Recent Advances in Plant Metabolites Analysis, Isolation, and Characterization

5

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Abstract

Metabolites from natural sources either from terrestrial or marine sources serve as unmatched resources for new drug leads or diverse chemical identity. Due to ever-rising requirement for new pharmacophore in high-throughput screening and discovery for therapeutic drugs from metabolites, there has been motivated interest particularly in edible plants around the globe. Bioactive compounds are indispensable component present in different forms of botanicals, nutraceuticals, and herbal preparations used for the various medicinal applications. The prime focus in present chapter is to enlighten and discus diverse analytical methodologies which have been applied during extraction, isolation, and characterization of active constituents in botanicals, nutraceutical, and herbal preparations.

Keywords

Plant Metabolites · Extraction · Chromatographic techniques · Spectroscopy · Quality Control

5.1 Introduction

Natural products in the form of standardized extracts or pure compounds act as reservoir capable of providing new pharmacophore for drug discovery due to availability of chemical diversity (Cos et al. 2006). It has been claimed that more than 80% of the world's population derives its primary healthcare needs solely from traditional system of medicine as per report of World Health Organization (WHO).

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S. K. Yadav et al. (eds.), *Recent Trends and Techniques in Plant Metabolic Engineering*, https://doi.org/10.1007/978-981-13-2251-8_5

Asians have a long history of using herbal medicines which represents a human connection with the environment. Plants from terrestrial as well as marine sources which have been used in various traditional system of medicine contain eclectic assortment of constituents that can be used for the treatment of infectious as well as chronic diseases (Duraipandiyan et al. 2006). The modern system of medicine which comprise of drugs from synthetic origin suffers a drawback of adverse effects and resistance which instigated turning of men toward ethnopharmacognosy. It has been reported that thousands of phytochemicals isolated from plants are broadly considered as safe and effective alternative with fewer adverse effects. Natural products have a wide range of biological activities ranging from antioxidant, antimicrobial, antidiarrheal, analgesic, anticancer, and wound healing. Traditionally, people claim the benefit of general well-being promoted by utilization of certain natural or herbal products.

Recent reports by World Health Organization (WHO) acclaimed that at least 20,000 medicinal plants exist in 91 countries. The utilization of biologically active compound from plant resources is a multistep process. The premier step includes extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation. A brief summary of the general approaches in extraction, isolation, and characterization of bioactive compound from plants extract can be found in Fig. 5.1. The current chapter discusses various techniques involved during extraction, isolation, and characterization of bioactive compound from natural products of terrestrial as well as marine origin using various phytochemical screening assays and chromatographic and spectroscopic techniques.

5.2 Extraction of Plant Metabolites

Natural source is a vast reserve for bioactive compounds. Each following step involved in the determination of phytochemicals from solid samples is of utmost importance. If any protocol is not followed correctly, then there will be decreased performance of the analysis and errors will be introduced which will consequently pile up and lead to inconsistency in the results. During the extraction process from the matrix, a single compound or cluster of compounds gets transported into a different phase, generally a liquid phase, and is prepared for further analysis in chromatographic systems. The primary goal of extraction may include exhaustive recovery of target compound, selectively retrieving of target compound.

During the extraction process, solvent is transferred to the solid phase, where it pervades it by molecular diffusion. By desorption the soluble material is solubilized from the matrix to extraction solvent (Fig. 5.2). There are numerous extraction procedures developed with the advancement in technology and automation. These include hydro-distillation and Soxhlet and supercritical fluid extraction. Other widely used extraction procedure involves accelerated liquid extraction, microwave-assisted extraction, and ultrasound-assisted extraction. The adoption of extraction procedure depends on the character of the source matter, nature of the target compounds, and objective of the extraction.

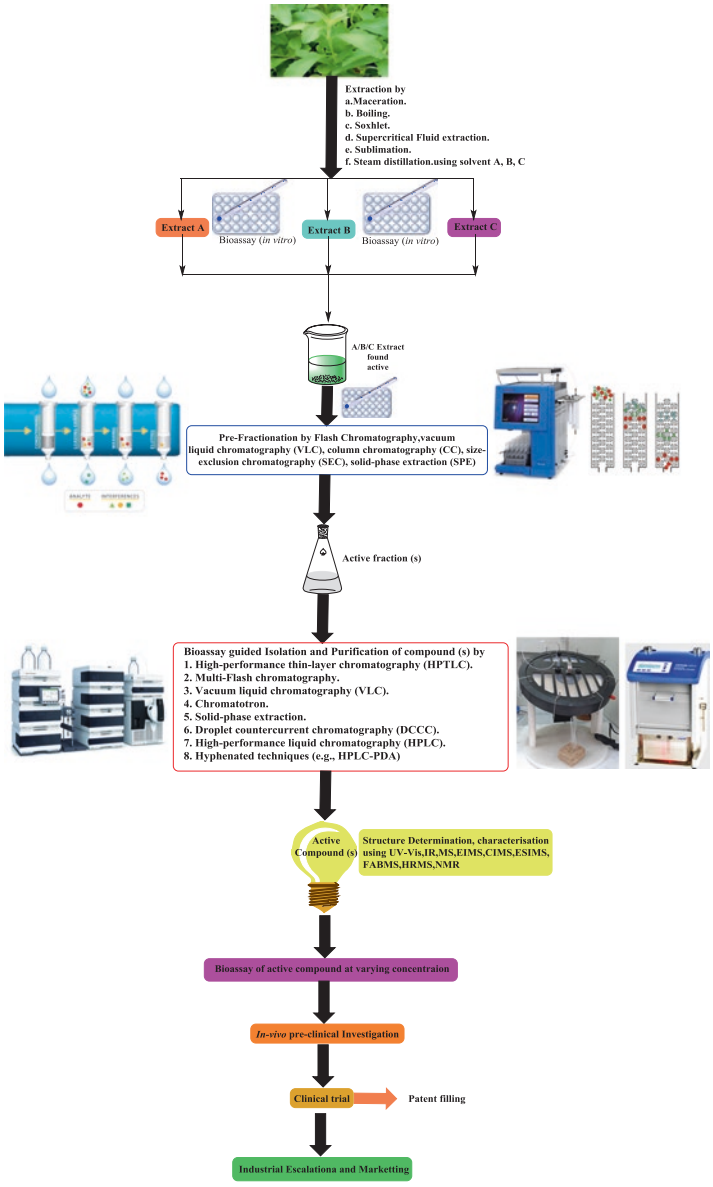


Fig. 5.1 Natural product drug discovery process

5.2.1 Hydro-Distillation Method

It is a distinctive type of distillation for temperature sensitive materials like natural aromatic compounds (Table 5.1) (Guleria et al. 2011b; Saini et al. 2012). In this technique volatile compounds are carried and are subsequently condensed in the

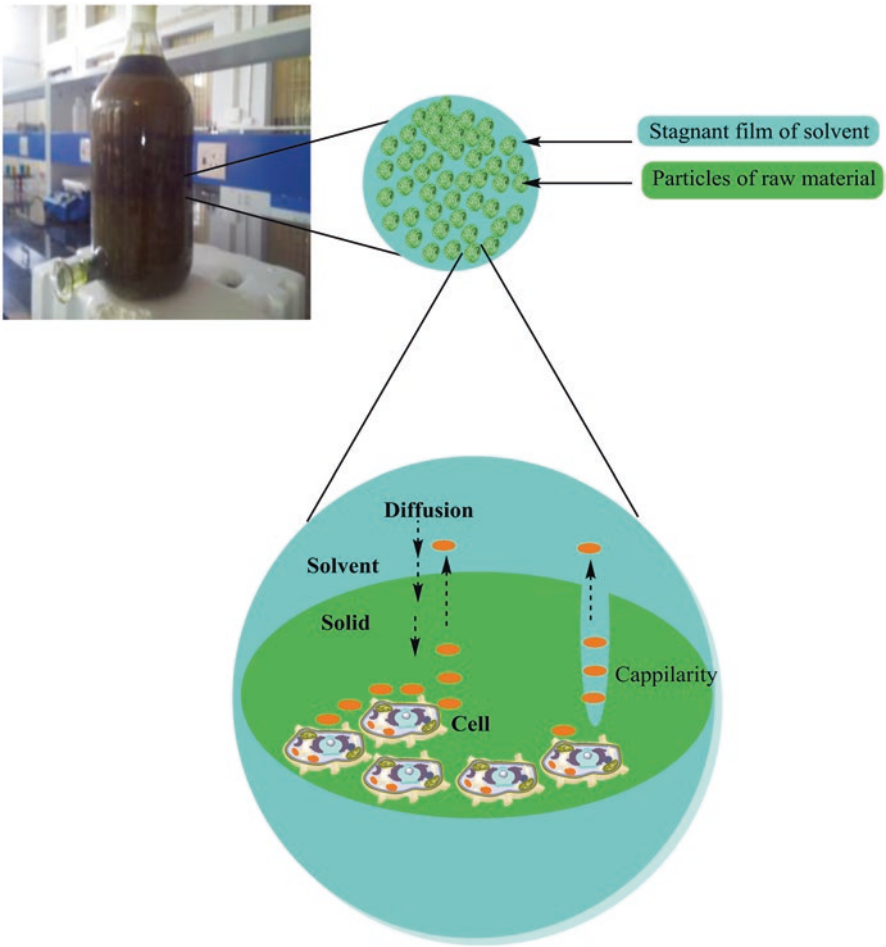


Fig. 5.2 Mechanism of extraction of phytoconstituents from the matrix

condensation flask and get separated from the condensed liquid phase and collected. This process can be effectively applied for the heat-sensitive metabolites under reduced pressure, thereby reducing the operating temperature. The basic principle that operates behind this process is when a mixture of two immiscible liquids is heated and agitated in order to expose the surface of each liquid to the vapor phase, and then each constituent individualistically exerts its own vapor pressure as if the other constituent were not present. Consequently, the vapor pressure of the whole system increases. When the sum of the vapor pressure of two immiscible liquids just exceeds the atmospheric pressure, boiling occurs.

