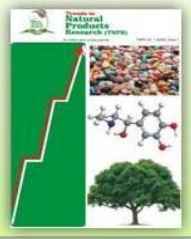
Trends in
Natural Products Research



Basics and Fundamentals of Natural Product Research

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Abstract: Historical evidence shows that plant-derived agents have had therapeutic relevance in the lives of humans providing different classes of drugs. Many natural products and synthetically modified natural product derivatives have been successfully developed for clinical use to treat human diseases. Drug discovery from medicinal plants continues to provide new and important leads against various pathologies targets including cancer, malaria, cardiovascular diseases and neurological disorders. Proper sample preparation can increase the extraction efficiency of biologically active compounds. Extraction is the separation of the pharmacologically active, chemical distinct non-matrix components of a plant, microbial, or animal material from the matric (structural) parts. Natural extracts are often extremely complex and contain many unknown compounds. In this situation, the use of an effect-related analytical approach is a real relief. Information about biological effects of natural complex materials in humans, is a necessity for natural product research to be meaningful and useful. This brings to the fore effect-directed analysis which identifies or isolates substances of biological relevance. Data bases and books were consulted for information contained in this review. This review discusses the fundamentals of natural product research from a wide range of methods of preparing plant material, extraction, concentration, separation, isolation, pharmacological activity screening, toxicity profiling, virtual screening, and data analysis

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INTRODUCTION

Sample preparation is critical in obtaining accurate analytical data and reliable interpretation of results of plant analysis. Proven procedures should be followed during decontamination, drying, particle-size reduction, storage. The drying process is primarily aimed at inhibiting metabolic processes which result in stopping changes in the plant's chemical composition. This occurs by removing water from the plant material, which is necessary for plant enzymes to function properly. The lack of water and a high drying temperature contribute to the inhibition of enzymes that can break down the active ingredients. Proper drying also reduces the number of microorganisms in the final product. It significantly reduces the weight and volume of plant material, reducing the costs of packaging, transport, and storage of plant substances (Lewicki, 2006; Stepien *et al*., 2008). The drying of the plant material may take place under natural or artificial conditions. The former includes drying in the open air or semi-open rooms, where free air circulation is used, but there is no direct sunlight. Drying carried out in natural conditions is the oldest method of preserving raw material. Nowadays, this method is abandoned because the process is long and does not allow for the adjustment of drying parameters, which means that the quality of the obtained dried material is low. Drying in artificial conditions includes ventilated rooms with various heating methods, where conditions such as temperature range and air pressure can be adjusted (Lewicki, 2006; Roshanak *et al*., 2016; Thamkaew *et a*l., 2021).

The method of drying plant materials largely depends on the active ingredients it contains.

Air-drying, microwave-drying and oven-drying can be utilized in drying plant materials. The air-drying method does not force dried plant materials using high temperature; hence, heat-labile compounds is preserved. However, air-drying take longer time in comparison to microwave drying and freeze drying and may be subjected to contamination at unstable temperature condition. Oven-drying uses thermal energy to remove moisture from the samples. Oven-drying at 44.5°C for 4 hours does not cause any significant reduction in antioxidant activity of most plant materials (Mediani *et a*l., 2013).

Steps in extraction of natural products involve (1) size reduction, (2) extraction, (3) filtration, (4) concentration, and (5) drying. After drying, samples should be ground to pass a 1.0-mm (20 mesh) screen using the appropriate mill. A 20-mesh sieve is adequate if the sample aliquot to be assayed is > 0.5 g. However, if the sample aliquot to be assayed is < 0.5 g, a 40-mesh screen should be utilized (Jones and Case, 1990). After grinding, the sample should be thoroughly mixed. After particle size reduction and homogenization, samples should be stored in conditions that will minimize deterioration and maintain sample integrity for weighing and follow-up analytical work. These may be air-tight plastic storage containers, storage cabinet located in cool, dark, moisture-free environment, or a refrigerator. Containers should be stored under cool, dry conditions. For long-term storage, ground samples should be thoroughly dried, sealed, and placed under refrigerated conditions (4^{o}C) until analyses can be completed (Campbell and Plank, 1998). If samples are placed in a cool (4^{o}C), dark, dry environment, storage life is significantly extended.

Extraction

Extraction is the separation of the pharmacologically active, chemical distinct non-matrix components of a plant, microbial, or animal material from the matric (structural) parts. Extraction and recovery of a solute from a solid matrix may be regarded as a five-stage process: (i) desorption of the compound from the active sites of the matrix, (ii) diffusion into the matrix itself, (iii) solubilization of the analyte in the extractant, (iv) diffusion of the compound in the extractant, (v) collection of the extracted solutes (Sticher, 2007). Extraction is a major determinant of the success, and the quality of any natural product phytochemical analysis. The handling, collection and processing, of the material and appropriateness of extraction procedure tremendously affect the outcome of a natural product research. The nature of the metabolite to be extracted and the nature of the extracting solvents have significant effect on the yield and composition of extracts. The quantity and quality of an extraction procedure are affected by the nature of plant material, extraction technology applied, sample particle size, and experimental conditions, especially temperature, solvent polarity concentration, and solvent-to- sample ratio. Extraction using polar organic solvents (e.g. ethanol, methanol, or an aqueous alcoholic mixture) is employed in an attempt to extract as many compounds as possible. It is based on the ability of alcoholic solvents to increase cell wall permeability, facilitating the efficient extraction of large amounts of polar and medium- to low-polarity constituents. The components of such a mixture are separated by partitioning with water immiscible solvents of varying polarities (Sticher, 2008). Extraction can also be performed through liquid-liquid extraction method. This method takes advantage of the immiscibility of liquids and the differential solubility of compounds in different liquids. Addition of a second immiscible solvent causes compounds which are more soluble in it to move from the initial solution into it. The factors that contribute to the efficiency of solvent extraction are

polarity of solvent or solvent mixtures, pH, temperature, and particle size.

