatile and sensitive technique. Its versatility is due to the variety of adsorbents which can be spread on glass plate. Generally silica gel is used, but ciliate, polyamide, ion-exchange resin, calcium hydroxide, magnesium phosphate, aluminum oxide, sephadex, cellulose, polyvinylpyrrolidone, and a mixture of two or more of the above materials can also be used. In this technique, time taken in running the sample is less in comparison to PC due to the more compact nature of adsorbent. In ancient times, a major drawback with the TLC was the labor of spreading glass plates with adsorbent, but nowadays automatic spreading devices as well as a variety of pre-coated plates of glass, plastic, or aluminum sheets are commercially available, which has eased the routine phytochemical work. In TLC, there is a sequence of steps to be followed with all the precautions for obtaining good results. First, the glass plates are cleaned properly, and slurry of silica and/or other materials which are used as stationary phase is prepared. Some additives like calcium sulfate

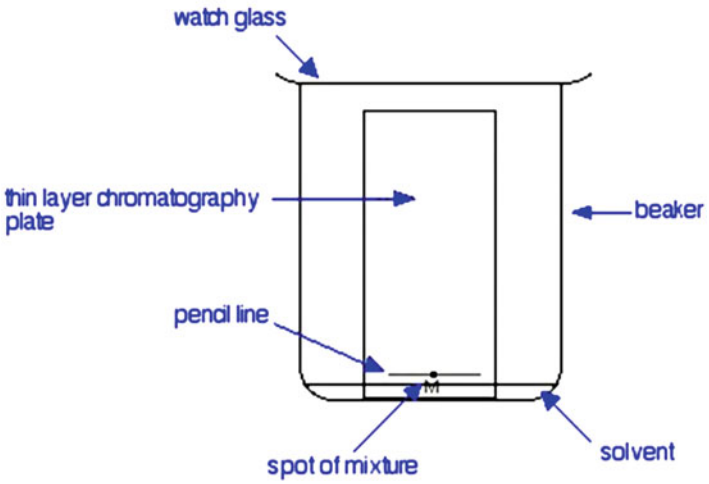

Fig. 14.4 Thin-layer chromatography

hemihydrate and inorganic salts can also be added into the slurry for effective binding and improving the adsorbents properties, respectively. Then the slurry is spread onto the glass plates, dried, and heated at 100–110 °C for 30 min for activation. Thereafter, loaded plates are put into an ascent tank saturated with the solvent system consisting of a combination of solvents in exact proportions. However, in this technique, Rf values are considerably less reproducible; thus it is essential to include one or more standard compounds for reference (Fig. 14.4).

In TLC, glass plates can be easily sprayed with concentrated H_2SO_4 for the detection of lipids and steroids. Also, the separated compounds can be recovered by scraping the adsorbent at the appropriate places on the developed glass plate, eluting the powder with a solvent like ether and centrifuging to remove the adsorbent. TLC plates can be repeatedly developed with one or several different solvent systems with intermittent drying (Archana Khale and Anubha 2011; Mohammad et al. 2010; Beckett and Stenlake 2005; Kasture et al. 2008; Chatwal and Anand 2008). Table 14.1 includes a few examples of analytes evaluated by TLC.

14.2.3 Gas-Liquid Chromatography

GLC is a well-developed analytical technique frequently used for the identification, quantification, and characterization of volatile substances. It is a highly efficient and sensitive technique, which can be used for the separation and detection of essential oils (Bombarda et al. 2008). GLC requires sophisticated and expensive apparatus (Fig. 14.5) consisting of four important components:

Table 14.1 Examples of analytes evaluated by TLC

S. No.	Analyte	Parameters of TLC	References
1	Harhra' (*Terminalia chebula* and gallic acid)	Stationary phase, i.e., silica gel Mobile phase, i.e., ethyl acetate with toluene and formic acid in the ratio of 5:5:1	Lalla et al. (2000)
2	*Azadirachta indica*, *Catharanthus roseus*, and *Momordica charantia*	Stationary phase, i.e., silica gel Mobile phase, i.e., methanol with dichloromethane in the ratio of 8:2	Habib et al. (2000)
3	*Strychnos nux-vomica*	Stationary phase, i.e., silica gel Mobile phase, i.e., diethylamine with chloroform and ethyl acetate in the ratio of 1:0.5:8.5	Jadhav et al. (2009)
4	Fruit of *Piper chaba* constituents are piperamine, piperine, methyl piperate, and piperlonnguminine	Stationary phase, i.e., silica gel Mobile phase, i.e., ethyl acetate with n-hexane in the ratio of 1:1	Richter et al. (2003)
5	Quinones	Stationary phase, i.e., silica gel 60 Mobile phase, i.e., n-hexane with dichloromethane in the ratio of 2:8	Pyka (2014)

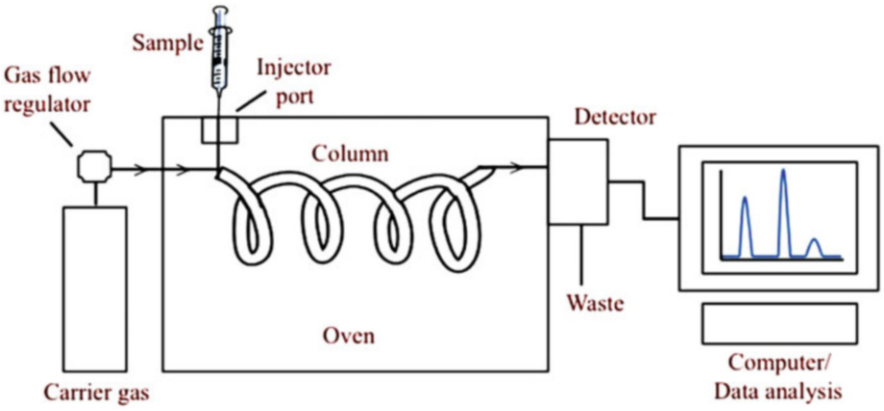

Fig. 14.5 Gas-liquid chromatography

1. Column—made up of metal in the form of a coil to conserve space, packed with a stationary phase like 5–15% silicone oil on an inert powder (Chromosorb W, celite, etc.).
2. Heater—provides heat to the column progressively from 50 to 350 °C at a standard rate and holds the temperature at the higher limit if needed. The temperature of column inlet is controlled separately so that the sample can be

vaporized rapidly as it is passed on to the column. The sample is dissolved in hexane or ether and is injected by hypodermic syringe into the inlet port through a rubber septum.
3. Gas flow—consists of an inert carrier gases, i.e., argon or nitrogen. Separation of the compounds on the column depends on passing this gas at a controlled rate.
4. Detector—based on either electron capture capacity or flame ionization, detects the compounds as they are swept through the column, and the potentiometric recorder generates peaks for different compounds with varying intensities.

The results of GLC are expressed as retention volume R", which is the volume of carrier gas required to elute a component from the column, or in terms of retention time Re, which is the time required for elution of the sample. These parameters are nearly always expressed in terms relative to a standard compound (as RR" or RRe), which may be added to the sample extract or which could take the form of the solvent used for dissolving that sample.

There are three important parameters, namely, stationary phase, the column, and the temperature of operation which varies with the volatility and polarity of the compounds to be isolated. Conversion of the compounds to derivatives can be a strategy to carry out separation at a lower temperature. GLC provides both quantitative and qualitative data on plant substances, since measurements of the area under the peaks shown on the GLC trace are directly related to the concentrations of the different components in the original mixture. There two general formulae for measuring these areas: (a) peak height $\times$ peak width at half height $= 94\%$ of the peak area (this only applies to symmetrical peaks), and (b) peak area is equivalent to that of a triangle produced by drawing tangents through the points of inflection. Peak areas can be determined automatically, e.g., by the use of an electronic integrator.