Table 5.1 Most recent published works dealing with the use of hydro-distillation for the extraction of essential oil components

S. no.	Matrix	Compound of interest/major components	References
1	*Eupatorium tremulum*	Volatile oil	deSouza et al. (2017)
2	*Pomelo flavedo*	Essential oil	Liu et al. (2017d)
3	*Calea ternifolia*	Essential oil/chromene 1	Escandon-Rivera et al. (2017)
4	*Tanacetum balsamita* L.	Essential oil/β-thujone	Baczek et al. (2017)
5	*Ligustrum obtusifolium*	Essential oil/hydroxyl anisole	Bhalla and Bajpai (2017)
6	*Ocimum gratissimum* L.	Linalool	Mohr et al. (2017)
7	*Cuminum cyminum*	γ-Terpinene	Minooeianhaghighi et al. (2017)
8	*Boswellia dalzielii*	3-Carene	Kohoude et al. (2017)
9	*Satureja avromanica*	n-Pentacosane	Abdali et al. (2017)
10	*Mentha piperita* L.	Linalool	da et al. (2017)
11	*Pistacia terebinthus*	α-Pinene	Piras et al. (2017)
12	*Citharexylum spinosum*	Cuparene	El et al. (2017)
13	*Dracocephalum kotschyi*	Limonene	Moridi Farimani et al. (2017)
14	*Lavandula x hybrida*	1,8-Cineole	Bajalan et al. (2017)
15	*Calocedrus decurrens*	δ-3-Carene	Ibrahim et al. (2017)

5.2.2 Soaking Method

During this process, the untreated or powdered plant material remains in direct contact with extracting solvent for stipulated period of time in a single container. Soaking solubilizes the soluble material from the solid sample to the solvent (Gupta et al. 2016; Jaitak et al. 2010b). Agitation is provided which facilitates diffusion by increasing the contact of solid with solvent, intensifies mass transfer rate, and processes fine particles avoiding bed compression and its channeling. The agitation continuously removes and redistributes extracting solvent and prevents supersaturation surrounding the surface of the solid to be extracted (Naviglio et al. 2007). Soaking is usually performed at room temperature, but elevated temperatures are applied if the target phytochemicals are not heat sensitive. In a patent, for the preparation of stable fat oilseed extract, the comminuted oilseed was soaked for 1 to about 20 min, and additional heat has applied to extract at temperature of about 140.55 °C for a brief period of 15 s, for destroying anti-nutritional factors such as antitrypsin in the extract (Oberg 1978). The method yielded stable, fat-rich, high-protein, debittered, and colored extract acceptable in the US region (Wilkens and Hackler 1969). The role of solvent is

also well recognized on the characteristics of phytoconstituent obtained after extraction besides the attributes of raw material. It was found that upon extraction of hazelnut woody with acetone yielded higher concentration of phenolic content than ethanol but during the extraction of its roasted kernels by ethanol yielded highest phenolic concentration as compared to the acetone (Contini et al. 2008). Frequently, temperature and solvent both collectively affect the nature, amount, and other characteristics of the phytochemicals. In an investigation-related extraction of resveratrol by solid–liquid from grape canes, it was found that both ethanol and temperature have concentration effect in resveratrol recovery. The effective diffusivity values get increased with temperature, thus leading to higher recovery of phenolic and phytochemicals rich in trans-resveratrol and trans-ε-viniferin. The temperature titration indicated that maximum yield was obtained at 83.6 °C (Karacabey and Mazza 2008). Soaking method suffers a drawback as it is time-consuming, labor intensive, and high energy demanding, the product quality is lost during solvent evaporation, and the mass transfer rate decreases with time.

5.2.3 Soxhlet Extraction

The classical Soxhlet apparatus was designed way back in 1879 by Franz von Soxhlet. It maintains its applicability until today with some modifications for reducing extraction period; decreasing quantity of amount of solvent and agitation is introduced for avoiding the possibility of thermal decomposition of the target compounds (Luque-Garcia and De Castro 2004a; Priego-Capote and de Castro 2005; Virot et al. 2007; Dutta et al. 2017). In a typical Soxhlet apparatus, the grounded plant material packed in a thimble composed of thick filter paper/porous frit glass. In a glass extraction chamber, thimble is positioned above a flask, which contains solvent and condenser attached below. In principle, boiling solvent fills extraction chamber gradually, and upon reaching maximum level, it is siphoned back extracting the solutes into the solvent reservoir below. This cycle is completed in 10–15 min and repeated several times. In this process, a film of filter paper separates the solvent and the ground plant material. The method has an edge over classical soaking extraction that supersaturation of the solvent does not occur even when using hot solvent. The phytochemicals under study should have lower volatility than the solvent as the solute and solvent are separated by distillation. The main advantage is applicability of high temperatures, which causes increased mass transfer rate, no need of a filtration after leaching, and displacement of assignment equilibrium. Few modifications for reducing thermal degradation, solvent consumption, and speeding up process have been applied, for example, focused microwave-assisted Soxhlet extraction (FMASE) system (Fernandez-Pastor et al. 2017a) and ultrasound-assisted Soxhlet extraction (UASE) (Table 5.2) (del Pilar Garcia-Mendoza et al. 2017; Dutta et al. 2017; Pereira et al. 2017).

Table 5.2 Most recent published works dealing with the use of Soxhlet for the extraction of bioactive components

S. no.	Matrix	Compound of interest	References
1	*Schinziophyton rautanenii*	Tocopherol	Gwatidzo et al. (2017)
2	*Cienfuegosia digitata*	Gossypol	Sidi Boune et al. (2017)
3	*Cicer arietinum* L.	Chickpeasaponin B1	Cheng et al. (2017)
4	*Leucas cephalotes*	Fatty acid	Verma et al. (2017)
5	*Olea europaea*	Triterpene acids	Fernandez-Pastor et al. (2017b)
6	*Hallea ciliata*	Lignans	Jack and Nwachoko (2015)
7	*Euterpe edulis* Mart.	Phenolic compounds and anthocyanins	Garcia-Mendoza et al. (2017)
8	*Citrus sinensis*	Phenolic compounds	Espinosa-Pardo et al. (2017)
9	*Leucas cephalotes*	Fatty acid	Verma et al. (2017)
10	*Lagerstroemia speciosa*	Quercetin	Sai et al. (2017)
11	*Fagopyrum esculentum*	Phenolic compounds	Mackela et al. (2017)
12	*Eugenia jambolana*	Flavonoids, tannins, triterpenoids, saponins and glycosides	Sasikala et al. (2016)
13	*Piper longum*	Piperine	Gigliarelli et al. (2017)
14	*Rubus fruticosus*, *Vaccinium myrtillus*, *Eugenia brasiliensis*	Polyphenols	Machado et al. (2017)
15	*Citrus sinensis*	D-Limonene	Negro et al. (2016)

5.2.4 Accelerated Solvent Extraction

In recent years, extraction from solid samples is facilitated by accelerated solvent extraction (ASE) which utilizes liquid phase at high temperature and/or pressure but below its critical point (Table 5.3). The process is efficient due to its automation as it reduces the process time and amount of solvent required for extraction. The term "accelerated solvent extraction" was coined and patented for extraction by Dionex Corporation (Richter et al. 1996). The ASE can be carried out in static mode or in a dynamic mode. The extraction cell in static mode is packed with the grinded material and filled with solvent and equilibrated till desired pressure and temperature are reached. During this process, diffusion and solubilization analytes are discharged from solid matrix and transferred to the solvent. Static mode is selected for avoiding the dilution of extract and transfer equilibrium (diffusion and solubilization) which

Table 5.3 Most recent published works dealing with the use of accelerated solvent extraction for the extraction of bioactive components

