Chapter 17

Methods in the Extraction and Chemical Analysis of Medicinal Plants

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Abstract

This chapter aims to give an overview of advanced techniques for the extraction, isolation, and analysis of natural products from medicinal plants. It is of great pharmacological interest to isolate and study bioactive natural products. Although sometimes the plants selected for study are chosen based on their traditional medicinal uses, this need not be the case as other attributes may justify study, such as chemical diversity and lack of previous study. Extraction techniques represent one of the earliest steps in natural products isolation, and as such can greatly impact results. Once a crude extract is obtained, compound isolation is achieved through the framework of bioassay-guided fractionation. Under this framework, chromatographic separations are used to iteratively generate fractions, each enriched with a compound or set of compounds of a certain attribute, until finally single compounds are isolated. Analysis of extracts, fractions, and single compounds is performed via spectroscopy, through which the chemical character of fractions and structural attributes of compounds of interest can be elucidated.

Key words Extract, Flash chromatography, Mass spectrometry, High-performance liquid chromatography, Nuclear magnetic resonance

1 Introduction

Extraction is the process of releasing natural products from a biomass [1]. The biomass is often a pulverized plant or plant part, but may also be a preparation of a fungus or other microorganism. It is important to begin with good quality starting material that meets the standards of the World Health Organization's guidelines on Good Agricultural and Collection Practices (GACP) for medicinal plants [2]. For example, collection of certain plants specimens should be avoided, such as those subjected to agrochemical runoff, those that are in close proximity to roadsides, and those that belong to threatened or endangered species. Furthermore, international collections need to incorporate consideration of appropriate collection and import/export permits and adhere to the principles of the Nagoya protocol [3]. After plant collection, a chief consideration is

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whether to dry the plant material. Dried material lasts longer at ambient temperature and can easily be shipped without decay, though drying may result in loss of some volatile compounds. If fresh material is to be extracted, freezing may be necessary for shipping. For all collected plants, a herbarium voucher specimen must be made.

Extraction of a biomass is usually undertaken with a liquid solvent, the polarity of which being one of the key determinants of the types of natural products that migrate and dissolve into the liquid [4]. Another key determinant is the length of time the biomass and solvent are in contact and the degree and type of agitation exerted. The initial extraction of a given biomass yields a crude extract, referred to as such because it is yet unrefined by chromatographic techniques. A crude extract may contain anywhere from a few to hundreds or even thousands of unique compounds and isomers. To isolate compounds from this composition, chromatographic methods are employed, such as partitioning, column chromatography, and high performance liquid chromatography (HPLC). The goal of chromatography is the separation of compounds in a sample based on attributes such as polarity, size, and chemical functionalization [5]. Fractions of a crude extract may be referred to as enriched extracts since they are enriched for chemicals with a certain set of attributes, or they may simply be referred to as "fractions."

Extracts and isolated compounds are analyzed via spectroscopic techniques such as ultraviolet-visible spectroscopy (UV-Vis), mass spectrometry (MS) and nuclear magnetic resonance (NMR). While only mass spectrometry is capable of providing the masses of analytes, both MS and NMR can be used to elucidate the structure and connectivity of functional groups, with NMR being more capable in this respect [6]. Compounds do not necessarily have to be isolated before analysis. With hyphenated techniques, separation and analysis of compounds can be coupled into one procedure [7]. This has made the quick identification of natural products possible and has opened many doors to the analysis of complex chemical samples. Nevertheless, the vast majority of natural products isolation and structural elucidation is performed without the aid of these hyphenated techniques, often due to the expense of the instrumentation. Figure 1 provides an example of a general workflow for identifying single compounds from plant material.

2 Extraction Techniques

2.1 Maceration and Decoction

Maceration is a simple and widely used extraction technique in which pulverized biomass is left to soak in a solvent in a closed container at ambient temperature [5]. Stirring may be incorporated to speed up the extraction process. Macerations are typically run for