The Hildebrand solubility parameter (δ) provides numerical estimate of the degree of interaction between materials and can be used as indicator of solubility. Materials with similar (δ) values are likely to be miscible**.** https://www.chemeurope.com/en/

The Hildebrand value of a solvent mixture can be determined by averaging the Hildebrand values of the individual solvents by volume.

To calculate the solvent polarity index for a mixture of two solvents, the following formula is used:

$P = \phi_A p_A + \phi_B p_B$========== Equation 1

where ΦA and ΦB are the volume fractions of solvents A and B (ΦA + Φβ = 1), PA and PB are the polarity index of pure solvents A and B. Equation 1 can be written as

$P_{tot} = \Psi_A P_A + \Psi_B P_B$ ==========

Equation 2

where P_{tot} is the solvent polarity index for a mixture of solvents A and B that have an individual polarity index of P_A or P_B, respectively, and Ψ_A or Ψ_B are the volume fractions of these solvents in the new mobile phase (Ravindranath, 1989).

A wide range of technologies are available for the extraction of active components and essential oils from medicinal and aromatic plants. The choice depends on the economic feasibility and suitability of the process to the particular situation (Singh 2008). General methods of extraction of medicinal plants include: maceration, infusion, digestion, decoction, percolation, Soxhlet extraction or hot continuous extraction, counter-current extraction, ultrasound extraction (Sonication), supercritical fluid extraction, microwave assisted extraction (MAE), accelerated solvent extraction (ASE), and phytonics. The type of extraction procedure also plays a decisive role in determining the qualitative and quantitative phytochemical constituents. Factors affecting the choice of extraction process include nature of the crude drug, stability of the crude drug, cost of the crude drug, solvent, concentration of the product and recovery of solvent. The spectrum of constituents may vary considerably depending on the hydrophilic or lipophilic nature of the solvent.

Another convenient approach is sequential solvent extraction in which solvents of increasing polarity are used in turns. Initial extraction with low-polarity solvents yields the more lipophilic components, while ethanolic solvents obtain a larger spectrum of non-polar and polar material (Sticher, 2008). Unwanted lipids or lipophilic materials in crude extract can be eliminated by washing it with non-polar solvents like hexane, or dichloromethane (Bligh *et al*., 2013). When different solvents are applied to the same plant material during the process of extraction the process is called sequential extraction. The residue from an extraction process is used as starting material for the next extraction. When the various solvents are applied to different portions of the plant material the process is referred to as parallel extraction. When seeking compounds from green plant material, the success of the extraction with alcohol depends on the extent to which chlorophyll is removed. It can then be assumed that all the low molecular weight compounds have been extracted during extraction it is advisable to obtain a crude extract, then fractionate into separate main classes of compounds which is then subjected to chromatographic analysis (Harborne, 1998). The factors that contribute to the efficiency of solvent extraction are polarity of solvent or solvent mixtures, pH, temperature, and particle size (Harborne, 1998).

Concentration

A rotary evaporator (sometimes abbreviated to rotavap) is a piece of equipment primarily used to remove solvent from a sample through "evaporation under reduced pressure. The presence of reduced pressure in the apparatus causes the solvent to boil at a lower temperature than normal. Rotating the round bottom flask increases the liquid's surface area and thus the rate of evaporation. The solvent vapor travels into the cooler water condenser, where it condenses and drips into a separate receiving flask, the solvent is removed, therefore leaving a concentrated compound in the original round bottom flask.

Lyophilization or freeze drying or molecular drying is a process in which water is frozen, followed by its removal from the sample, initially by sublimation (primary drying) and then by desorption (secondary drying). Freeze-drying is a process of drying in which water is sublimed from the product after it is frozen. Generally, this process removes water from a material using sublimation, i.e., a solid-to-gas transition bypassing the liquid phase. It is carried out in negative temperature conditions and significantly reduced pressure (1–50 Pa). The first and crucial step in this process is freezing at a temperature of −40 °C to −50 °C and is relatively short-lived to prevent the formation of large ice crystals. This procedure allows for the removal of up to 95% of the water and can take up to 2 days. In this process, moisture does not condense into a liquid but condenses in a volatile state. The last stage is re-drying, called desorption, which occurs at 40–50 °C. Its purpose is to eliminate the strongly bound (chemically) water that has not been frozen and remains in the dried material. As a result, only 1–2 % of water remains in the freeze-dried material (Kunal *et al*., 2015). It is a drying process applicable to certain pharmaceuticals and biologicals that are thermo-labile or otherwise

unstable in aqueous solutions for prolonged storage periods, but that are stable in the dry state. The quality of the obtained dried material is much higher than in other drying methods.