S. no.	Matrix	Compound of interest	References
1	*Theobroma cacao*	Polycyclic aromatic hydrocarbons	Belo et al. (2017)
2	*Passiflora* sp.	Flavonoids	Gomes et al. (2017)
3	*Spinacia oleracea*	Flavonoids, phenolic acids, carotenoids, vitamins A, C, and E	Singh et al. (2017)
4	*Morus atropurpurea* Roxb.	Phenolics	Yang et al. (2017)
5	*Trigonella foenum-graceum* L.	Linoleic acid, linolenic acid, and oleic acid	Gu et al. (2017)
6	*Diospyros kaki* L.	Carotenoid	Zaghdoudi et al. (2017)
7	*Panax ginseng*	Ginsenosides	Zhang et al. (2017e)
8	*Brassica oleracea*	Polyphenols	Jayaprakasha et al. (2017)
9	*Curcuma longa* L.	Curcuminoids	Yadav et al. (2017)
10	*Ocimum gratissimum*	Essential oil	Ma et al. (2017a)
11	*Salvia hispanica* L.	Omega-3 fatty acids	Castejon et al. (2017)
12	*Anvillea radiata*	Flavonoids and germacranolides	Boukhris et al. (2016)
13	*Carthamus caeruleus* L.	Phenolic compounds	Toubane et al. (2017)
14	*Impatiens glandulifera*	Phenolic acid	Szewczyk and Olech (2017)
15	*Platycodon grandiflorum*	Platycodin D	Chen et al. (2016b)

governs the extraction process which is mostly displaced under the superheated conditions. In case of dynamic mode, the packed solid material is continuously under circulation with superheated solvent at high pressure. The leaching process is accelerated under this mode as it favors displacement of the transfer equilibrium. Limitation of this mode is the dilution of extract, as there is a continuous use of clean solvent. Thereby, requires an additional steps of subsequent concentration (Priego-Capote 2013).

5.2.5 Supercritical Fluid Extraction

In supercritical fluid extraction (SFE) technique, solvents are used for extraction at temperatures and pressures above their critical points. At critical temperature, a homogeneous supercritical fluid which has zero heat of vaporization is formed at which properties of its gas and liquid phases converge, thereby resulting in only one phase. Green SFE solvents (i.e carbon dioxide behave supercritical fluid at temperature of 31.2oC and pressure 7.38 Mpa) have found a niche as a clean alternative to

hazardous processes due to restricted use of organic solvents after Montreal Protocol 1987. Since the first patent application of SFE for extraction in 1943 and its first industrial application was developed in 1978 (Zosel 1978). Factors including particle size, moisture content, location of the solute, solubility, porosity, and solvent flow rate largely affect SFE. Large particle size decreases the mass transfer area. On the other hand small particle size can make the bed compact, thereby enhancing the internal mass transfer resistance and instigating channeling inside the extraction bed (Nobre et al. 2006). The operating pressure and temperature governs the yield of solute and the separation selectivity. It has been observed that solvent solubilization capacity increases with pressure at constant temperature. The increased temperature at constant pressure eventually disseminates two conflicting effects. In the first instance, it reduces the solvation power of CO_2, and at the second, it intensifies the vapor pressure of solutes, thereby facilitating the transfer of solute to the supercritical phase (Zancan et al. 2002). Another factor is solvent flow rate (solvent-to-feed ratio), an important element as it directly linked to increased running and capital expenses (Brunner 1998; Hurtado-Benavides et al. 2004; Pereira and Meireles 2010). In a recent study, there was a comparative analysis carried out between the hydro-distillation and SFE of volatile oil from *Heracleum thomsonii*, *Rhododendron anthopogon* (Guleria et al. 2011a), and *Capillipedium parviflorum* (Saini et al. 2012). In these studies they observed a difference in the quantitative and qualitative aspect of the volatile oils obtained by the two methods (Guleria et al. 2011a). Therefore, choice of method depends on the target secondary metabolite (Table 5.4).

5.2.6 Ultrasound-Assisted Extraction

With the advancement in technology in twenty-first century, ultrasound technique is considered as innovative and promising. Ultrasound works on the principle of cavitation phenomena, in which microbubbles of gases are formed, get enlarged, and are subsequently imploded in the liquid phase (McClements 1995). When ultrasound is passed from the liquid medium, it causes the longitudinal displacement of the molecules acting as a piston creating a succession of compression and rarefaction phases.

The phenomenon creates voids from dissolved gases in medium, which are also referred to as cavitation bubbles (Schutt 1996). These cavitation bubbles formed have a tendency to grow by rectified diffusion. When they grow and reaches a critical point, these bubbles break down onto the surface of matrix. This causes formation of microjets and shock waves having high pressure and temperature on the solid matrix (Fig. 5.3). Microjets formed disrupt the cell walls of the plant matrix, subsequently leading to the exudation of contents into the extraction medium (Suslick 1989; Wang and Weller 2006). Factors like shape, frequency, power, and size of reactor affect the extraction process. Additionally, solvent type, temperature, and matrix also affect the extraction process. In industrial application, the power is optimized to reduce the operating cost of the extraction. Higher yields and shorter extraction time have been achieved by applying higher ultrasound power,

Table 5.4 Most recent published works dealing with the use of SFE for the extraction of plant metabolites

S. no.	Plant material	Compound of interest	Conditions	References
1.	*Ruta graveolens*	N-Tritriacontane and furanocoumarins	CO_2,120–300 bar and 40–60 °C dynamic method	Sovová et al. (2017)
2.	*Lonicera japonica*	Phenolics and Flavonoids	CO_2, 150–350 bar at 45 °C for 2 h,	Hsu et al. (2016)
3.	*Salvia hispanica*	Fatty acids and phenolic compounds	CO_2, CO_2 + ethanol, CO_2 + ethyl acetate, 50–300 bar, 40–50 °C	Guindani et al. (2016)
4.	*Butia catarinensis*	Phenolic	CO_2, 100–300 bar, 40–60 °C,	Cruz et al. (2017)
5.	*Myrtus communis* L.	Flavonoids and anthocyanins	CO_2, 230 bar, 45 °C	Pereira et al. (2016)
6.	*Satureja montana L.*, *Coriandrum sativum L.*, and *Ocimum basilicum* L.	Essential oil	CO_2, 100 bar, 40 °C	Elgndi et al. (2017)
7.	*Piper nigrum* L.	Piperine and terpenoids	CO_2, 200 bar and 40 °C	Grinevicius et al. (2017)
8.	*Corylus avellana*	Phenolic	CO_2 + ethanol, 300-350 bar pressure, at 40-50 °C	Barla Demirkoz and Karakas (2017)
9.	*Eryngium billardieri*	Essential oil	CO_2, 100-300 bar, 35–55 °C	Esquivel-Hernandez et al. (2016)
10.	*Paeonia* sp.	Essential oils	CO_2, 400 bar, and 55 °C	Yusong (2016)

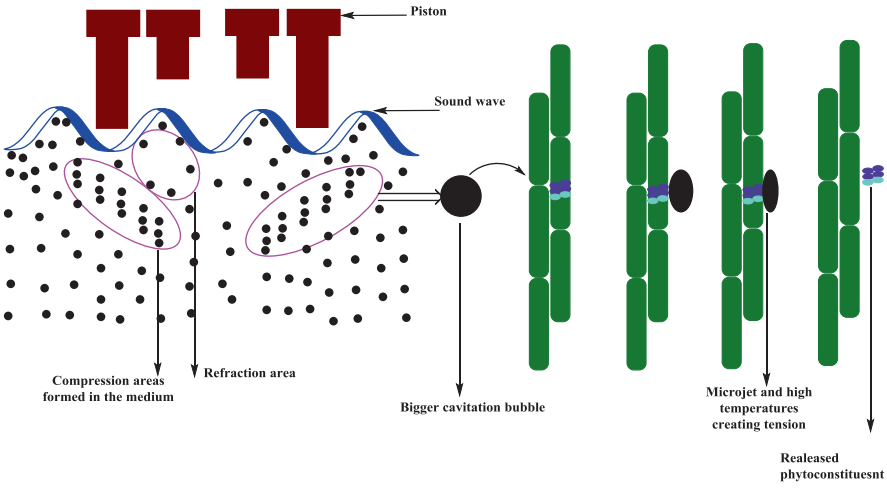


Fig. 5.3 Mechanism of ultrasound-assisted extraction process

Table 5.5 Recent application of ultrasound-assisted extraction process

S. no.	Matrix	Compound of interest	References
1.	*Pseudobulbus cremastrae seu pleiones*	Colchicine	Tang (2016b)
2.	*Polygonatum sibiricum*	Polysaccharide	Ruan and Wang (2017)
3.	*Dioscorea zingiberensis*	Diosgenin	Li et al. (2014)
4.	*Acer palmatum*	Flavonoids	Zhu (2014)
5.	*Fritillaria thunbergii*	Polysaccharide	Zheng et al. (2014)
6.	*Lonicera japonica*	Chlorogenic acid	Wang (2013)
7.	*Cynara cardunculus* L., *Cynara scolymus* L.	Phenolic	Kollia et al. (2017)
8.	*Carica papaya* Linn.	Fiber polysaccharide	Zhang et al. (2017c)
9.	*Xylocarpus granatum*	Limonoids	Shi et al. (2017b)
10.	*Chenopodium quinoa*	*Chenopodium* Quinoa polysaccharides. A and C	Hu et al. (2017)
11.	*Oryza sativa*	Tocols, γ-oryzanol, and ferulic acid	Truong et al. (2017)
12.	*Okra* sp.	Okra polysaccharide	Ni et al. (2017)
13.	*Tithonia diversifolia*	Tagitinin C	Silva et al. (2017a)
14.	*Nelumbo nucifera*	Hyperoside	Huang et al. (2017a)
15.	*Artemisia rupestris*	Polysaccharide	Liu et al. (2017a)