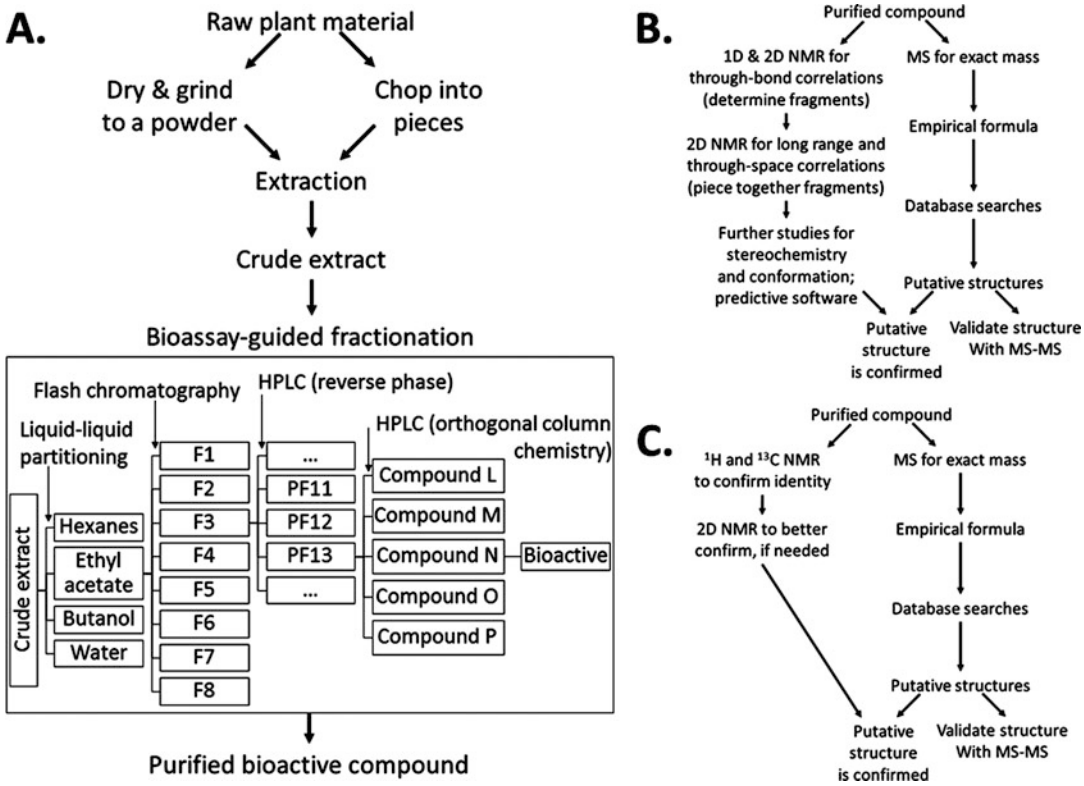

Fig. 1 Example of a general workflow for the identification of natural products from plants. (**a**) Workflow for isolating single compounds from plant material. In the bioassay-guided fractionation box, the vertical arrows indicate examples of chromatographic methods employed to obtain further fractions of a sample. For example, F1–F8 are example names of fractions obtained from the flash chromatography of the ethyl acetate partition of the crude extract. (**b**) Workflow for the identification of a previously undiscovered compound. (**c**) Workflow for the identification of a previously discovered compound

at least three days [8]. Eventually, the concentration of compounds in the solvent reaches equilibrium with the concentration of compounds in the biomass and the extraction effectively stops [5]. Maceration is usually followed by filtration, where the solvent containing the extracted compounds is completely separated from the extracted biomass (marc). In cases where the pulverized biomass is very fine and easily clogs up filter paper, centrifugation is performed beforehand to force the biomass to the bottom of the container prior to decanting for filtration. Often, a maceration is followed by a second or more macerations, where all residual biomass is returned to its container and fresh solvent is added.

Macerations are often used in traditional medicinal practices for chemical extraction, as are decoctions, where the biomass of interest is immersed in boiling water throughout the boiling process [9]. Decoctions are usually performed for 15–20 min, although different types of decoctions can run for much longer [9, 10]. An infusion is different from a decoction, where boiling solvent, also

usually water, is poured onto the biomass. For the purposes of analyzing plants used in traditional medicine, the traditional extraction technique is often replicated so as to most accurately assess the traditional preparations under study.

2.2 Reflux, Soxhlet Extraction, and Percolation

Reflux extraction is a continuous solvent extraction usually performed in a round bottomed flask or boiling flask containing both the extracting solvent and the material undergoing extraction [11]. It utilizes elevated, controlled temperatures at ambient pressure to increase extraction efficiency. As such, reflux is a heated extraction technique, and thermolabile components are at risk of degradation. The elevated temperatures result in reflux and gradual evaporation of the extracting solvent; as such, the round bottomed flask neck is connected to a condenser that preserves the volume of extracting solvent in the system. Common solvents for natural products reflux extractions include: ethanol, methanol, and ethyl acetate; 8 h is a common extraction time.

Soxhlet extraction is also a continuous solvent extraction, but the extracting solvent and material are placed in separate compartments [1]. The extracting solvent is present in a round flask at the base and is heated to a boil. The Soxhlet apparatus is placed into the mouth of the flask and allows the vapors of the solvent to be condensed by a condenser above and the now cold solvent to drip into the thimble, where the material is stored. Drops of solvent gradually fill the thimble until it automatically siphons back down to the round flask, carrying extracted compounds with it. This way, as with reflux extraction, the total volume of extracting solvent is preserved. Unlike reflux extraction, Soxhlet is a cold extraction technique because it is the condensed solvent that contacts the material. After siphoning, however, the extract is transferred into the solvent being heated, which may lead to degradation of thermolabile components. For more reading on Soxhlet, evolutions thereof, and applications, a review has been written on the topic [12]. Commonly, extraction time is determined by the number of cycles (fill/siphon) completed per hour, and 4–6 cycles per hour are often observed. A total of 72 cycles is often used as a benchmark, though this can be shortened, especially if it is a fraction of a crude extract that is being extracted.