14.2.4 High-Performance Liquid Chromatography

High-performance liquid chromatography is analogous to gas-liquid chromatography as it can provide qualitative and quantitative data in a single operation. Presently, HPLC is the most accepted technique for the phytochemical separation (Faghihi et al. 2001; Lu et al. 2005; Li et al. 2010).

HPLC has high rates of precision, resolution, sensitivity, and selectivity. It has the column filled with small spherical particles enclosed in stationary phase held in a narrow bore stainless steel column making it especially sensitive to impurities, so it is essential to purify and filter the plant extracts before injecting into the column (Fig. 14.6). The liquid mobile is a combination of solvents and is forced under considerable pressure. It either remains constant (isocratic separation) or may be changed continuously in its proportions by including a mixing chamber into the setup called as gradient dilution. The HPLC apparatus is more costly than GLC because of the pumping system which can withstand the high pressure. In this technique, elution process can be monitored by a detector using ultraviolet light. A computing integrator may be added to handle the whole operation, which can be

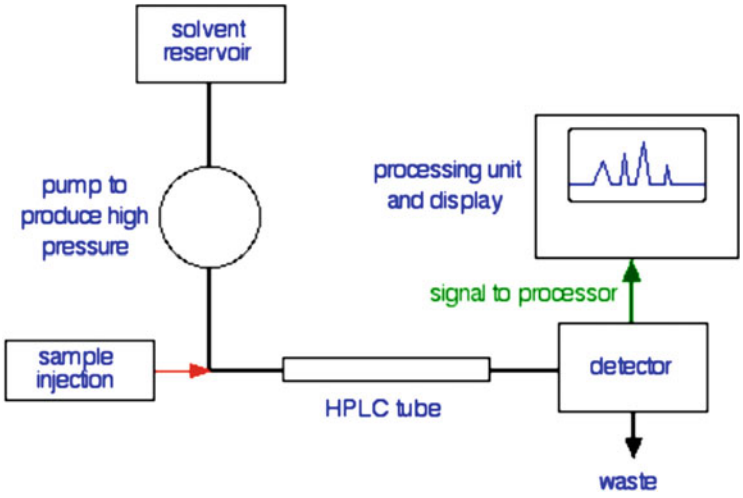

Fig. 14.6 Instrumentation of HPLC

controlled through a microprocessor. The major difference between HPLC and GLC is that the former operates at ambient temperature and the compounds are not subjected to rearrangements during separation. Moreover, the thermostatically controlled jacket of HPLC column provides effective temperature control. HPLC is used for those classes of compounds which are nonvolatile in nature, e.g., phenolics, higher terpenoids, lipids, alkaloids, and sugars. It is more advantageous for compounds which can be detected in the visible or ultraviolet regions of the spectrum. Proteins can be separated by HPLC on columns of modified silica gels, sephadex, or ion exchangers.

Preparative HPLC and analytical HPLC are used extensively in pharmaceutical industry for the isolation as well as purification of herbal compounds. The objective of the former is to purify and isolate the compounds, whereas the latter aims to get information regarding the sample. There are commonly two types of preparative HPLC, i.e., low-pressure HPLC (pressure under 5 bar) and high-pressure HPLC (pressure $>$ 20 bar). In preparative high-pressure HPLC (pressure $>$ 20 bar), larger stainless steel columns and packing materials of (particle size 1030 μm) are required. Applications of HPLC for evaluation of herbal drug are enlisted in Table 14.2.

14.2.5 High-Performance Thin-Layer Chromatography

High-performance thin-layer chromatography is commonly employed in the herbal pharmaceutical industries for identification, quality control and quality assurance of herbs and herbal products. Also the pesticide content, mycotoxins and adulterants in health food products are detected by HPTLC (Patel and Patel 2016).