decreasing moisture, enhancing solvent–solid contact, and optimizing temperature condition (Chen et al. 2007; Riera et al. 2004; Weiss et al. 2011). Upon increasing the temperature, it causes rise in vapor pressure and reduction in viscosity and surface tension, which facilitates entry of solvent vapors in the bubble cavity; consequently bubbles collapse less violently, thus reducing the sonication effect (Santos et al. 2008). The process has been applied during the extraction of a vast array of plant metabolites from the plant sources (Table 5.5).

5.2.7 Microwave-Assisted Extraction

In the era of green technology, microwave-assisted extraction (MAE) have attracted considerable interest and emerged as promising techniques for extraction (Jaitak et al. 2009). Effect of microwave energy is strongly dependent on the nature of both solvent and plant matrix. In case of microwave-assisted extraction, it is possible to transfer heat only to sample matrix, thereby releasing the solutes in cold solvent which leads to prevention of degradation of thermolabile compounds. Heating phenomenon of water causes liquid vaporization within the matrix core cells leading to rupturing of cell walls and plasma membrane. As the majority of phytochemicals

are localized inside cell walls/cytoplasm, hence its disruption often leads to shortened diffusion path. It also facilitates mass transfer of solvent into the matrix of plant material. As a result, the phytochemicals gets diffused into the solvent, thus improving the efficiency of extraction. The methods suffer a disadvantage due to application of elevated temperature, higher power of irradiation, and longer irradiation time which can eventually lead to degradation of thermolabile compounds. In order to counter this drawback, MAE has been carried out under an inert atmosphere or vacuum, thereby restricting the aerial degradation by oxygen in the reactor (Destandau et al. 2013); such a process is called nitrogen-protected microwave-assisted extraction (NPMAE). The reduced extraction time and improved energy efficiency are attributed by fast microwave heating of solvent–sample mixture. Thereby, microwave energy is primarily used for accelerating the heating and refining the conventional extraction method efficiency. Some recently developed modifications of MAE include focused microwave-assisted Soxhlet extraction (FMASE) (Luque-Garcıa and De Castro 2004b), microwave hydro-distillation (MWHD or MAHD) (Lucchesi et al. 2004), and microwave steam distillation (MSD) (Sahraoui et al. 2017). Solvent-free microwave extraction (SFME) is the only technique which has been used for the extraction of secondary metabolites from fresh/moistened matrix without any solvent/water (Benmoussa et al. 2016). Firstly these developments were designed for essential oil extraction, but today other compounds soluble in water can also be extracted without solvent. The different variations in the process have been applied in the extraction of secondary metabolites from the natural products (Table 5.6).

5.3 Isolation of Plant Metabolites

The vital factor that needs to be taken account before designing an isolation protocol is identifying the nature of the crude extracts or fractions. The typical features of target compound which govern the selection criterion for the isolation process include acid–base properties, solubility (hydrophobicity or hydrophilicity), stability, charge, and molecular size. Numerous chromatographic techniques have been developed which have been used in the isolation of various types of secondary metabolites. Among these techniques low-pressure column chromatography (LPCC), ion-exchange chromatography (IEC), thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), high-speed countercurrent chromatography (HSCCC), and high-performance liquid chromatography (HPLC) have been widely used.

5.3.1 Low-Pressure Column Chromatography

In this procedure, the separation occurs via selective distribution of the solutes between a mobile phase and a stationary phase. The equilibrium that exists between solvent and stationary phase is referred as distribution constant. It depends on the

Table 5.6 Recent application of microwave-assisted extraction process

S. no.	Matrix	Compound of interest	References
1.	*Arctium lappa*	Arctiin	Yao and Sun (2017)
2.	*Radix et rhizoma glycyrrhizae*	Flavonoids	Chen et al. (2016a)
3.	*Cortex phellodendri*	Active ingredients	Wang (2016)
4.	*Brassica rapa*	Essential oil	Saka et al. (2017)
5.	*Citrus reticulata*	Essential oil, pigment, pectin, flavone	Zhou et al. (2017a)
6.	*Bursera graveolens*, *Ribes nigrum*, *Rosmarinus officinalis*	Resins, anthocyanins, Essential oils	Patrascu and Kumbakisaka (2016)
		Fats and glyceridic oils	
		Polyphenols (nonpolymeric), proteins	
		Sesquiterpenes, terpenes	
7.	*Zingiber officinale*	Gingerol	Zhang et al. (2016c)
8.	*Stevia rebaudiana*	Steviol glycosides	Jaitak et al. (2009)
9.	*Petroselinum crispum*	Essential oil	Dong et al. (2017)
10.	*Berberis jaeschkeana*	Polyphenolics	Belwal et al. (2017)
11.	*Nerium oleander*	Oleandrin	Jablonksi and Green (2017)
12.	*Ziziphus joazeiro*	Betulinic acid	Fonseca et al. (2017)
13.	*Polygala tenuifolia*	Sibiricose A5, 3,6′-di-O-sinapoyl-sucrose, glomeratose A, tenuifoliside B, and tenuifoliside C	Li et al. (2017d)
14.	*Zingiber officinale*	Gingerols and shogaols	Guo et al. (2017a)
15.	*Myristica fragrans*	Essential oil	Bouchachia et al. (2017)

chemical nature of separation system. Some factors that regulate the separation process include physiochemical nature of mobile phase, stationary phase, and solutes which control the numerous interactions between the solutes and two phases. The interactions between solute and stationary phase depend on the particle size and distribution of the stationary phase. Number of interactions is directly proportional to the surface area of stationary phase. In this method, there is column that is compactly packed with very small porous polymer beads having pore size designed specifically for the target analyte. When the mixture travels down, the column smaller particles enter into the pores, whereas larger particles are not able to enter pores. Therefore, larger particles escape easily and eluted first. Because larger particles flow through the column more quickly and smaller molecules gets trapped inside small pores of the stationary phase and have prolonged retention time. It has been found that enhanced separations can be achieved by stationary phase which has high surface area (Salituro and Dufresne 1998). This technique can be divided into adsorption chromatography and size-exclusion chromatography. In adsorption chromatography, the separation is primarily based on the distinctive adsorption

affinities of the analyte molecules for topography of the stationary phase (Snyder 1968). Interactive forces that come into play include hydrogen bonding, dipole–dipole interactions, van der Waal forces, complexation, acid–base properties, and charge transfer.

Low-pressure column chromatography can be classified on the basis of solvent used. One is size-exclusion chromatography (SEC) or gel permeation chromatography (GPC) in cases when organic solvents are utilized, and the other is gel filtration chromatography (GFC) (aqueous solvents are used). This technique solely works molecular size and shape of the analyte molecules, and there is no interaction involved between solute and stationary phase (Mori and Barth 2013). These techniques have applied in the extraction and purification of commercially and biologically important secondary metabolites (Table 5.7).