A similar technique to the reflux and Soxhlet extractions is percolation, which is performed using a percolator, an apparatus similar to a separatory funnel [13]. The material to be extracted sits above the drain valve, sometimes packed in between filters or sometimes isolated from the valve by a cotton plug. The material is first soaked with extraction solvent, and then additional solvent is poured so as to allow the extract to percolate dropwise through the drain valve [1]. Successive percolations can be performed for exhaustive extraction. The main drawback of a percolation extraction is high consumption of solvent and time due to lack of agitation and

dependence on gravity for flow. In order to reduce the overall solvent consumption, the extract can be reused in subsequent percolations, rather than fresh solvent. In order to have a successful percolation, the particle size of the ground material must be coarser than material used in other extraction techniques.

2.3 Ultrasound-Assisted Extraction

Ultrasound-assisted extraction (UAE) is an extraction technique where, typically in a maceration-type setup, ultrasound waves are used to assist in the extraction process [14]. The employment of ultrasound reduces the extraction time to minutes, reduces the volume of solvent needed, and consumes less energy as compared to other extraction techniques. Commonly used UAE systems include: Erlenmeyer flask in an ultrasound bath, an ultrasound reactor with stirring, and use of an ultrasound probe. For the extraction of chemicals from plant material, the ultrasound bath is preferred, though high power ultrasonic probes are preferred for most other applications. In both systems, transducers serve as the source of ultrasound waves. A number of mechanisms have been identified as aiding in the effectiveness of ultrasound to increase extraction efficiency: fragmentation, erosion, sonocapillary effect, sonoporation, local shear stress, and destruction–detexturation of plant structures. These mechanisms, as well as parameters that affect the extraction process, have been thoroughly discussed in a recent review of the field [14].

2.4 Essential Oil Extraction

An essential oil is a concentrated hydrophobic liquid obtained from a plant that contains the volatile aroma compounds (e.g., terpenoids) that yield its characteristic fragrance. The most common method of essential oil extraction is steam distillation, where steam from boiling water passes through the plant material of interest, releases its essential oil, carries it through a condenser, after which the water and oil deposit and form two layers that can be separated [15]. Hydro-distillation is also common and follows the same principle, except that the plant material is placed in the boiling water. Other methods of essential oil extraction are discussed in a number of review articles [15, 16]. Of note, enfleurage, specifically the hot enfleurage, is considered the oldest known procedure for extracting essential oils. In this technique, solid odorless fats are heated and stirred with plant material, with spent plant material removed and fresh material added repeatedly until the fat is satisfactorily saturated with fragrance. In the cold enfleurage, plant material is placed on solid, odorless fat in a chassis, and diffusion of essential oils into the fat is allowed to take place over the course of 1–3 days. Fresh plant material is repetitively replenished until saturation is achieved.

2.5 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is a chemical extraction process in which the extracting solvent is a supercritical fluid (SCF)

[17]. An SCF is any substance at a temperature and pressure above its critical point. As a result, an SCF has no distinct liquid or gas phase but is rather in a state between these two extremes, able to both effuse through solids (like a gas) and dissolve compounds (like a liquid). CO_2 is the most widely used SCF for extraction, usually employed for large-scale decaffeination of coffee beans [18]. A dedicated instrument is required to perform SFE. The instrument first withdraws liquid CO_2 from a CO_2 tank, heats and pressurizes it beyond the critical temperature (31 °C) and pressure (74 bar), and then either flows the supercritical CO_2 through the biomass to be extracted or fills a vessel containing the biomass for a static extraction. After a predetermined contact period, the instrument separates the extract from the now gaseous CO_2, condenses the gas back to its liquid form, and returns it to the storage tank. Alternatively, the used CO_2 can be vented, leaving the extract behind in a collection vessel.

There are some limitations to SFE. CO_2 is a poor solvent for polar molecules due to its hydrophobicity. This can be somewhat circumvented by pumping cosolvents such as methanol through the biomass along with the supercritical CO_2, thus increasing the range of compounds extracted. On the other hand, this limitation gives SFE a niche in the extraction and analysis of hydrophobic compounds such as essential oils, with several reviews written on the topic [19, 20]. Another limitation is cost, which primarily arises from the need to pressurize the CO_2. SFE is often used when extraction selectivity and speed are very important. Because different cosolvents can be used and the properties of an SCF can be adjusted by changing the temperature and pressure, selective extractions can be performed. For instance, it was found that by varying temperature, pressure, and cosolvents in an extraction of *Plantago major*, selective enrichment of triterpenic acids, α-LNA, and oleanolic and ursolic acids could be achieved [21]. Additionally, an SFE can be completed in relatively short time due to the enhanced diffusion properties of SCFs as compared to liquids. Another advantage of utilizing SCFs is that they largely circumvent oxidizing and thermally degrading extracted compounds. They are also less costly to dispose of (e.g., CO_2 can be released into the air) and are generally nontoxic, contaminant-free, and inexpensive. A commonly cited advantage of SFE is its environmental friendliness. However, in applications that require the full range of polar compounds to be extracted, substantial amount of modifier such as 30% methanol must be used, negating this benefit [13].