Table 14.2 Applications of HPLC for evaluation of herbal drugs

Active compounds	Flow rate	Mobile phase	Column	Gradient detector	References
Atropine	1.0 mL/min	A is 0.05M KH2P04 in water of pH 3, B is acetonitrile	Zorbax Eclipse XDBC18, 3.5 µm	UV [diode array detector]	Pharmaceutical Applications with HPLC (2000)
Quercetin Kaempferol	2.0 mL/min	A is 0.5% H2.0 mL/min 3P04 in water, B is methanol	Hypersil ODS, 5 µm	Diode array detector	Pharmaceutical Applications with HPLC (2000)
Rhein Emodin	1.0 mL/min	A is 0.05M NH 40Ac in water of pH 2.5, B is acetonitrile	Hypersil ODS, 5 µm	Diode array detector	Pharmaceutical Applications with HPLC (2000)
Ephedrine Norephedrine	1.0 mL/min	A is 0.025M KH2PO4 in water of pH 3, B is acetonitrile	Zorbax SBC18, 3.5 µm	UV [diode array detector]	Pharmaceutical Applications with HPLC (2000)
Quinidine Quinine	0.8 mL/min	A is 0.05M KH2P04 in water of pH 3, B is acetonitrile	Purospher RP-18, 5 µm	UV [diode array detector]	Pharmaceutical Applications with HPLC (2000)

It is also known as planer chromatography or HPTLC. Conventional TLC is modified in order to utilize the full potential of the method. The principle of HPTLC is the same as that of TLC. The samples to be chromatographed are applied to the self-coated plates in the spot or a band without damage to the layer. The volume is governed by disposable glass capillaries or sample applicator. The sample is concentrated into a narrow band of selectable length, which needs precision and exact positioning. The growth of chromatogram takes place in the same way as that of TLC. As the solvent migrates on the plate by capillary, the samples are separated into fractions. For the evaluation of chromatogram, the peaks are scanned in a densitometer with a light beam in the visible or ultraviolet range of spectrum. The fluorescence is calculated by diffused reflectance. HPTLC systems are provided with the photo and video documentation systems. Densitometric scanning of individual or fractions can be repeated with the same or different parameters, stored on the plate operators, and evaluated or documented before and after derivatization. This technique is largely used in the study of natural product botanicals and herbal cosmetics. Some mobile phases and common derivatization used in HPTLC are tabulated in Tables 14.3 and 14.4. Applications of HPTLC in the evaluation of herbal drugs are mentioned in Table 14.5.

Table 14.3 Some mobile phase used in HPTLC for herbal compounds (Srivastava and Springer Verlag 2011; Atlas 1996; Shivatare et al. 2013)

Chemical constituents (herbal)	Mobile phase used
Hydrophilic constituents: Aanthraglycosides, arbutin, alkaloids, bitter principles, cardiac glycosides, saponin, flavonoids	Ethyl acetate with methanol and water in the ratio of 100:13.5:10
Lipophilic constituents: Eessential oils, coumarin, naphthoquinones, terpenes	Ethyl acetate with toluene in the ratio of 7:93
Flavonoids	Ethyl acetate with glacial acetic acid, formic acid, and water in the ratio of 100:11:11:26
Alkaloids	Toluene with ethyl acetate and diethylamine in the ratio of 70:20:10
Cardiac glycosides	Ethyl acetate with methanol and water in the ratio of 100:13.5:10 or 81:11:8
Terpenes	Chloroform with water and methanol in the ratio of 65:4:25
Triterpenes	Ethyl acetate with toluene and formic acid in the ratio of 50:50:15 Toluene with chloroform and ethanol in the ratio of 40:40:10
Essential oil	Ethyl acetate with toluene in the ratio of 7:93
Saponins	Chloroform with glacial acetic acid, methanol, and water in the ratio of 64:32:12:8

14.3 Recent Advances in the Chromatographic Techniques with Their Applications

The necessity to identify the components of complex mixtures stimulated the development of combination of chromatographic techniques with mass spectrometry (MS).