5.3.2 Ion-Exchange Chromatography

In this technique the separation of ions and polar molecules is based on their affinity for ion exchanger. It has been applied to a wide range of charged molecules like large proteins (Lenhoff 2016), small nucleotides (Li et al. 2017c), amino acids (Rustandi et al. 2016), and natural compounds (Klejdus et al. 2017) (Table 5.8). A straight intention in the application of the ion-exchange method is concentrating, and selective extraction of the desired secondary metabolite is targeted. The

Table 5.7 Recent application of low-pressure column chromatography

S. no.	Matrix	Compound of interest	References
1.	*Sabia parviflora*	Alkaloids	Yan and Zhu (2017)
2.	*Polygonum capitatum*	Flavonoid glycoside	Qi et al. (2016)
3.	*Geigeria alata*	Phenolic acids	Zheleva-Dimitrova et al. (2017)
4.	*Phaseolus lunatus* L.	Lectin	e. Lacerda et al. (2017)
5.	*Geigeria alata*	Acylquinic acids	Zheleva-Dimitrova et al. (2017)
6.	*Raphanus raphanistrum*	Radish thioglycoside	Kuang et al. (2017)
7.	*Ammi majus* L.	Methoxyfuranocoumarins	Bartnik and Mazurek (2016)
8.	*Panax quinquefolius*	Polysaccharides	Yu et al. (2017)
9.	*Ficus simplicissima*	Pinocembrin-7-O-β-D-glucoside	Wan et al. (2016)
10.	*Panicum maximum*	Peroxidase	Centeno et al. (2017)
11.	*Herba hedyotidis corymbosae*	Paederosidic acid	Lin et al. (2016)
12.	*Oryza sativa*	Vitamin E	Li et al. (2017b)
13.	*Armillaria albolanripes*	Essential oil	Zhang et al. (2016b)
14.	*Idesia polycarpa*	β-amyrin and β-sitosterol	Zhang et al. (2016a)
15.	*Dicliptera chinensis*	Polysaccharides	Xu et al. (2017)

Table 5.8 Recent application of ion-exchange methods

S. no.	Matrix	Compound of interest	References
1.	*Momordica dioica*	Trypsin inhibitor protein	Varghese and Gajbhiye (2016)
2.	*Allium sativum*	Garlic polysaccharide	Peng (2017)
3.	*Eriobotrya japonica*	Polysaccharides	He et al. (2016)
4.	*Ziziphus jujuba*	Polysaccharides	Zhang et al. (2017a)
5.	*Corylus heterophylla*	Hazelnut ACE inhibitory peptide	Min et al. (2016)
6.	*Ginkgo biloba*	N-methylated tyramine derivatives	Konczol et al. (2016)
7.	*Flax protein powder*	Trypsin inhibitor	Shi and Chen (2016)
8.	*Artemisia annua*	Artemisinin	Mantri (2016)
9.	*Stevia rebaudiana*	Rebaudioside B	Liao et al. (2016)
10.	*Hibiscus sabdariffa*	Polysaccharide	Jiang et al. (2017)
11.	*Pennisetum glaucum*	Lipoxygenase	Sharma et al. (2017)
12.	*Cicer arietinum*	Protease	Shamsi et al. (2017)
13.	*Ananas comosus*	Bromelain	Ramli et al. (2017)
14.	*Colocasia esculenta*	B-Prism lectin	Vajravijayan et al. (2016)
15.	*Dendranthema indicum*	Polysaccharide	Wang et al. (2016)

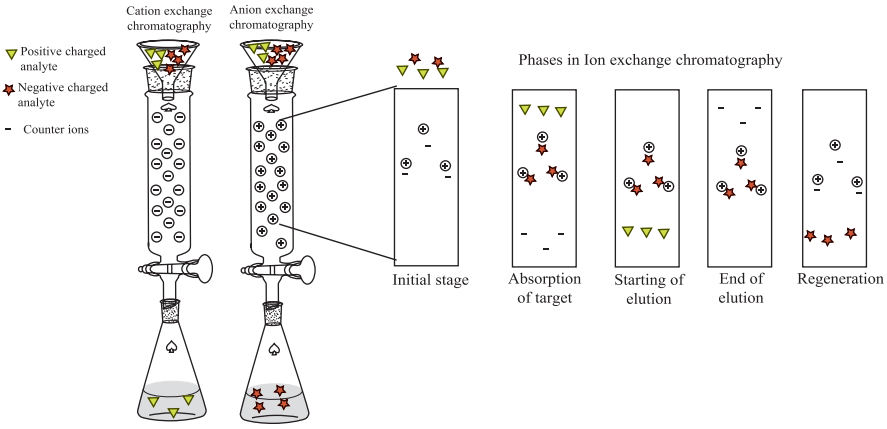


Fig. 5.4 A positively charged anion exchanger particle is shown, with counter ions on its surface in the starting state. Next, the binding of the ions to be separated takes place. At the starting of the elution, positive-charged ions are eluted. Till the end of elution, only target analyte is present binding to matrix. During regeneration, target analyte is desorbed, and the starting state can be reconstituted via washing the column with the starting buffer

imperative need of the separation by this technique is the availability of charged or ionizable group within the molecular structure. Ionizable group enables reversible binding of charged molecules to an oppositely charged insoluble matrix (Harland 1994) (Fig. 5.4). A charged group in target molecule binds to oppositely charged

group present in the supporting resin matrix and is in turn retained by displacement of a counterion.

5.3.3 Thin-Layer Chromatography

It is a simple, low-cost, swift, and extensively used technique for the investigation and isolation of natural and synthetic products. The TLC uses adsorption, partition, size inclusion/exclusion and ion exchange. A variation in the conventional TLC is HPTLC (high-performance thin-layer chromatography) which is an evolved form of TLC, which uses many chromatographic layers for maximum separation efficiency. This technique employs of contemporary instrumentation during all the steps involved in procedure starting from the accurate sample application to reproducible chromatogram development and standardized and software-assisted analysis. In comparison to erstwhile isolation techniques, the upfront cost for an HPTLC system, maintenance, and analysis cost per sample is comparatively small. The most helpful aspects of TLC, which is a visual assessment of separated analytes on the plate, reach an entirely new dimension in HPTLC by the using of advanced techniques generating and evaluating digital images (Ankad et al. 2015; Fuchs et al. 2008). Various validated HPTLC techniques (Jaitak et al. 2008a) has been utilized for both for quantification and qualitative analysis of secondary metabolites (Table 5.9).

5.3.4 High-Speed Countercurrent Chromatography

The technique has been evolved over the last 20 years. It is the serenest form of chromatography as it uses no solid support, which reduces the chances of loss of the substrate by binding to column. Solvent and Teflon tubing is the only media encountered by the sample. This method is frequently used in the isolation of similar compounds having identical polarity (Ito 2005), as it has been found that they can have remarkably different specific partition coefficients in a two-phase system, resulting in efficient separation by HSCC (Oka et al. 1991). In this system among the two liquid phases which come in contact, one phase is pumped through a column or a series of connected chambers containing both the phases. The components which are needed to be isolated are seperated on the basis of there solubilities and vigorous mixing and settling actions in the two phases (Weisz et al. 2000). A broad range of two-phase solvent systems has been developed which consist of at least two immiscible liquids for selective separation of desired principle/target compounds. In this method, the role of stationary phase is played by one fluid and the pumped fluid through the column serves as mobile phase. The liquid in the stationary phase remains stationary by gravity or by centrifugal force. Droplet countercurrent chromatography (DCCC) utilizes a gravity for keeping the stationary phase in place (Ito and Tanimura 1974). Under the influence of centrifugal force, two modes such as hydrostatic and hydrodyanamic mode of

Table 5.9 Recent application of high-performance thin-layer chromatography

S. no.	Matrix	Compound of interest	References
1	*Stevia rebaudiana*	Steviol glycosides	Jaitak et al. (2008a)
2	*Blumea mollis*	Essential oil	Ravindran et al. (2016)
3	*Cymbopogon distans*	Kaempferol, scopletin, and vanilla acid	Ji et al. (2016)
4	*Barleria cristata*	Phenol, flavonoid, alkaloid and steroid	Devaki and Naramadha (2016)
5	Herbs of Gramineae family	Ferulic acid and p-coumaric acid	Dhande et al. (2016)
6	*Ocimum sanctum*	Eugenol, ursolic acid, oleanolic acid, and β- sitosterol	Ghani and Khan (2016)
7	*Crocus sativus* L.	Crocins and picrocrocin	Kabiri et al. (2017)
8	*Hippophae rhamnoides turkestanica*	Nucleobases	Mishra et al. (2017)
9	*Stevia rebaudiana*	Jasmonic acid	Kilam et al. (2017)
10	*Aristolochia indica*	Aristolochic acid	Agrawal and Laddha (2017)
11	*Colocasia esculenta*	Flavonoids	Lebot et al. (2017)
12	*Centratherum punctatum* Cass	Flavones	Shankaran et al. (2017)
13	*Fallopia japonica*	Flavan-3-ols and proanthocyanidins	Glavnik et al. (2017)
14	*Ipomoea batatas*	Polysaccharides	Lebot (2017)
15	*Leea indica*	Quercetin and gallic acid	Patel et al. (2017)

stationary phase are retained. Hydrostatic instruments are often advertised as centrifugal partition chromatography (CPC). In this method a series of connected chambers are rotated around a central axis (Marchal et al. 2003). Hydrodynamic instruments, for example, high-speed or high-performance countercurrent chromatography (HSCCC and HPCCC, respectively), operate on the priciple of Archimedes' screw force (Ito 2005). These methods have high recovery of the analyte which is resultant of elimination of permanent adsorption of the analyte onto the solid column supports (Sutherland 2007). Another advantage of this technique is easy switching between normal-phase chromatography and reversed-phase chromatography merely by changing mobile and stationary phases (Berthod et al. 2003). The scaling up of experiments conducted in the laboratory to industrial volumes is easily possible in countercurrent chromatography. During the technology transfer in case of GC or HPLC, the resolution is lost when working on large sample size due to issues with surface-to-volume ratios and flow dynamics. These two factors does not have an infuence when both phases are liquid (Liang et al. 2008) (Schwarz et al. 2003). A various recent application of high-speed countercurrent chromatography in natural products has been discussed in Table 5.10.