2.6 Accelerated Solvent Extraction

Accelerated solvent extraction (ASE) is a fully automated rapid extraction technique that enjoys widespread use due to rapid extraction times requiring little solvent. The technique was developed by Dionex Corporation and introduced in 1995, and they remain the sole manufacturer of ASE equipment [22]. It employs

common solvents at elevated temperature and pressure, which increases extraction efficiency. Because the temperatures used are usually above the boiling point of the solvent, the increased pressure (1000–2000 psi) functions to maintain the liquid state. Commonly used organic solvents include *n*-hexane, dichloromethane, acetone, and methanol. ASE is capable of extraction for sample sizes of 100 g or less in minutes while consuming very small volumes of solvent. As a result, the cost per sample tends to be lower than that of other extraction techniques. A main advantage of ASE is that it aims to maximize the extraction of compounds present at low concentrations in the matrix, and it generally is an exhaustive extraction technique. A disadvantage is the increased likelihood of thermal degradation of susceptible compounds. While most ASE extractions utilize a temperature between 75 and 125 °C, 100 °C is the most commonly setting chosen. Temperature is the dominant factor in ASE's exhaustive extraction, with selectivity tunable by lowering extracting temperature and choosing an appropriate solvent. ASE is increasingly used in the extraction of contaminants in foods for analysis, with a review having been written on the topic, giving a deeper overview of the technique [23].

2.7 Microwave-Assisted Extraction

Microwave-Assisted Extraction (MAE) is a rapid extraction technique in which solvent extraction is supported by heating with microwaves [24]. As in ASE, the heating yields the benefit of increased extraction efficiency. This brings the advantage of lowering volume of solvent and cost, but also the limitation of thermal degradation of susceptible compounds. MAE can be performed in ways as simple as inserting a maceration flask into a microwave oven to ways as complex as utilizing a dedicated instrument with a variety of controls and vessel options. Many different types of MAE instruments have been developed. For high-throughput sample preparation, for example, an instrument may enclose numerous maceration capsules in a microwave box for heating. MAE can also be used to assist reflux and Soxhlet extractions by physically placing the biomass-containing piece of glassware in a specially made microwave unit [25, 26]. There are several examples of the literature of natural products having been extracted more efficiently from a biomass of interest via MAE, such as luteolin and apigenin from tree peony pod and fucoidan from marine algae [27, 28].

2.8 Extraction Efficiencies

In order to provide insight into the relative extraction efficiencies of some of the most popular extraction methods, a few examples from the literature are given where extraction techniques have been optimized. Interestingly, most studies of comparative extraction efficiencies study the extraction of pollutants rather than natural products, though conclusions may be drawn from these studies. Here, Soxhlet extraction has been considered as the reference. ASE and Soxhlet extraction have been found to extract volatile and

phenolic compounds with higher efficiency than SFE from mint (Lamiaceae) species [29]. Soxhlet was also found to achieve the higher extraction yield of terpenoids and sterols from tobacco than ASE, though the latter was faster and used less solvent [30]. However, for the extraction of polycyclic aromatic hydrocarbons and organochlorine pesticides from soils, Soxhlet and MAE were found to have similar efficiencies, though ASE proved more efficient than the two, and both MAE and ASE consumed less solvent and time [31]. In a study on the extraction of twelve polychlorinated biphenyls (PCBs) from algae samples, Soxhlet and SFE were found to have same extraction efficiencies for most PCBs, though SFE had the advantage of leading to the detection all PCBs at lower concentrations and reducing extraction time and solvent consumption [32]. In a study on the extraction of N-nitrosamines and aromatic amines from various soil matrices, MAE was found to be superior to Soxhlet while also requiring just 3 min for extraction [33]. It is the opinion of the authors based on the literature and experience that Soxhlet is often capable of extraction efficiencies similar to ASE and MAE. However, other extraction methodologies are often chosen for increased throughput, reduced solvent usage, or available processing time. A comparison of other attributes of extractions techniques is summarized in Table 1. The following section will discuss chromatographic techniques, and a comparison of their attributes is summarized in Table 2.

3 Chromatographic Techniques

3.1 Flash Chromatography

Flash chromatography is a type of preparative (as opposed to analytical) liquid chromatography used for the separation of organic compounds, which is widely used for natural product extract separation. While flash chromatography began as a low-pressure technique, now vacuum pressure or, more often, pumps are employed to achieve medium pressures for faster flow rates and quicker separation [34]. Numerous types of columns (and hence separation methods) exist. Although columns can be packed in the laboratory, one of the key advantages to flash chromatography is that columns can be purchased as prepacked, one-time use columns. One of the most commonly used columns is packed with a silica adsorbent with a particle size usually between 40 and 60 μm [35]. Particle shapes are often irregular, and the particle size distribution in any particular column tends to be relatively wide.