14.3.1 Liquid Chromatography-Mass Spectroscopy (LC-MS)

Liquid chromatography-mass spectrometry or high-performances liquid chromatography-mass spectrometry (HPLC-MS) is an analytical technique joined together for high-resolution separation with sensitive and specific mass spectrum detection. Combination of LC with MS is a significant development in the chromatography techniques. Mass spectrometry in LC-MS helps to find out the elemental composition and elucidate the structure of a sample (Pitt 2009).

Typical LC-MS system is a combination of HPLC with MS using interface, i.e., ionization source. The sample is separated using liquid chromatography, and the separated sample is sprayed into atmospheric pressure ionic source, which transformed ions into the gas phase. There is a mass analyzer which is used to sort ions depending on their mass-to-charge ratio, and a detector is used to count the ions

Table 14.4 Common derivatization agents used in HPTLC (Jork and Funk 1990; Knap et al. 2006)

S. No.	Reagent	Chemical compounds (herbal)	Color
1.	**Dragendorff reagent** This reagent forms complex reaction with nitrogen-containing compounds	Alkaloids	Red-brown zone (visible)
2.	**Natural products** PEG reagent, i.e., diphenylboric acid 2-aminoethyl ester, forms complexes via condensation reaction with 3-hydroxyflavones	Flavonoids	Intense yellow, green, and Orange Fluorescent zones at UV 366 nm
3.	**Iodine** It produces iodine reaction which results in oxidative products	Quinolone derivative, indole, thiols, and organic compounds	Dark zone at UV 254
4.	**Ethanolic KOH (10%)**	Anthrones (cascarosides, aloin)	Yellow zone (visible) Yellow fluorescence at UV 366 nm
5.	**Ninhydrin reagent**	Amines, peptides, amino acids, and amino sugars	Yellow, brown to pink, and violet (visible)
6.	**Vanillin sulfuric acid or Aanisaldehyde sulfuric acid**	Bitter principle, saponins, essential oils	Red-brown, yellow-brown, dark green zone (visible) Colored zones (visible) Blue, brown, or red zones (visible)

rising from the mass analyzer, and it may also amplify the signals generated from each ion. As a consequence, spectrum of mass is obtained, which is used to determine the mass of molecules and elemental or isotopic nature of a sample and to elucidate the chemical structure of molecules (Korfmacher 2005; Lim and Lord 2002).

Generally the LC-MS consists of an assembly of ion generation unit or ionization source, liquid chromatography, mass analyzer, and mass spectrometric data acquisition. The effluent mobile phase with separated compound from the liquid chromatography is interfaced with the ionization source of the mass spectrometer. Ionization sources include matrix-assisted laser desorption/ionization (MALDI), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI). Apart from this chemical ionization (CI), electron impact (EI), or negative chemical ionization is also used as a source of ionization in MS (Sparkman 2006).The analyzer is a component of the mass spectrometer which takes ionized molecules and separates it on the basis of charge-to-mass ratios to the detector to be detected and later converted to a digital output (Fig. 14.7).

Table 14.5 HPTLC application in the evaluation of herbal drugs (Knap et al. 2006)

Herbal constituents	Reagent for spraying	Quantification
Panaxadiol and panaxatriol (ginseng)	10% H_2SO_4 acid in CH_3OH	UV spectroscopy absorbance at 544 nm and 522 nm, silica gel is used as an adsorbent with solvent system ether/chloroform (1:1)
Flavonol glycosides (ginkgo biloba)	8% $AlCl_3$ in Ethanol	UV spectroscopy absorbance at 370 nm, silica gel is used as an adsorbent with solvent system chloroform/benzene/ethanol/water/acetic acid (11:4:2:2:1)
Glycyrrhetinic acid (liquorice)	None	UV spectroscopy absorbance at 260 nm, silica gel is used as an adsorbent with solvent system ethyl acetate/ammonia/methanol: (10:1:3)
Aloin (aloe vera)	None	UV spectroscopy absorbance at 350 nm, silica gel is used as an adsorbent with solvent system ethyl acetate/acetic acid/water (17:2:3)
Carvone (*Cuminum cyminum*)	Anisaldehyde sulfuric acid	UV spectroscopy absorbance at 410 nm, silica gel is used as an adsorbent with solvent system acetone/chloroform (2:100)
Cholesterol (bear gall bladder)	10% H_2SO_4 acid in alcohol	UV spectroscopy absorbance at 400 nm, silica gel is used as an adsorbent with solvent system ethyl acetate/acetone/petroleum ether (2:1:11)