Drying

Methods of drying include air-drying, microwave-drying, oven-drying. Drying can cause substantial changes in the physical and organoleptic properties of dried foods (Romanik *et a*l., 2007; Moldoveanu *et a*l., 2015:). Several changes to food occur when it is subjected to this processing method. There is increasing concern that some thermal methods negatively affect the bioactive components of herbs. Oven drying is considered one of the easiest and most rapid thermal processing methods that can preserve phytochemical content. Numerous studies have been performed to study the influence of oven drying on phytochemical compounds of herbs (Kong et al., 2010). Plant materials can be dried in an oven set at between 30^{o}C and 45^{o}C without loss of quality or activity (Mediani *et al*., 2013).

Separation, Isolation and Characterization

Most of the times, phytochemical evaluation presents with mixtures whose components have to be characterized. An ideal technique to achieve this is chromatography. It is a physical separation technique based on differential affinity of various compounds for the stationary and mobile phases. This differential affinity gives rise variation in speed of movement of the separating compounds, thereby resulting in their separation. Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction (IUPAC, 2014). The two phases can be a solid and liquid, a liquid and a liquid gas and a solid, or a gas and a liquid. Separation of two or more compounds or ions is based on their molecular interactions with two phases- one moving and one stationary. The partitioning of the analytes between these two phases determines the rate at which they pass through the system, and allows them to be separated from one another.

The fundamental principle of chromatography is the distribution equilibrium that forms when a compound is either dissolved in a mobile phase or adsorbed on a stationary phase.

Without qualitative and quantitative information, study of the phytochemistry of plants is of little or no value. This information can only be gotten when the chemical constituents of plants are identified and separated. Chromatography is an inevitable undertaking in phytochemical evaluation. No level of sophistication in instrumentation and techniques will make chromatography irrelevant in phytochemical evaluation. Methods and techniques involved in preparative chromatography include quantitative thin layer chromatography (quantitative TLC), High Performance Liquid Chromatography (HPLC), Vacuum Liquid Chromatography (VLC), Flash Chromatography (FC), gas chromatography (GC), gas chromatography-mass spectroscopy (GC-MS), among others. According to Williamson and Masters (2011), the separation and purification of plant constituents is mainly carried out using one or a combination, of four chromatographic techniques: paper chromatography (PC), thin layer chromatography (TLC), gas liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). The choice of technique depends largely on the solubility properties and volatilities of the compounds to be separated. PC is particularly applicable to water-soluble plant constituents, GLC finds its main application with volatile compounds, (non)less volatile constituents are separated by HPLC, a method which combines column efficiency with speed of analysis. It is also possible to use a liquid as the stationary phase. This might seem odd at first, because it would seem as if the flowing mobile phase would somehow push along a liquid stationary phase. The way to use a liquid as the stationary phase is to coat a very thin layer of it onto a solid support (Figure 1),

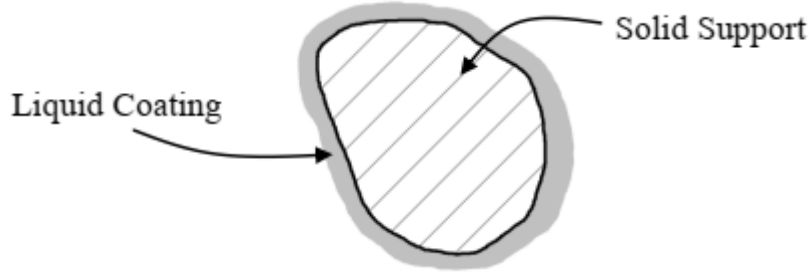

Figure 1. Liquid phase coated onto a solid support.

The stationary phase may be held in a column or coated on a planar cheet (Figure 2). In principle, column and planar chromatographic processes represent different separation methods, each with its own strengths and weaknesses (Spangenberg et al., 2011).

Figure 2. Liquid phase coated onto the inside walls of a capillary column

Liquid/liquid extraction techniques

In liquid chromatographic technique the solute separates by being distributed between two immiscible liquids one of which. acting as the stationary phase is coated on a solid support. If the stationary phase is polar the operation is referred to as Normal Phase (NP), while if it is non-polar it is Reversed Phase (RP) chromatography (Inczedy *et al*., 1997; Majors and Carr, 2001)

Partitioning of solutes between a stationary and a mobile phase is the fundamental principle of all kinds of chromatographic methods. Separation methods are the tools of choice to determine liquid–liquid partition coefficients (Berthod and Carda-Broch, 2004).

If a third substance is added to a system of two immiscible liquids in equilibrium, the added component will distribute itself between the two liquid phases until the ratio of its concentrations in each phase attain a certain value; the distribution constant or partition coefficient (Majors and Wilmington, 2013).

The compounds are partitioned between a largely water-immiscible alcoholic solvent (e.g. n-butanol) and water. The classic solvent mixture, n-butanol-acetic acid-water (4:1 :5), (abbreviated as BAW) was indeed devised as a means of increasing the water content of the n-butanol layer and thus improving the utility of the solvent mixture. Indeed, BAW is still widely applicable as a general solvent for many classes of plant constituent (Harborne, 1998). Liquid /liquid extraction involves two layers: the organic layer and the aqueous layer. The solvent used for extraction possess a number of properties including the following; it should be immiscible with water, it should readily dissolve the substance to be extracted at room temperature, it should not react with the solute or the other solvent, it should not be highly inflammable and it should be relatively inexpensive.

The net amount of analyte extracted depends on the value of the distribution coefficient, and on the ratio of the volumes of the two phases used. More analyte is extracted with multiple portions of extracting solvent than with a single portion of an equivalent volume of the extracting phase. Recovery is independent of the concentration of the original aqueous sample.