The smaller the silica particle size, the more tightly packed a column is, the more backpressure builds in response to a given flow rate of a mobile phase. As compared to high-performance liquid chromatography (HPLC), flash chromatography uses larger particle sizes and thus generates lower backpressures than HPLC. Flash chromatography is often performed on a flash instrument, which

Table 1
Comparison of attributes of different extraction methods

	Maceration	Decoction	Infusion	Reflux	Soxhlet	Percolation	UAE	SFE	ASE	MAE
Often takes <1 h		✓	✓				✓	✓	✓	✓
Hot extraction		✓	✓	✓					✓	✓
Requires little solvent			✓	✓	✓		✓	✓	✓	✓
Limited risk of compound degradation	✓					✓	✓	✓		
Cold extraction					✓					
Exhaustive in most cases	✓			✓	✓		✓		✓	✓
Requires instrumentation								✓	✓	✓

Table 2
Comparison of attributes of different chromatographic methods

	Flash	HPLC	GC	SFC
Can be analytical or preparative		✓	✓	✓
More amenable to larger sample sizes	✓	✓		
More amenable to compound isolation	✓	✓	✓	✓
Variable separation chemistries	✓	✓		✓

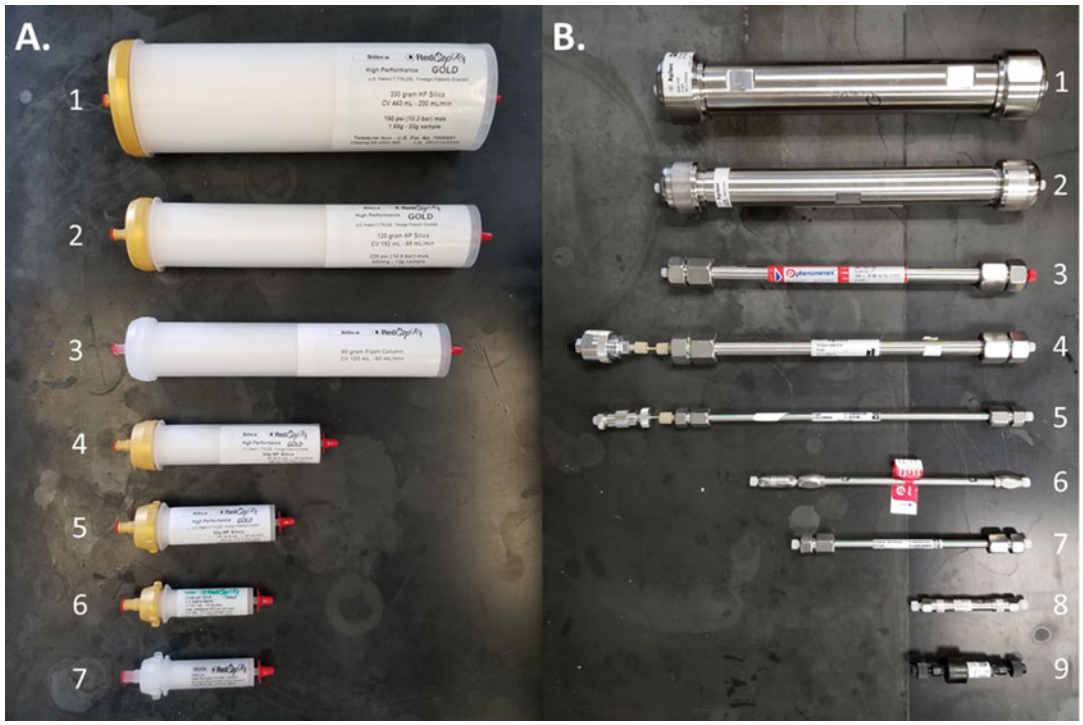

Fig. 2 Examples of column employed for flash chromatography and HPLC. (**a**) Commercially available Teledyne Isco flash columns arranged from high to low (1–7) loading capacities ranging from 33 g to 5.5 mg. Most of the columns are silica columns; column 6 is a diol column. (**b**) HPLC columns arranged from large to small (1–9) sizes ranging from 30 × 250 mm to 6.4 × 30 mm. Columns 1 and 2 are preparative columns, 3 and 4 are semipreparative, and the rest are analytical. Columns 4 and 5 are fitted with compatible guard columns. Most of the columns are C-18 columns, and particle sizes range from 2.6 to 5 μm. Columns 3 is a C-5 column, while columns 7 and 9 phenyl columns

not only pumps the mobile phase but also detects column eluate, usually via a UV-Vis absorbance detector and sometimes with an evaporative light scattering detector (ELSD) and produces a chromatogram of the run. In addition to normal and reverse phase columns, amine, cyano, diol, ion exchange, and many more types

of columns are available. Figure 2a shows examples of different flash columns. Flash chromatography has been used extensively as a step in bioassay-guided fractionation and to isolate single compounds [36–40]. Given the importance of flash chromatography as a separation technique, efforts have been made to construct flash apparatuses with inexpensive setup costs [41].