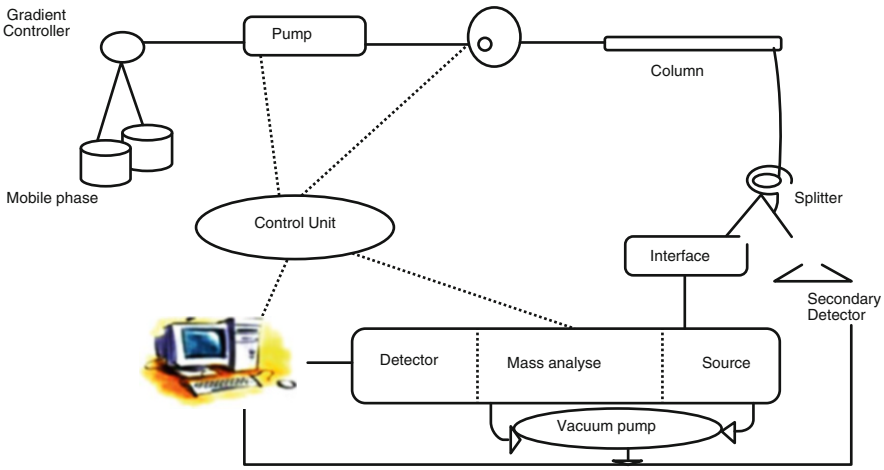

Fig. 14.7 Instrumentation of LC-MS

14.3.2 Gas Chromatography-Mass Spectroscopy (GC-MS)

The most extensively and effectively used combination technique is the GC coupled with MS. The combination of GC and MS was first reported in 1958, and it was made available commercially in 1967 (Ardrey 2003).

GC-MS is a synergistic union, extremely favorable as the compounds which can be analyzed by GC (low molecular weight, medium or low polarity in ppb-ppm

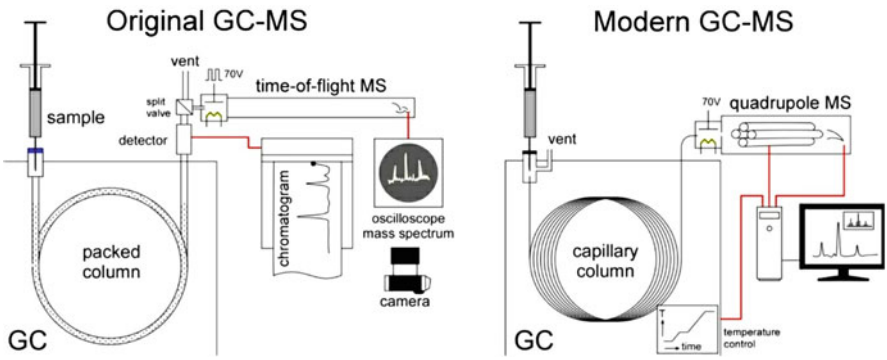

Fig. 14.8 Instrumentation of GC-MS

concentrations) are also compatible with the MS requirements. In addition, both analyses proceed in the same aggregation state, i.e., vapor phase. But both the techniques differ in the working pressures, i.e., pressure in the GC column exists low at 10.5–10.6 Tor than in the ionization chamber. To solve this problem, efficient vacuum pumps (gas jet pump and turbo molecular pump) and gas chromatography capillary columns (having internal diameter of 0.18–0.32 mm, traditionally used in GC-MS) directly inserted into the ionization chamber of a mass detector (Hubschmann 2009; McMaster 2011) are used.