A quantitative measure of how an organic compound will distribute between aqueous and organic phases is called the distribution or partition coefficient. It is the ratio, K, of the solubility of solute dissolved in the organic layer to the solubility of material dissolved in the aqueous layer. It is an equilibrium constant with a certain value for a given substance, pair of solvents, and temperature.

$$\text{Distribution constant, K} = \frac{\text{Solubility in organic (g/100ml)}}{\text{olubility in water (g/100ml)}} \quad ==========$$

Equation 3

By using the correct solvent system, a molecule can be specifically selected and extracted from another solvent. Since the distribution coefficient is a ratio, unless K is very large, not all of a solute will reside in the organic layer in a single extraction. Usually two, three, or four extractions of the aqueous layer with an organic solvent are carried out in sequence in order to remove as much of the desired product from the aqueous layer as possible (Wells, 2003).

Many smaller extractions are more efficient than one large extraction. This phenomenon can be proved mathematically and the applicable equation is as follows:

$$\%\ Rx = \frac{100K_D}{K_D+(\frac{V_O}{V_E})} \text{ ==========Equation 4}$$

where V_O is the volume of the original sample and V_E is the extraction solvent volume. % RX = percent of analyte extracted into organic solvent. (The recovery factor of analyte X, expressed as a percent). The recovery factor can also be expressed in the equivalent form

$$\%\ Rx = 100\left[\frac{K_D(V_E/V_O)}{1+K_D(V_E/V_O)}\right] = \quad 100\left[\frac{K_D(V)}{1+K_D(V)}\right]$$

==========Equation 5

where V / VE=VO is known as the phase ratio.

The formula for expressing repeated extractions is

$$\%\ Rx = \langle 1 - \left[\frac{1}{1+K_D\left(\frac{V_E}{V_O}\right)}\right]^n x\ 100\rangle$$

========== Equation 6

Investigation of the principle of repeated extractions demonstrates that (i) the net amount of analyte extracted depends on the value of the distribution coefficient (ii) the net amount of analyte extracted depends on the ratio of the volumes of the two phases used. More analyte is extracted with multiple portions of extracting solvent than with a single portion of an equivalent volume of the extracting phase, and (iii) recovery is independent of the concentration of the original aqueous sample (Wells, 2003).

Important considerations in liquid-liquid extraction are miscibility, density, and solubility. Solvents that are denser than water will form the lower layer while solvents that are less dense will form the upper layer or flat on the water. Although solvent form visibly distinct phases when mixed together, they are often somewhat soluble in each other, and will in fact become mutually saturated when mixed together. For example, dichloromethane is 1.6% soluble in water while water is 0.24% soluble in dichloromethane (Wells, 2003). In other words, the organic solvent used for extraction dissolves not only the compound being extracted but also water resulting in desired compound contaminated with water (Williamson and Masters, 2011).

Evaporation or use of drying agents is used to remove the water contaminant. Many choices of chemical drying agents are available for this purpose and the choice of which one to use is governed by four factors; (1) the possibility of reaction with the substance being extracted, (2) the speed with which it removes water from the solvent, (3) the efficiency of the process, and (4) the ease of recovery from the drying agent (Williamson and Masters, 2011). Commonly used drying agents in organic laboratories are calcium chloride ($CaCl_2$), sodium sulfate (Na_2SO_4) calcium sulfate ($CaSO_4$, also known as *Drierite*) and magnesium sulfate ($MgSO_4$), and molecular sieves (sodium-alumino silicates with well-defined pore sizes) (Williamson and Masters, 2011).

Drying agents are used in their anhydrous forms and form hydrates at low temperatures as follows:

Drying agei $A + H_2O \longleftrightarrow A^*(H_2O)$ n.

efficiency. Capacity is the maximum number of moles of water (n) the drying agent can bind. Efficiency (e) expresses the amount of water remaining in the organic phase at the end of the drying process

Table1: Some drying agents and their corresponding solvents

Class of Compounds	Recommended Drying Agent
Hydrocarbons and others	$CaCl_2$, $CaSO_4$, P_2O_5, Na Metal (in the form of wire)
Aldehydes, ketones, esters	$CaSO_4$, $MgSO_4$, Na_2SO_4, K_2CO_3
Bases (Amines)	NaOH (pellets), KOH (pellets), CaO, BaO, K_2CO_3
Acidic compounds	Na_2SO_4, $MgSO_4$, $CaSO_4$
Aromatic hydrocarbons, ethers	$MgSO_4$, $CaCl_2$, $CaSO_4$, P_4O_{10}, Na-metal
Alcohols	$MgSO_4$, K_2CO_3, $CaSO_4$, CaO, BaO

(Azogu, 2010)

High Perfrmance Liquid Chromatography (HPLC)

HPLC is a separation technique that involves; the injection of a small volume of liquid sample into a tube packed with tiny particles (3-5 micron in diameter) called the stationaryy phase where the componenets are separated from one another by the column packing that involves various chemical and/or physical interactions between their molecules and the packing particles. These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. An output from this detector is called a liquid chromatogram.