3.2 High-Performance Liquid Chromatography (HPLC)

HPLC is a type of liquid chromatography that can be used for both analytical and preparative purposes. Analytical HPLC and larger scale preparative HPLC each require separate, dedicated instruments, although hybrid systems are available, most commonly for analytical and semi-preparative HPLC. As compared to flash chromatography, HPLC columns contain silica with smaller particle sizes ranging from 2 to 50 μm [35]. Additionally, particles are spherical in shape, and in any given column the particle size distribution is narrow. Particle size plays a role in resolution. Consider the fundamental resolution equation

$$R = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{K'}{1 + K}$$

where N = efficiency, α = selectivity, and K = retention capacity. Since separation efficiency increases with decreased particle size, HPLC has the advantage of higher efficiency as compared to flash chromatography, allowing for higher resolutions to be reached. This concept is modeled by the theoretical plate model of chromatography, which supposes that a chromatographic column consists of a large number of imaginary separate layers called theoretical plates (N). As such, decreased particle size results in decreased height equivalent to a theoretical plate; thus, more theoretical plates are present in a given column length, increasing separation efficiency.

Modern HPLC instruments nearly always include a UV/Vis or a photodiode array (PDA) detector as a spectroscopic component for the characterization of column eluate. For natural product extracts, analytical HPLC is used specifically for chemical characterization, where chromatograms are compared for different runs, be they same-extract batches, different extracts, chemical standards, etc. This function utilizes small amounts of extract or standards; therefore, column eluate is usually not collected because it amounts to very little material. In preparative HPLC, on the other hand, column eluate is collected because the purpose of the separation is the production of large amounts of extract fractions. Therefore, column length and especially diameter for preparative HPLC are larger than those for analytical HPLC (typically 2.1–4.6 mm diameter, 30–250 mm length), as these attributes yield higher column throughput and capacity. Column particle sizes are often slightly larger than those of analytical HPLC columns, where resolution is

more highly emphasized. Beyond these factors, several differences between analytical and preparative HPLC result in the need for separate instrumentation for each. Chief among these differences is the capacity to accommodate higher flow rates in preparative HPLC while maintaining an acceptable backpressure that will not damage the instrument of the chromatographic column.

It is possible to use an analytical HPLC instrument for fraction production; this is often called semi-preparative HPLC. This usually calls for a larger analytical column—still much smaller than preparative columns—and many more runs are required as compared to preparative HPLC to produce a given amount of a fraction. Additionally, an even higher resolution form of analytical HPLC is UPLC (ultra-performance liquid chromatography). UPLC is chiefly characterized by even smaller particles sizes (sub 2 μm), resulting in a higher theoretical plate count and thus increased separation efficiency. Because of this, UPLC columns sizes are often shorter to reduce run times, and UPLC instruments require the capacity to accommodate even higher backpressures. Numerous types of columns exist for all HPLC instruments. In addition to normal phase and reverse phase columns, there are size-exclusion, ion-exchange, chiral, and bioaffinity columns, among many others. Figure 2b shows examples of different HPLC columns. Columns may be fitted with a guard column on the inlet end to protect them from particulate matter in a sample by trapping it in the sacrificial guard column. Besides the column, the schematic of a HPLC instrument usually consists of a degasser, sampler, pumps, and a detector, usually a UV/Vis or a photodiode array (PDA) detector.

There are established methods for the separation of compounds for botanicals and herbs of commerce. Refer to pharmacopoeia of the United States [42], Europe [43], and China [44] for methods on specific materials.

3.3 Gas Chromatography (GC)

GC is a group of analytical separation techniques used to separate volatile compounds in the gas phase [45]. While the success of GC has largely been associated with analytical purposes, GC can be successfully used for preparative purposes as well [46]. Unlike liquid chromatography where the sample, dissolved in liquid, is pushed by a liquid mobile phase through a packed column, in GC the sample, also dissolved in liquid, is vaporized and pushed by an inert gas mobile phase through a heated column, usually hollow. Separation occurs only due to the interaction of the sample components with the coating inside the hollow column (the stationary phase). Because the mobile phase is inert, separation is not due to differential chemical interactions with the mobile phase (none occur). The stationary phase can be either a liquid on an inert support (gas–liquid chromatography) or, more commonly, a solid adsorbent (gas–solid chromatography). A very typical GC column is a fused-silica capillary of varying length from 5 to 100 m, in

which the inner surface of a silica capillary is coated with one of several polymer stationary phases and the outside of the capillary is coated with a polyamide polymer to add strength. A review of many of the types of GC columns describes them in detail [47]. Usually, nonpolar samples are analyzed by GC, interacting with a similar stationary phase to achieve sufficient separation. As such, in the realm of natural products, GC is most adept to separation of components of nonpolar, volatile samples such as essential oils.