Table 5.10 Recent application of high-speed countercurrent chromatography

S. no.	Matrix	Compound of interest	References
1.	*Erigeron breviscapus*	Breviscapine	Liu et al. (2017c)
2.	*Nelumbo nucifera*	Flavone glycoside	Wu et al. (2017a)
3.	*Eurycoma longifolia*	Polyacetylenes	Wang et al. (2017c)
4.	*Radix Isatidis*	R,S·epigoitrin	Huang et al. (2017c)
5.	*Herba peristrophis roxburghianae*	Glycosylflavone	Xie and Jiang (2016)
6.	*Rhodiola rosea*	*Rhodiola rosea* rhodioside	Chen (2016)
7.	*Ormocarpum kirkii*	Phenolic	Kamto et al. (2017)
8.	*Gastrodia elata*	Gastrodin	Tang (2016a)
9.	*Chrysanthemum indicum*	Flavonoids	Li and Su (2016)
10.	*Lonicera japonica* Thunb.	Flavonoid glycosides and caffeoylquinic acid	Wang et al. (2017a)
11.	*Juglans mandshurica*	Gallic acid	Zhang et al. (2017d)
12.	*Eurycoma longifolia*	Polyacetylenes	Wang et al. (2017b)
13.	*Curcuma longa*	Sesquiterpenoids	Zhou et al. (2017b)
14.	*Isatis tinctorial*	R,S-goitrin	Huang et al. (2017b)
15.	Red wine	Polyphenols	Li et al. (2017e)

5.3.5 High-Performance Liquid Chromatographic Separation Methods

The application of preparative high-performance/pressure liquid chromatography (prep HPLC) is an integral part of drug discovery. Over the last 10 years, this technique has been used for the standardization, quantification, and isolation of most diverse classes of natural products (Table 5.11). The prep HPLC differs from other forms of "lower-pressure" column chromatographic system in consistency, distribution, and size of the particles packed in stationary phase. The stationary phase in HPLC has a lower average particle size in the range of 3–10 mm, which is considerably lower than the particle size used in other techniques. Particles of the stationary phase are manufactured in a spherical shape, and the size distribution is kept to be narrow as much possible which results in extremely uniform packing and in a reproducible manner. It has been observed that poor resolution and isolation occur if voids or channels are present in stationary phase due to disrupted uniformity in the flow of mobile phase. Due to use of small particle size, this technique requires the application of relatively high controlled pressure (up to 3–4000 psi) in order to drive the mobile phase through the stationary phase. However, this is efficiently achieved

Table 5.11 Recent application of high-performance liquid chromatography

S. no.	Matrix	Compound of interest	References
1.	*Perilla* sp.	Dipeptide	Hong et al. (2017)
2.	*Ilex pubescens*	Secoiridoid glucosides	Zhang et al. (2017f)
3.	*Camellia sinensis*	Aflatoxins	Zeng et al. (2016)
4.	*Paeonia suffruticosa*	Phenolic acids	Fu et al. (2016)
5.	*Empetrum hermaphroditum*	Anthocyanins and flavanols	Lavola et al. (2017)
6.	*Gloriosa superba*	Colchicine	Zawahir et al. (2017)
7.	*Psidium guajava*	Quercetin	Liu et al. (2017b)
8.	*Polygonum cillinerve*	Anthraquinones and resveratrol	Wu et al. (2017b)
9.	*Tritici fructus*	Vitamin B2	Xu et al. (2017)
10.	*Hertia cheirifolia* L.	Phenolic compound	Majouli et al. (2017)
11.	*Maclura pomifera*	Protease inhibitor	Indarte et al. (2016)
12.	*Nepeta leucophylla*	Polyphenolic compound	Sharma et al. (2017)
13.	*Camellia japonica* L.	Flavonoid glycosides	Sato et al. (2017)
14.	*Trigonella* sp.	Flavonoids, phenolics, and mineral contents	Güngör et al. (2017)
15.	*Coix larchryma-jobi L.*	Peptides	Li et al. (2017a)

by the available sophisticated technology. The chromatography separation with high resolution is attributed by small particle size yielding high surface area hence enabling the efficient interaction of analytes with the stationary phase. Currently there are six modes of HPLC which are been in use for the purification and analysis of complex mixture secondary metabolites. These include HP-RPC (high-performance reversed-phase chromatography), HP-HILIC (high-performance hydrophilic interaction chromatography), HP-NPC (high-performance normal-phase chromatography), HP-IEX (high-performance ion-exchange chromatography), HP-AC (high-performance affinity chromatography), and HP-SEC (high-performance size-exclusion chromatography). HP-SEC (high-performance size-exclusion chromatography) is usually performed under isocratic conditions, but all other methods can also be maneuvered under step gradient or gradient elution conditions (variable step or continuous changes in eluent composition). All the modes can be used in analytical, semipreparative (Mazzei and Antonio d'Avila 2003), or preparative (Ben Mansour et al. 2017; Udompaisarn et al. 2017) situations. HP-SEC separates the analytes on the basis of their molecular mass or their hydrodynamic volume (Striegel et al. 2009). There is an existence of hydrophobic interaction between nonpolar surface regions of the stationary phase and analytes in case of RPC which drives the chromatographic separation (Horváth et al. 1976) and this principle is also referred to as solvophobic theory (Molnar 2005), whereas HP-HILIC separates analytes on

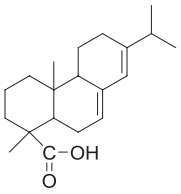
the basis of hydrophilic interaction between the immobilized hydrophilic ligands on porous stationary phases and analytes (Pelekani et al. 1999). Electrostatic interactions are the driving forces in case of HP-IEX hence, separating the analyte on the basis of charged surface of the analyte(s) and complementary immobilized charged surface of the sorbent stationary phase (Kopaciewicz et al. 1983). Presence of chirality in phytochemicals led to the development of chiral HPLC techniques in natural products. One form is HP-AC that is used for the resolution of enantiomeric compounds. In this technique stationary phase has immobilized biomimetic/biospecific ligands and separates the analytes on the principles of molecular recognition (Abbott et al. 2001; Miao et al. 2017a). With the advent of bio-polymerization, another variation of HPLC is HPMC (high-performance membrane chromatography), in which separation is achieved by using 1-mm-thick layers made from macroporous methacrylate polymer. This technique has an technological edge over other forms of HPLC as high resolution is obtained at much lower pressure, eliminating the need of heavy duty hardware and also can be operated at higher loading volume (Tennikova et al. 1990).

5.4 Characterization of Plant Metabolites

Structure elucidation and characterization of compounds isolated from natural resources including plants, bacteria, fungi, or other organisms are time-consuming and often can be a "bottleneck" in drug discovery. In the past, structure elucidation was done by several synthetic modification and derivatization of the target compound. It has led to the misinterpretation of several compounds which were corrected after the discovery of spectroscopic techniques (Lamb 2015; Nicolaou and Snyder 2005). A wide range of spectroscopic techniques have been developed for the requisition of about structural information, but the deciphering of these spectra depends on the experience and deep knowledge of the specialists in natural product chemistry. Remarkable advancement in the field of artificial intelligence and computing has led to development of automated structure elucidation programs. An array of spectroscopic techniques have been developed which include ultraviolet-visible spectroscopy (UV-Vis), infrared (IR) and Raman spectroscopy, mass spectrometry (MS), nuclear magnetic resonance (NMR), circular dichroism spectroscopy, polarimetry, and X-ray crystallography.