HPLC can be classified based on the polarity of the stationary phase. This classification broadly divides HPLC techniques into reverse phase (RP) and normal phase chromatography. Reverse phase is an elution procedure used in liquid chromatography where the mobile phase is significantly more polar than the stationary phase. Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity (Figure 3). At discovery chromatography involved the use of polar stationary phases and non-polar mobile phases. Over time innovations which made the use of non-polar stationary phases possible. The former is referred to as normal phase chromatography (NPC) while the latter is reversed phase chromatography (RPC). When the stationary phase is polar and the mobile phase is less polar or nonpolar, the system is called normal phase. If the sorbent is chemically altered (e.g., by bonding non-polar groups to the surface of

the silica gel or by coating or impregnating the stationary phase with a nonpolar organic solvent), a nonpolar stationary phase can result (Gasparic, 1992).

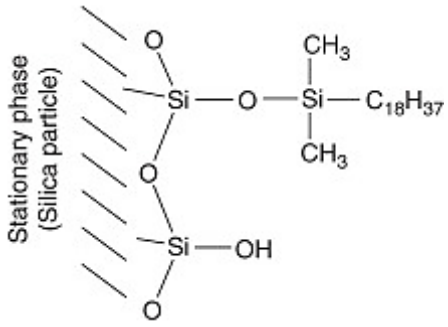

Figure 3. Typical surface of RP silica (Evans, 2008)

There are two modes of analysis depending on the operation techniques viz. isocratic and gradient. Isocratic analysis is the procedure in which the composition of the mobile phase remains constant during the elution process. In gradient elution, the composition of the mobile phase changes continuously or stepwise during the elution process (Gupta and Shanker, 2008)

HPLC can be characterized depending on column diameter, which is the governing factor for flow rate from microscale to industrial scale chromatography (Figure 4). Column internal diameter (i.d.) defines the sample load and flow rates (Gupta and Shanker, 2008). In general, HPLC columns range from 20 mm to 500 mm in length [L] and 1 mm to 100 mm in internal diameter [i.d.]. As the scale of chromatography increases, so do column dimensions, especially the cross-sectional area. To optimize throughput, mobile phase flow rates must increase in proportion to cross-sectional area. If a smaller particle size is desirable for more separation power, pumps must then be designed to sustain higher mobile-phase-volume flow rates at high backpressure. Table B presents some simple guidelines on selecting the column i.d. and particle size range recommended for each scale of chromatography (Sirard,2012) .

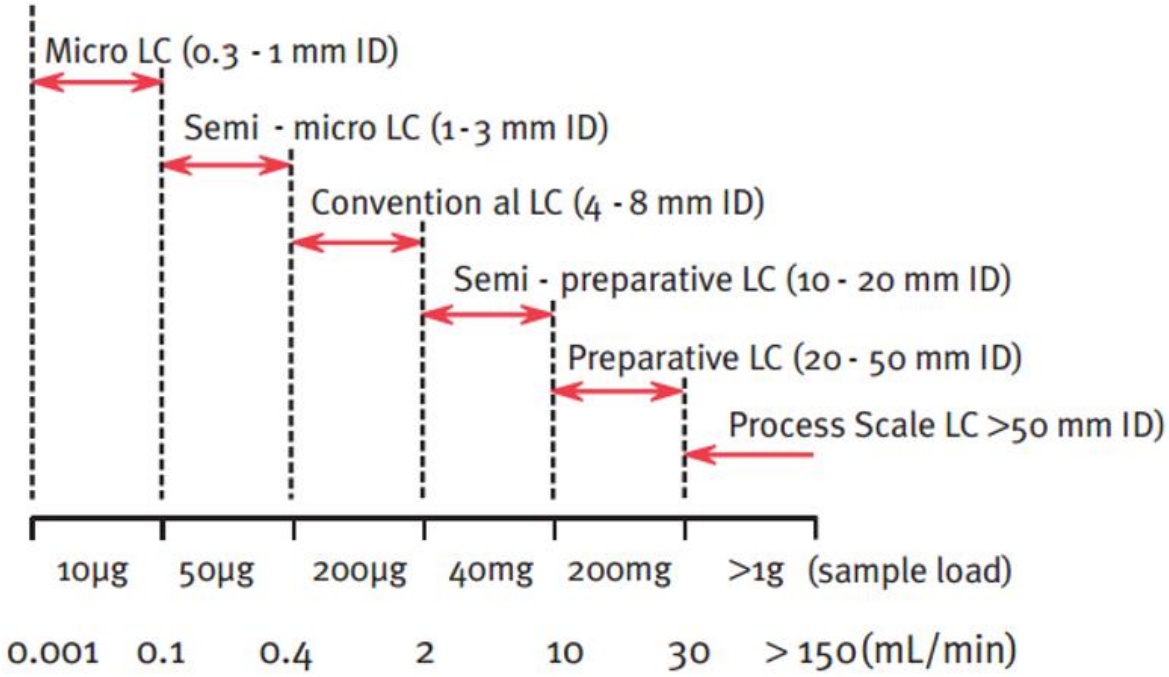

Figure 4. Classification of HPLC according to column diameter.

HPLC can also be divided into analytical and preparative. The difference between the analytical and preparative methodologies is that analytical HPLC does not rely on the recovery of a sample, while preparative HPLC is a purification process and aims at the isolation of a pure substance from a mixture (Hostettmann, and Marston 2006). In preparative LC the separated compounds are collected in individual containers for further processing, whereas in analytical LC the separated compounds earn no further attention. The need to enrich or purify target compounds from mixtures for further investigation or commercial purposes is the key motivation to adopt and deploy preparative LC (Schulnberg-Schell and Tei, 2015).