The schematic of a GC instrument begins with a source of carrier gas connected to a flow controller [45]. The most common carrier gases are helium, nitrogen, argon, and hydrogen, but the exact gas to use is usually determined by the detector being employed. Carrier gas then flows to the column inlet (or injector), which provides the means to introduce a sample into a continuous flow of carrier gas. The inlet is a piece of hardware attached to the column head. The most common inlet type is an S/SL (split/splitless) injector, where a small, heated chamber facilitates volatilization of the sample solution. Once the sample is swept by the carrier gas, it flows through a column heated by an oven, after which the sample finally hits a detector. The flame ionization detector (FID) and the thermal conductivity detector (TCD) are the most typically employed detectors. Both can accommodate a wide range of sample concentrations and are sensitive to a wide range of components. Other detectors have a shorter range of usability but are used when dealing with specific types of substances.

3.4 Supercritical Fluid Chromatography

Supercritical fluid chromatography (SFC) is very similar to LC, where the defining feature is that the mobile phase is a supercritical fluid, usually CO_2. The instrumentation is nearly identical to that of LC, and there are both analytical and preparative instruments. The pumping system consists of two pumps: one delivers supercritical CO_2, and the other delivers a cosolvent, such as a simple alcohol, acetonitrile, chloroform, or ethyl acetate. The cosolvent and CO_2 are homogenized by a static mixer, and the mobile phase is delivered to the autosampler, equipped with an injection value for delivery to the front of the chromatographic column. The columns are present in an oven to maintain a temperature of above 40 °C for supercritical conditions to be achieved with CO_2. Column eluate then reaches the detector. Until this point, the instrumentation is almost identical to that of liquid chromatography. An additional component is the automated backpressure regulator, which provides a control parameter not found in LC—pressure. In terms of operation, SFC is as simple and robust as LC, and fraction collecting is even easier due to the evaporation of the CO_2 component of the mobile phase. For natural products, supercritical fluid chromatography is mainly utilized for the separation of nonpolar compounds such as carotenoids, fatty acids, and terpenes, though it is has also been successfully used with more polar natural products [48–51].

3.5 Prefractionation

Of great importance, flash chromatography and HPLC are often employed in the prefractionation of crude extracts so as to remove chemicals unlikely to be pharmacologically active prior to screening [52–63]. Prefractionation prior to high-throughput screening (HTS) results in higher hit rates, such as in screening campaigns that revealed pharmacological activity of fractions where the crude extracts were previously identified as inactive [56, 57]. A review has been written with an expanded discussion on this topic and the place of natural products in drug discovery [64]. Prefractionation is also used to "clean up" a fraction or extract so as to remove compounds that may bind to a stationary phase to be used for subsequent chromatographic separation [65]. In addition to chromatographic methods of prefractionation, a manual method of liquid–liquid partitioning is commonly employed prior to flash chromatography or HPLC. The extract is suspended in a solvent (e.g., water) and successively partitioned against solvents of differing polarity (e.g., hexanes, ethyl acetate, n-butanol) using a separatory funnel apparatus to create two distinct layers of solvent and extract constituents at each step. The advantage of this method is avoidance of loss of compounds to the column matrix, but a disadvantage is the manual and time-consuming nature of the technique.

3.6 Solid-Phase Extraction

Solid-phase extraction (SPE) is a sample preparation technique where compounds in a liquid mixture are separated from each other based on chemical and physical characteristics. The most common SPE format consists of a syringe cartridge that contains 50 mg to 20 g of stationary phase [66]. After pouring the sample solution into the cartridge, a plunger or vacuum can be used to push it through the stationary phase. Any type of stationary phase can be used, except those used for exclusion chromatography. As such, SPE allows for rapid, simple, and reproducible analyte purification. While liquid–liquid partitioning can be used for similar purposes, it is more time consuming and emulsions may form between different liquid phases. SPE is also often employed for analyte concentration. Very commonly, a stationary phase is selected such that it retains the analytes of interest; subsequently, the cartridge is rinsed with a small volume of a strong solvent to elute the analytes [67]. Opposite to this retentive mode, SPE can be utilized in non-retentive mode, where it is the desired solutes that pass through the stationary phase [66]. When SPE is used to prepare a sample prior to HPLC, the need for a guard column may be negated [65]. SPE can also serve as a concentration step for compounds which are retained; a relatively large volume containing a small amount of the analytes of interest can be loaded onto the column. Then the analytes are eluted with a small volume of very strong solvent, thus increasing their concentration for analysis without the time required to dry down the initial extraction solvents.