There are several advantages of GC-MS including the capillary column, which provides effective separation capacity, producing clear chemical fingerprint. Another is the coupling with mass spectroscopy and corresponding mass spectral database provides the complete information of the components of the plant extract which can be used for exploring phytoconstituents and their properties especially the volatile compounds (Guetens et al. 2002; Gong et al. 2000a, b; Gong et al. 2001a, b, c; Li et al. 2003; Li and Liang 2003; Wang et al. 2003; Tsai et al. 2002; Vieira and Simon 2002; Gherman et al. 2000; Velasco-Negueruela et al. 2003). Instrumentation of original and modern GC-MS is included in Fig. 14.8.

14.4 Summary

The aim of chromatographic techniques was to separate the individual substances into a mixture depending on their color as in the case of herbal pigments. With time, its application was extended numerously. Nowadays, chromatographic techniques are widely acceptable for separation of phyto-constituents. Advanced chromatographic techniques like HPLC, HPTLC, and GC are extremely reliable for quantification and purification of carbohydrates, nucleic acids, amino acids, steroids, hydrocarbons, proteins, antibiotics and many other phyto constituents due to their high sensitivity, accuracy and reproducibility. GC is a separation technique which is used to analyze thermally stable volatile substances. For example, gas chromatography is used for the determination of fatty acids, flavored compounds, triglycerides,

and several other food components, as well as aroma compounds, pesticides, polychlorinated biphenyls, and other volatile components.

High-performance thin-layer liquid chromatography is effective for quantification of biomarkers in extracts and fractions. Hyphenated techniques like LC-MS and GC-MS are highly authenticate for quantification and molecular weight determination of phyto-pharmaceuticals. The GC-MS is also used for quantification of volatile compounds too. Phytomedicine research has a very important role in contributing to the development of new and better drugs for effective phytotherapy. Fortunately, chromatography offers very powerful separation ability, such that the complex chemical components in HM extracts can be separated into many relatively simple subfractions. Further more, the recent hyphenated chromatographic techniques for separation, isolation, purification and quantification of the phytoconstituents are the stepping for global acceptance of phyto medicines. Separation, isolation, and purification of the phytoconstituents are the stepping stones to achieve this goal. Therefore, development and constant improvement of the existing chromatographic techniques is very crucial to mine the magical compounds from the huge plant repositories.

References

Archana Khale A, Anubha B (2011) An overview on thin layer chromatography. IJPSR 2 (2):256–267

Ardrey RE (2003) Liquid chromatography-mass spectrometry: an introduction, vol 4. Wiley, Hoboken, pp 182–189

Atlas WH (1996) Plant drug analysis: a thin layer chromatography. 2nd edn. Springer, p 87–92

Beckett AH, Stenlake JB (2005) Practical pharmaceutical chemistry, thin layer chromatography. 4th edn. CBS Publishers, p 115–128

Bombarda I, Dupuy N, Le Van Da JP (2008) Comparative chemometric analysis of geographic origins and compositions of lavandin var. Grosso essential oils by mid infrared spectroscopy and gas chromatography. Anal Chim Acta 613:31–39

Chatwal GR, Anand SK (2008) Instrumental methods of chemical analysis, vol. 2. 5th edn. Himalaya Publishing House, p 599–616

Coskun O (2016) Separation techniques: chromatography. North Clin Istanb 3(2):156–160

Faghihi J, Jiang X, Vierling R (2001) Reproducibility of the high- performance liquid chromatographic fingerprints obtained from two soybean cultivars and a selected progeny. J Chromatogr A915:61–74

Fan XH et al (2006) Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. Anal Chim Acta 555:217–224

Gherman C, Culeaand M, Cozar O (2000) Comparative analysis of some active principles of herb plants by GC/MS. Talanta 53:253