HPLC is optimum for the separation of chemical and biological compounds that are non-volatile. The principle involves partition of analytes between mobile phase and stagnant phase inside the pore space, and adsorption on the surface of bonded phase. In HPLC the stationary phase is either a solid, porous, surface active material in small

particle form, or a liquid which is coated onto micro-particulate beads of an inert solid support (usually silica). www.chromacademy.com
The analyte is injectied as a small volume of liquid sample into a tube packed with tiny particles the (stationaryy phase-3-5 micron in diameter). The componenets are separated from one another by the column packing through various chemical and/or physical interactions between their molecules and the packing particles (Figure 5). These separated components are detected at the exit of this tube (column) by a flow-through device (detector) that measures their amount. An output from this detector is called a liquid chromatogram. The resolving power of HPLC is ideally suited to the rapid processing of such multi component samples on both an analytical and preparative scale (Martin and Guiochon, 2005).

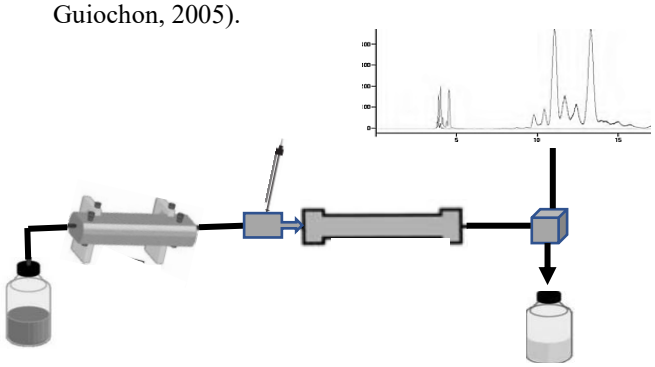

Figure 5. High Performance Liquid Chromatography Schematic Diagram

The resolving power of HPLC is ideally suited to the rapid processing of such multi component samples on both analytical and preparative scales (Martin and Guiochon, 2005).

Vacuum liquid chromatography (VLC)

VLC can be viewed as a preliminary TLC run performed in a column with the addition of vacuum to enhance eluent flow rates. In contrast to flash chromatography, each fraction is collected and the column is then allowed to run dry. The plates can be dried after a run and then re-eluted in preparative TLC, which is identical to this. Due to its ease of use, VLC has been more and more popular over the past ten years in the field of natural products. It is possible to separate up to 30 g of extract. In VLC, a variety of chromatographic supports have been used, including polyamide, Al_2O_3, CN, diol, and silica gel (both normal and reversed-phase). Hexane with ethyl acetate blended in increasing amounts is the most widely used eluent.

Flash chromatography (FC)

Flash chromatography, also known as medium pressure chromatography, is a rapid form of preparative column chromatography that uses optimized, prepacked columns through which a solvent is pumped at a high flow rate. Flash chromatography differs from conventional techniques in two ways. First, slightly smaller silica gel particles (250-400 mesh) are used. Second, due to the limited flow of solvents caused by the small gel particles, pressurized gas (10-15 psi) is used to drive the solvent through the column of stationary phase. The net result is rapid ("over in a fl ash") and high-resolution chromatography.
Flash chromatography is an easy and simple purification technique that requires minimal method development. Even though there are only a few factors to consider when preparing for a Flash chromatography purification, they all need to be selected thoughtfully in order to achieve a successful separation. Mobile phase, stationary phase, type of gradient elution, column loading capacity, and sample loading technique are some of these factors.

Gas-liquid chromatography or GLC

Gas-liquid chromatography or GLC is used to separate a wide variety of organic compounds. The basic requirements for GLC are that the sample be volatile and that it not decompose in the vaporization process. Since the vaporization occurs in an inert atmosphere, decomposition of the sample is generally not a problem. The main variables in G LC are the nature of the stationary phase of the column and the temperature of operation. These are varied according to the polarity and volatility of the compounds being separated. 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 (Harborne, 1998). Separation of a mixture into its components depends on the solubility differences of the sample vapor in a liquid (stationary phase). The stationary phase is coated in a thin layer on solid particles (solid support) of large surface area and then packed uniformly into a column. A constant flow of an inert carrier gas passes through the column and transports solute molecules in the gas phase. A sample of the analyte is introduced by syringe injection into the heated injector tube, where it is vaporized and mixed with the carrier gas. As the sample vapor/carrier gas mixture flows onto the column, the analyte partitions between the gas and liquid phases according to the analyte component's solubility in the liquid at the column operating temperature. This equilibrium partitioning continues as the sample is moved through the column by the carrier gas. The rate at which the sample travels through the column is determined by the sample solubility in the stationary phase, the carrier gas flow rate, and the temperature gradient (temperature program) applied. Each component travels at a characteristic rate, and if the

column has sufficient length and resolving power, the sample will be completely separated by the time it reaches the detector https://chem.libretexts.org.

It is the most popular and useful analytical tool in the research field of volatile plant secondary metabolites (Adams, 1995). The advantages of GC clearly lie in its high sensitivity of detection for almost all the volatile chemical compounds present in the a polar plant extracts or essential oils. Furthermore, the high selectivity of capillary columns guarantees high resolution of many volatile compounds simultaneously within comparatively short times. This fact is extremely important in the case of essential oils, generally contain a hundred of terpenes and derivatives. In addition, the unambiguous identification of plant metabolite is possible by computer matching with commercial and experimental databases of mass spectra and retention indices. The method of ionization generally used in conjunction with gas chromatography is Electron Impact, a hard-ionization method which provides reproducible mass spectra and allows library searching (Schauer, *et al*., 2005). The volatility and thermal stability of the analytes represent sometimes a great limit to GC. Another limitation of GC-MS is its inability to handle polar or high molecular plant metabolites and cofactors are often a problem too (Bertoli, *et al*., 2010). The challenge encountered when handling polar and high molecular weight compounds can be overcome through derivitalization which improves their thermal stability and peak shape (Majors and Wilmington, 2013).