5.4.1 UV-Visible Spectroscopy

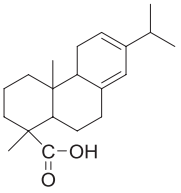
In the case of IR and Raman spectroscopy, it causes molecules to undergo vibrational transitions, whereas, in the case of UV-visible spectroscopy, the shorter wavelength having higher energy causes molecules to undergo **electronic transitions**. The functional group present in the analyte molecules that has an capacity to absorb light in the UV-vis region strongly are referred to as **chromophores.** A fantastic example is β-carotene; its structure has 11 conjugated double bonds. β-carotene



Abietic acid

heteroannular diene = 214 nm
4 alkyl substitution (4 x 5) = 20 nm
1 exo C=C = 5 nm
239 nm

Observed: 235, 241.5, 250 nm



Levopimaric acid

homoannular diene = 253 nm
4 alkyl substitution (4 x 5) = 20 nm
1 exo C=C = 5 nm
278 nm

Observed : 272 nm

Fig. 5.5 Application of Woodward–Fisher rule in differentiating abietic acid and levopimaric acid

absorbs wavelengths in the area of blue light of visible spectrum and emits its complementary red-yellow region which is the reason of the observed orange color of carrots (Boun and Huxsoll 1991). Various other anthocyanin has been characterized and quantified using the UV-visible spectroscopy (Giusti and Wrolstad 2001). Woodward–Fisher rule is applied for the calculation of empirical prediction of the wavelength for the lowest energy $\pi \rightarrow \pi^*$ electronic transition. It can be applied even for differentiating between abietic acid and levopimaric acid (Fig. 5.5) (Moore and Lawrence 1959). Apart from the characterization, it has also been applied in the quality control application by identifying the adulteration (Shimamoto and Tubino 2016). Recently, the technique is used, and the protocol was developed for detecting the adulteration in the sauces produced by different brands (Di Anibal et al. 2016). The technique is also implemented for classifying the samples of pure peaberry and real standard classes of coffee. The method provides a basis of straightforward and reliable method for the authentication of peaberry coffee (Suhandy and Yulia 2017). In a routine experimental procedure, the enzymatic de-glycosylations were assessed using HPLC, but recently, a protocol has been developed for the quantification of the enzymatic activity using the UV–visible spectrometry (Weiz et al. 2017).

5.4.2 Infrared Spectroscopy and Raman Spectroscopy

Infrared spectroscopy (IR) and Raman spectroscopy techniques are based on the principle of vibrational energy. IR spectroscopy is a resultant of absorption phenomenon. Infrared radiation is passed through the sample, photons are absorbed which match the energy difference of vibrational levels, and there is transition from ground to an excited state. The absorption of energy by vibrating chemical bonds generates a spectrum which primarily consists of stretching and bending modes. The prerequisite condition for obtaining IR spectrum of analyte under investigation

should possess a permanent or induced dipole moment. Characteristic for the bond present in the sample corresponds to the frequency or wave number that is absorbed (Table 5.12). Raman spectra are based on the inelastic scattering of monochromatic light. The source of light used is usually in the range of visible, near-infrared, and near-UV region. Raman spectroscopy helps in understanding the chemical structures, bonding of molecules, and the electronic environment around the molecules by analyzing the scattered light. The information obtained from IR and Raman spectroscopy gives corresponding information based on the fact that atoms in the molecule vibrate with a few certain sharply defined distinctive frequency (Singh et al. 2014). Both the techniques have been used widely for the qualitative and

Table 5.12 IR absorption frequency ranges of functional groups

S. no.	Functional group	Characteristic absorption(s) (cm^{-1})	Intensity
1.	Alkyl C–H stretch	2950–2850	Strong
2.	Alkenyl C–H stretch	3100–3010	Medium
3.	Alkenyl C=C stretch	1680–1620	Variable
4.	Alkynyl C–H stretch	~3300	Strong, sharp
5.	Alkynyl C≡C stretch	2260–2100	Variable, not present in symmetrical alkynes
6.	Aromatic C–H stretch	~3030	Medium
7.	Aromatic C–H bending	860–680	Medium
8.	Aromatic C=C bending	1700–1500	Medium-weak, multiple bands
9.	Alcohol/phenol O–H stretch	3550–3200	Strong, broad
10.	Carboxylic acid O–H stretch	3000–2500	Strong, very broad
11.	Amine N–H stretch	3500–3300	Medium (primary amines have two bands; secondary have one band, often very weak)
12.	Nitrile C≡N stretch	2260–2220	Medium
13.	Aldehyde C=O stretch	1740–1690	Strong
14.	Ketone C=O stretch	1750–1680	Strong
15.	Ester C=O stretch	1750–1735	Strong
16.	Carboxylic acid C=O stretch	1780–1710	Strong
17.	Amide C=O stretch	1690–1630	Strong
18.	Amide N–H stretch	3700–3500	Unsubstituted have two bands

quantitative (Musingarabwi et al. 2016) characterization of natural products. IR has been employed in the analysis of secondary metabolites. Recently, an innovative approach MYCOSPEC has been developed which can be utilized in the analysis of mycotoxin, which is released by the fungi in spoilt food (Sieger et al. 2017). In another example real-time qualitative and quantitative profiling of grapes berries at various stages of development was achieved by IR spectroscopy. It is an innovative approach as compared to HPLC, due to its robustness, rapidity, and accuracy (Musingarabwi et al. 2016). Fourier transform IR spectroscopy is also applied for evaluating the changes in the cellular composition of Gram-negative bacterial cells after their exposure to *Crocus sativus* L. extracts (Anastasaki et al. 2016). Raman spectroscopy is a nondestructive technique and is efficient in the determination of carotene content (Liu et al. 2016) and ranking of carrots according to carotenoid content (Lawaetz et al. 2016). Surface-enhanced Raman spectroscopy has been utilized in the molecular fingerprint identification with high analytical sensitivity and low processing time for the detection of B2 and B12 in cereals (Radu et al. 2016).

5.4.3 Mass Spectrometry

Identification and quantification of phytochemicals have been invariably performed by using mass spectrometry (MS) in natural product research. MS has proved its applicability in the field due to increased speed, sensitivity, and selectivity. MS has an advantage of versatility for analyzing solids, liquids, and gases. There has been a considerable advancement in the mass spectrometry due to continuous technological revolution in the ion source, ionization method, and mass analyzers. In general, the choice of ionization method hugely depends on the range of molecular mass and nature (neutral, ionic, polar) target compound. In recent years, mass spectroscopy is widely used not only in the determination of molecular weight but also applied in the differentiation of chemotypes of plant species like *Cinnamomum camphora*. DAPCI-MS (desorption atmospheric pressure chemical ionization mass spectrometry) was able to differentiate five chemotypes: isoborneol-type, camphora-type, cineole-type, linalool-type, and borneol-type, a type of *Cinnamomum camphora* (Guo et al. 2017b). In another invention, gas chromatography–electron impact ionization mass spectrometry (GC-EI-MS) method was patented for detecting pesticide silthiofam and penthiopyrad (Guo 2016) in fruits and vegetables (Guo et al. 2016). In a recent patent application, matrix-assisted light desorption ionization (MALDI) mass spectroscopy was used for the detection and protein fingerprinting of *Burkholderia gladioli* (Wu et al. 2016). HRMS fragmentation pattern in combination with NMR spectroscopy is widely applied in the identification of new plant metabolites. Recently, coccinone B which was isolated from *Symphonia globulifera* was identified by matching the HRMS fragmentation pattern with the METLIN database (https://metlin.scripps.edu/index.php) and an in-house database (containing metabolites isolated from *S. globulifera* (Cottet et al. 2017). Mass spectroscopy is also applied in the food science. Recently a technique involving the use of electron spray ionization mass spectroscopy (ESI-MS) was utilized for the

detection of azaperone and its metabolite azaperol which is used as a tranquilizer in pork (Du et al. 2017). Gas chromatography–mass spectroscopy (GC-MS) is widely used in the analysis of volatile flavor constituents (Stappen et al. 2015a; Stappen et al. 2015b; Stappen et al. 2015c). GC-MS was used in determining the variability of volatile constituents in *Artemisia maritima* collected from three different high-altitude locations in western Himalayan region (Jaitak et al. 2008b). In a recent patent, it was used for the identification of chemical constituents in rapeseed flavor (Bu et al. 2017). The quality control of natural products has also been done by GC-MS, for example, it has been used in the identification of fresh cocoon silk and dried cocoon silk (Ma et al. 2017b).