Gong F et al (2000a) Chin Anal Chem 28:860

Gong F, Song YQ et al (2000b) Acta Pharm Sin 35:394

Gong F et al (2001a) J Chromatogr 193:905

Gong F et al (2001b) Chem J Chin Univ 22:1481

Gong F et al (2001c) Anal Chim Acta 99:450

Guetens G et al (2002) J Chromatogr A 6:229–308

Habib MY et al (2000) J Nutr Pak 4:17

Hamburger MO, Cordell GA (1987) A direct bioautographic TLC assay for compounds possessing antibacterial activity. J Nat Prod 50, 19:–22
Hubschmann HJ (2009) Handbook of GC-MS: fundamentals and applications, 2nd edn. Wiley, Hoboken, pp 124–132
Jadhav MV et al (2009) J Int J Chem Tech Res 1:826
Jork H, Funk W (1990) LC reagents & detection methods-physical & chemical detection methods: fundamentals, vol 4. Wiley, Hoboken, pp 15–40
Kasture AV, et al (2008) A textbook of pharmaceutical analysis. Instrumental methods. Niraliprakashan, 13th edn, p 148–164
Knap H, Jork L, Liang Y, Fastre S (2006) Low-pressure gas chromatography: recent trends and developments. J Pharm Anal 23(3):212–315
Korfmacher WA (2005) Foundation review: principles and applications of LC-MS in new drug discovery. Drug Discov Today 10(20):1357–1367
Lalla JK et al (2000) J Planar Chromatogr 13:390
Li XN, Liang YZ (2003) Phytochem Anal 14:23–34
Li XN et al (2003) Phytochem Anal 14:23
Li Y, Wu T, Zhu J (2010) Combinative method using HPLC fingerprint and quantitative analyses for quality consistency evaluation of an herbal medicinal preparation produced by different manufacturers. J Pharm Biomed Anal 52:597–602
Lim CK, Lord G (2002) Current development in LC-MS for pharmaceutical analysis. Biol Pharm Bull 25(5):547–557
Lu GH, Chan K, Liang YZ (2005) Development of high- performance liquid chromatographic fingerprints for distinguishing Chinese Angelica from related umbelliferae herbs. J Chromatogr A 1073:383–392
McMaster M (2011) GC/MS: a practical user's guide, vol 4, New York, Wiley, pp 38–45
Mohammad A et al (2010) Analysis of herbal products by thin-layer chromatography: a review. Int J Pharma Biosci 1(2):1–50
Patel R, Patel K (2016) Significance of various chromatographic techniques in herbal drug analysis. J Chem Pharm Res 8(7):877–882
Pharmaceutical Applications with HPLC (2000) Agilent library
Pitt JJ (2009) Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev 30(1):19–34
Pyka A (2014) Detection progress of selected drugs in TLC. Biomed Res Int 2014:732078
Raaman N (2006) Phytochemical techniques, vol. 1. New India Publishing Agency, p 19–25
Richter J et al (2003) J Pharm Anal 23(3):167
Shivatare RS, Nagore DH, Nipanikar SU (2013) HPTLC: an important tool in standardization of herbal medical product: a review. J Sci Innov Res 2(6):1086–1096
Silva GO et al (2017) Extraction methods: qualitative and quantitative techniques for screening of phytochemicals from plants. Am J Essential Oils Natural Prod 5(2):29–32
Sparkman OD (2006) Mass spectrometry. J Am Soc Mass Spectromet 17(6):873–884
Srivastava MM, Springer Verlag (2011) High performance thin-layer chromatography (HPTLC), vol. 6. Berlin, p 32–60
Tsai TR et al (2002) Identification and determination of geniposide contained in Gardenia jasminoides and in two preparations of mixed traditional Chinese medicines. J Chromatogr A 961:83–88
Velasco-Negueruela MJ et al (2003) J Chromatogr A 1011:241
Vieira RF, Simon JE (2002) Econ Bot 54:207
Wang XS et al (2003) Am J Chin Med 31:927
Ye M et al (2007) Analysis of phenolic compounds in rhubarbs using liquid chromatography coupled with electrospray ionization mass spectrometry. J Am Soc Mass Spectrom 18:82–91
Zygmunt B, Namiesnik J (2003) Preparation of samples of plant material for chromatographic analysis. J Chromatogr Sci 41:109–116