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 are two general formulae for measuring these areas: *(a)* peak height x peak width at half the 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 use of an electronic integrator (Harbome, 1998).

Reference compounds are used to aid the identification of components of a mixture. For quantitative work with differential detectors, the areas enclosed by the peaks are proportional to the quantities of compounds which they represent. To obtain the percentage composition of components within a mixture, without the necessity of placing known amounts of sample on the column, internal standards can be used (Evans, 2009).

Gas Chromatographic Separation Methods

Gas chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one being a stationary bed of large surface area, and the other a gas that percolates through the stationary bed.

This technique is generally used to separate gases in a gaseous solution. The more common technique is gas-liquid chromatography in which the stationary phase is a porous solid covered with an absorbing liquid. Gas-liquid chromatography or GLC is used to separate a wide variety of organic compounds. The basic requirements for GLC are that the sample be volatile and that it not decompose in the vaporization process. Since the vaporization occurs in an inert atmosphere, decomposition of the sample is generally not a problem. Separation of a mixture into its components depends on the solubility differences of the sample vapor in a liquid (stationary phase). The stationary phase is coated in a thin layer on solid particles (solid support) of large surface area and then packed uniformly into a column. A constant flow of an inert carrier gas passes through the column and transports solute molecules in the gas phase. A sample of the analyte is introduced by syringe injection into the heated injector tube, where it is vaporized and mixed with the carrier gas. As the sample vapor/carrier gas mixture flows onto the column, the analyte partitions between the gas and liquid phases according to the analyte component's solubility in the liquid at the column operating temperature. This equilibrium partitioning continues as the sample is moved through the column by the carrier gas. The rate at which the sample travels through the column is determined by the sample solubility in the stationary phase, the carrier gas flow rate, and the temperature gradient (temperature program) applied. Each component travels at a characteristic rate, and if the column has sufficient length and resolving power, the sample will be completely separated by the time it reaches the detector https://chem.libretexts.org.

For volatile compounds, the separation by gas chromatography is unrivalled, due to its extreme efficiency and excellent detection sensitivity in combination of mass spectrometry. Another advantage is the availability of huge data libraries, which allows the tentative identification of compounds in minutes. Unfortunately, not many biochemical systems can be used in the gas (Weller, 2012).The detection step in an effect-related analysis system has a twofold purpose. First of all, it shows the presence of a compound, for example in a chromatographic elution. Here, a (relatively) non-specific detector is most suitable, such as UV, refractive index (RI), evaporative light scattering (ELSD) or charged aerosol detectors (CAD). A semi-quantitative or a quantitative determination is often desirable. The second purpose, which often might be the primary, is the elucidation of the structure of an unknown compound. Here, mass-

spectrometry (particularly based on ion traps) is dominant, since they deliver plenty of structural information with only a minute amount of substance required. The most valuable approach seems to be GC/MS in combination with a mass spectral library (e.g., from NIST), which facilitates the (tentative) identification of an unknown compound in a complex sample. However, extreme care is needed in this assignment step. Only after a validation step, e.g., by comparison with a reference compound, the compound can be considered to be identified. Very helpful are GC retention indices, which are available as large libraries, too. Another approach is the use of high-resolution MS, which allows the assignment of an elemental formula instantly Weller, 2012).

Gas chromatography mass spectroscopy (GC/MS)

Gas chromatography mass spectroscopy (GC/MS) is a materials analysis method that employs gas chromatographs (GC) fitted with mass selective detectors called mass spectrometers (MS). Gas chromatography and mass spectrometry are both standalone analytical techniques, but together they bring depth and versatility to solving contamination and chemical composition questions. The analysis of different unknown mixtures will be performed via high resolution capillary gas chromatography (GC) is coupled with an iron trap detector (ITD). The ITD is a variation of a quadrupole mass spectrometer and is designed to function specifically as a GC detector.

Combining the two processes, gas chromatography and mass spectrometry, decreases the possibility of error and increase certainty that a particular analyte is in the sample. The mass spectrum is used to identify the components by comparing each to reference libraries of over 275,000 unique spectra. To quantify compounds within the analyzed sample, analysts establish a standard curve of known concentrations of each material.

The coupling of GC to mass spectrometry (GC-MS) affords valuable structural information for reliable identification of authenticity markers and/or to get insight into the adulterants employed (Ruiz-Matute et al., 2018). It is the most popular and useful analytical tool in the research field of volatile plant secondary metabolites (Adams 1995).

The analysis of different unknown mixtures is performed via high resolution capillary gas chromatography (GC) coupled with an ion trap detector (ITD). The ITD is a variation of quadruple mass spectrometer and is designed to function specifically as a GC detector. The mass spectrum is used to identify the components by comparing each to reference libraries of over 275,000 unique spectra. To quantify compounds within the analyzed sample, analysts establish a standard curve of known concentrations of each material. (https://chem.libretexts.org). Unambiguous identification of plant metabolites is possible by computer matching with commercial and experimental databases and mass-spectra and retention indices. The method of ionization generally used in conjunction with gas chromatography is Electron impact, a hard-ionization method which provides reproducible mass spectra and allows library searching (Schauer *et al*., 2005; Ryding, 2020). Unknown compounds can be identified by matching full mass spectrum of unknown peaks with a mass spectral library or a database (Rockwood *et al*., 2018).