5.4.4 Nuclear Magnetic Resonance

The advancements in the modern spectroscopic techniques have reformed the process of compound identification and quantification (Jaitak et al. 2010a; Singla and Jaitak 2016). Earlier multigram quantities of isolated natural products were required for their identification. The isolated compounds were derivatized and degraded, and the resulting derivatives or fragments were analyzed for identifying the structure. The requirement multigram quantities and series of experimentation have been eliminated with the tremendous technological improvement in sensitivity and resolution NMR. Now it is possible to identify the precise structure in microgram quantities. NMR exploits the magnetic properties of individual atomic nuclei. Under the influence of applied magnetic field, NMR active nuclei (most commonly analyzed ^{1}H or ^{13}C) absorb electromagnetic radiation at a frequency characteristic of the isotope (Shah et al. 2006). The energy of the absorption, resonant frequency, and intensity of signal is proportional to the strength of applied magnetic field. Free induction decay (FID) is obtained when the sample is excited with a radio-frequency (60–1000 MHz) pulse (Bottomley 2016). A Fourier transform extracts frequency-domain spectrum from raw time-domain FID. Initially, spectrum obtained from single FID has a low signal-to-noise ratio, but it is improved by averaging large number of repeated acquisitions (Levitt 2001). NMR is often linked with other spectroscopic or isolation techniques for enhancing the data acquisition and acquiring more informative data. Internal standard recovery constant high-performance liquid chromatography–quantitative nuclear magnetic resonance (ISRC-HPLC-qNMR) is widely used in cases where the compounds with lower purity primarily molecular weight more than 500 qNMR are at risk of error of the purity because the impurity peaks are likely to be incompletely separated. From the peak of major component (Zhang et al. 2017b). A novel differential offline LC-NMR approach (DOLC-NMR) was developed to capture and quantify nutrient-induced metabolome alterations in *Saccharomyces cerevisiae* (Hammerl et al. 2017). Multiple hyphenated techniques are also prepared for the accelerated structural identification of natural products in complex mixtures. HPLC-PDA-HRMS-SPE-NMR (high-performance liquid chromatography, photodiode array detection, high-resolution mass spectrometry, solid-phase extraction, and NMR spectroscopy) is utilized in the analysis of coumarins in

Coleonema album using analytical scale pentafluorophenyl column for orthogonal separation, followed by fractionation using preparative-scale C_{18} column (Lima et al. 2017). A similar application was also applied in *Ziziphus nummularia*, *Ziziphus spina-christi* (Tuenter et al. 2017), *Solanum americanum* (Silva et al. 2017b), *Ziziphus oxyphylla* (Tuenter et al. 2016), *Eremanthus crotonoides* (Silva et al. 2016), and *Eremophila lucida* (Tahtah et al. 2016). Supercritical fluid chromatography (SFC) has been combined with online NMR. It enables the use of a hydrogen-free solvent which circumvents the problems associated normally with solvent signals. Braumann et al. separated and identified five acetate isomers of vitamin A by using supercritical CO_2 as the eluent and 400 MHz NMR spectrometer with a specially designed pressure-proof probe for detection (Braumann et al. 1997). In a patent, gel permeation chromatography was coupled with NMR that was developed for the analysis of polymer-containing samples (Kamlowski et al. 2011). An innovative procedure which combines NMR, GPC, and analytical pyrolysis coupled with gas chromatography/mass spectrometry was applied for the characterization of whole cell wall and evaluation of its state of preservation (Zoia et al. 2017). In comparison to the HPLC-SPE-MS-NMR better sample recovery was obtained when using (capillary liquid chromatography) CapLC-NMR. This indicated that CapLC-NMR is best suited for samples that are truly mass limited (Lewis et al. 2005). There are considerable improvements in the stand-alone as well as in hyphenation techniques where NMR has played an imperative role in the structural investigation. These techniques not only widened the range of separation methods but also have enabled analysis of single components from the complex mixtures. The advancements have reduced the analysis time and improved the precision without laborious preparative-scale separation and purification procedures.

5.4.5 Circular Dichroism Spectroscopy

Linearly polarized light upon passing through a medium containing chiral material and plane of the transmitted light is rotated by an angle "α" as compared to the angle of incident light. The linearly polarized incident light now becomes ellipsoidal polarized. The observed phenomenon prompted by the change in absorption intensity for left and right circularly polarized light is referred to as circular dichroism (CD). In nature, it has been observed that natural products/phytochemicals are chiral, which have a profound effect on their biological activity (Mason 1982). Therefore, determination of the absolute configuration of chiral compounds in natural product research and biomolecular systems is of utmost importance. Electronic circular dichroism (ECD) is a sensitive diagnostic tool which not only helps in assigning the absolute conformation and configuration but also have been applied in determining the intermolecular interactions involved in chiral systems (Berova and Nakanishi 2000; Eliel and Wilen 2008). Recently, eight stereoisomeric 2,3-dihydrobenzo[b]furan neolignans isolated from *Gardenia ternifolia* called as gardenifolins A–H (1a(I)-d and 2a-d) were structurally characterized by CD (Tshitenge et al. 2017). It has also been patented for use as a diagnostic technique for the

prediction in severe alcoholic hepatitis by measuring optical activity of albumin–bilirubin ([A-B]) compound in blood plasma (Das et al. 2016). In a patent by Ping He, the absolute configuration of schizandrin present in the drug was found to be (+)-(7S, 8R)-schizandrin, by circular dichroism spectroscopy (He et al. 2014).

5.4.6 Polarimetry

Optically active samples exhibit circular birefringence, a phenomena in which plane polarized light rotates as it passes through the sample. To measure this rotation, a polarimeter consists of a long tube with flat glass ends to place the samples. The angle of rotation is then record using a scale. Sex pheromone ((2,2-dimethyl-3-isopropylidene cyclobutyl) methyl 3-methylbut-2-enoate) of cotton mealybug, *Phenacoccus solenopsis*, was identified by polarimetry and found to be the (R)-(−)-enantiomer (Tabata and Ichiki 2016). In another study the polarimetry in combination with GC/GC-MS was used in the identification of five compounds having antimicrobial activity from the fruit of *Kundmannia sicula* L. (Chouitah et al. 2015). Similarly, a new spermidine macrocyclic alkaloid (2S)-9-benzoyl-1-methyl-2-phenyl-1,5,9-triazacyclotridecan-4-one was identified from *Gymnosporia arenicola* and found to be optically active using polarimetry $[\alpha]_D$ given in −13.27 deg. $cm^2\ g^{-1}$. In addition to the determination of optical activity, polarimetry has also been used for the quantitative determination of menthol in the preparation of camphor liniment (Ling et al. 2014). In another study xanthine oxidase inhibitors nudibaccatumin A and B were identified from *Piper nudibaccatum* by polarimetry and 1D and 2D NMR, HRESIMS, UV, IR spectroscopy (Liu et al. 2015).

5.4.7 X-Ray Crystallography

In 1941, Max von Laue was awarded Nobel Prize for the breakthrough invention of diffraction of X-rays by crystals. Since then the technique has become an essential part of natural product discovery for structural elucidation. X-ray crystallography has been employed for defining the atomic and molecular structure of a crystal. In this technique the crystalline atoms diffracts a beam of incident X-rays to many precise directions. The crystallographer analyzes the angles and intensities of these diffracted beams. This helps in deducing three-dimensional picture of electrons density within the crystal. The distribution of electron density and mean positions of atoms in the crystals are also determined. Further nature of chemical bonds and disorders if any is also identified. In certain cases, due to unavailability of single crystals of sufficient size, various other X-ray methods have been developed. However, in these cases less detailed information is available; hence these techniques are continuously in the phase of active development. Such methods include fiber diffraction, powder diffraction, and small-angle X-ray scattering (SAXS). Another method of electron crystallography is applied for determining the atomic structure of the material which is only available in the form of nanocrystalline

powders or suffers from poor crystallinity. Recently, a patent discloses a new kind of crystal of docetaxel trihydrate having a characteristic peak at 4.30 ± 0.2 °, 8.74 ± 0.2 °, 10.32 ± 0.2 °, 11.04 ± 0.2 °, 13.92 ± 0.2 °, and 17.70 ± 0.2 ° as determined by powder X-ray diffraction (Zheng et al. 2017). Several patents for the determination of crystal structure of important commercial biomolecules have been granted in past years, for example, imazapyr and diclosulam, herbicides (Bristow 2017a, c); nelzarabine, vandetanib antitumor drugs (Yang 2017; Zhou et al. 2017b), dequalinium an antiseptic (Huang et al. 2017b), sulconazole an antifungal agent (Shi et al. 2017a), brexpiprazole a serotonin–dopamine activity modulator (Miao et al. 2017b), pyraclostrobin a fungicide (Tian and Zimmerman 2017), and isoxadifen (Bristow 2017b). X-ray crystallography has helped in the correction of misassigned natural product. A classical example is the structure of cholesterol for which Weiland and Windaus were awarded Nobel prize, but the right structure was reported later on (Lamb 2015). Another example is kinamycin C which is isolated from *Streptomyces murayamaensis*: it was realized after 21 years of discovery that the cyano group in the structure was actually a diazo group (Gould et al. 1994; Mithani et al. 1994).

5.5 Conclusion

In the present chapter, we have tried to portray the most significant and beneficial applications of plant metabolites and illustrated their importance in recent era. Extraction processes governs the bioactivity and composition of the extracts. Hence, it is crucial to comprehend the mechanisms implicated in the extraction processes. In the next step, selection of isolation and analysis hugely depend on the nature of extract obtained during extraction process. Purified compounds can be isolated more efficiently from the complex natural sources by employing a continuum of separation techniques and applying multistep isolation procedures. In the final step, characterization of purified compounds was elaborated by spectroscopic techniques. With the advancements in technology and evolution of spectroscopy has eliminated need of derivatization for structure elucidation. Artificial intelligence and advanced database mining have increased in the accuracy in characterization. Therefore, for any natural product chemist, it is therefore indispensable to acclimatize and experience newer approaches and techniques used in the natural product discovery.

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