It is imperative that the compounds identified in a plant after isolation and characterization, be subjected to pharmacological activity investigation. The last 75years have been a period of astounding growth in our insight of the molecular function of the human body. This has led to discovery of medicines to treat diseases that were not even recognized before the mid-20th century (Pudipeddi *et al.,* 2020). Effect-directed analysis tries to interconnect instrumental analytical techniques with a biological/biochemical entity, which identifies or isolates substances of biological relevance. Successful application has been demonstrated in many fields, either as proof-of-principle studies or even for complex samples (Weller, 2012).

In silico docking

Using in silico techniques to choose potential compounds from chemical databases is known as virtual screening (Lyne, 2002). It can be thought of as the computational equivalent of high-throughput screening and other experimental biological evaluation techniques (Stahura and Bajorath, 2004). One of the most popular approaches in drug development is the use of sizable and chemically varied compound libraries for biological and computational screening (Kodadek, 2011). Essentially, the goal of molecular docking is to use computational techniques to anticipate the structure of the ligand-receptor complex. Molecular docking is one of the most widely utilized methods in SBDD because of its capacity to predict, with a high degree of accuracy, the conformation of small-molecule ligands within the proper target binding site (Meng *et al*., 2011). Molecular docking studies are utilized to discover how two molecules interact and to identify the optimal ligand orientation that would result in a compound with the lowest possible total energy. Two contrasting paradigms are usually used in the computational approach: ligand-based (ligand centric) and protein structure-based (target centric). (Vanek and Tropsha 2008; Basith *et a*l., 2018). The former makes use of information about pocket anatomy and the protein's three-dimensional structure. (Lionta *et al*., 2014). One of the most critical factors is intermolecular recognition,

determined by the geometric and physicochemical complementarity as well as the steric fit (Böhm 1994; Raschka *et al*., 2018). Several computational techniques and in vitro/in vivo experimental procedures are used to identify the possibility for intermolecular recognition. SM ligand libraries are screened for chemical and spatial fitness against three-dimensional protein structure pockets using computational structure-based approaches. Typically, computerized docking methods are used to calculate the fitness between the protein drug pockets and chemical structure (LaBute *et al*., 2014). Molecular docking is widely used in modern drug designing to predict the binding orientation of small molecule drug candidates to their protein targets in order to predict the affinity and activity of the small molecule. It also provides valuable information about drug receptor interactions (Vijesh *et al*., 2013). Scoring functions are used by molecular docking systems to approximate the binding energetics of the anticipated ligand-receptor complexes. Negative ΔG binding values, as demonstrated by Guimarães *et al*., (2014) signify advantageous interactions between the ligand-receptor complex. This method involves computationally querying the target molecule against a panel of protein structures (sometimes more than 100,000 structures) to see whether an electronic or steric fit might occur. Potential off-targets are protein locations with a strong steric/electrostatic fit. Protein sites with a significant steric/electrostatic fit are considered possible off-targets. The best compound can be selected based on the least glide energy/docking score/both (Subasri *et al*., 2011).

DISCUSSION

Natural product research is a wide muti-discipline and multi-faceted undertaking which is expanding and increasing in scope and techniques with time. The broad emergence of effect-directed analytical concepts might lead to a true paradigm shift in analytical chemistry, away from ever growing lists of chemical compounds. The connection of biological effects with the identification and quantification of molecular entities leads to relevant answers to many real life question (Toffler 1970). Once the novelty or utility of a given constituent is established, it is then necessary to process the plant extract in the usual manner, to isolate samples for full structure elucidation and biological testing. The combination of biological and chemical screening provides important information about plant constituents but will not be a sufficient condition for the discovery of potent new drugs if suitable pharmacological models (or disease-specific assays in agro-chemistry) are not available. It is thus essential to adopt a multidisciplinary approach when working in this field. Efficient collaborations with pharmacologists and medical doctors, plant pathologists and biologists are crucial for development of lead compound into a exploitable product. This will result in an acceptable measurement and evaluation techniques and result interpretation. Drug discovery scientists must confirm activity in an assay in order to validate a “hit” as a statistically significant reaction. Many reactions will give false positives for reasons such as organic impurities, reader interference, and nonspecific binding (Hermann *et al*. 2013). When a hit is identified, a confirmation screen is done. This confirmation screen establishes the reproducibility of the hit by repeating the test with the same compound (Pena *et al*., 2020). Recent advances in the isolation, purification, and structure elucidation of naturally occurring metabolites have made it possible to establish appropriate strategies for the determination and analysis of quality and the process of standardization of herbal preparations (Kunle *et al*., 2012). Natural products are frequently isolated following the evaluation of a relatively crude extract in a biological assay in order to fully characterize its properties. The resolving power of HPLC is ideally suited to the rapid processing of such multi component samples on both an analytical and preparative scale (Thirumal and Laavu, 2017). Only very few medications are taken as a single, one-dose course. Hence, it is important to access the effect(s) of a taking them for a reasonable length of time. Some agents such as drugs, nutritional supplements and herbal remedies can adversely affect kidney and liver structure and function. Acute and chronic toxicity studies help in boosting confidence in the safety and use of herbal medicinal items.

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