4 Spectroscopic Techniques

4.1 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique for determining the mass of individual compounds that comprise a given sample. This is achieved through the ionization of chemical species and their sorting based on their mass-to-charge ratio (*m/z*). Solids, liquids, or gasses may be analyzed by mass spectrometery. There are three main components of a mass spectrometer: ion source, mass analyzer, and detector. The ion source ionizes the chemical species present in the sample. For gaseous samples, the most common ionization techniques are electron ionization and chemical ionization, whereas for liquid samples, the most common are *e*lectro*s*pray *i*onization (ESI) and *a*tmospheric-*p*ressure *c*hemical *i*onization (APCI). For solid samples and some proteins, *m*atrix-*a*ssisted *l*aser *d*esorption/*i*onization (MALDI) is employed. In ESI, a high voltage is applied to a liquid being sprayed in order to create an aerosol of ions, whereas in APCI the nebulized effluent passes over a coronal discharge needle and as the liquid evaporates due to elevated temperature the remaining compounds acquire a charge. On the other hand, there is an array of ambient ionization techniques, where ions are produced outside of the mass spectrometer in ambient conditions from samples that require little to no pretreatment [68]. Since the development of the first reported technique, desorption electrospray ionization (DESI), dozens more have been developed [69].

After ionization, ions are transported by magnetic or electric fields to the mass analyzer, which separates ions based on their *m/z*. The *t*ime-*o*f-*f*light (TOF) analyzer is a mass analyzer that uses an electric field to accelerate ions toward a detector, measuring the time they take to reach it. Velocities of the ions depend solely on their masses due to identical charge, which tends to make all kinetic energies constant. As such, ions with lower masses will travel faster and reach the detector sooner. Quadrupole mass analyzers utilize a quadrupole field created between four parallel rods. Oscillating electrical fields produced by the rods stabilize or perturb the paths of ions passing such that, at any time, only the ions in a certain range of *m/z* are passed through. The quadrupole ion trap is very similar, except that the ions are trapped and sequentially ejected toward the detector. The state-of-the-art of mass analyzers is the Orbitrap, where ions are electrostatically trapped in an orbit around a central, spindle-shaped electrode such that they also oscillate along the electrode's long axis. Through detector plates, the instrument records image currents generated by the oscillations, the frequencies of which depend on the *m/z* values of the ions. Fourier transformation is used to translate the image currents to mass spectra.

*I*maging *m*ass *s*pectrometry (IMS) is a powerful method for the analysis of chemicals in a biological sample of interest. IMS was first popularized by the MALDI ionization method [70] and it can be used on a tissue section, bacterial colony, or even a whole organism. The sample is mounted on a conductive support, a thin layer of matrix is applied, and the sample is positioned on a stage in a vacuum [71]. The surface of the sample is scanned by a laser, ionizing compounds, and then a 2D image of the sample is constructed with mass data. 3D images can also be constructed using cross-sections of samples. MALDI is the most commonly used ionization method for imaging, and it has the advantage of allowing for the detection of molecules in a wide *m/z* range. What may be the key shortcomings of using MALDI in natural product research is interference by low mass-to-charge molecules present in the MALDI matrix as well as reproducibility, contingent on the quality of the sample [72]. Ambient ionization methods such as DESI circumvent these shortcomings due to the ability to ionize samples under ambient conditions and the lack of sample preparation [71].

Tandem mass spectrometry (MS/MS) is an important analytical tool in the field of natural products research. After a first stage of MS where, as described above, ions are formed and separated by their *m/z* ratio, a second stage of MS is performed. In this second stage, ions of a certain *m/z* are fragmented. Each molecule of an individual compound always fragments into a characteristic pattern that depends on the fragmentation technique employed, allowing mass spectra to be used as "fingerprints" for identifying compounds. Indeed, functional groups such as alkyl chains may be structurally elucidated based on fragmentation of the group specific to its chemical characteristics and the fragmentation technique. Throughout the 1960s–1980s hundreds of proposed structures were determined for natural products, many identified due in part to fragmentation studies [73]. MS/MS is also the backbone of molecular networking, an informatics approach used for both dereplication and identification of structural analogs [74–76]. Through this approach, all MS/MS data in an experiment is mapped according to mass spectral structural space, resulting in the clustering of molecular families with related MS/MS spectra.

4.2 Nuclear Magnetic Resonance Spectroscopy

*N*uclear *M*agnetic *R*esonance (NMR) spectroscopy, or simply NMR, is a spectroscopic technique for observing local magnetic fields around atomic nuclei. Because these fields are highly unique to individual atoms in any given compound, NMR is primarily employed for the elucidation of chemical structures in natural products research. Over the past several decades, improvements to NMR hardware and methodology have reduced the amount of sample required for analysis from tens of milligrams to often under 1 mg. A NMR experiment operates by first applying a constant magnetic field (B_0) to the sample such that the magnetic nuclear

spins align with B_0 either with the field (low-energy state) or 180° against the field (high-energy state). Because resolution depends directly on magnetic field strength, modern NMR spectrometers are fitted with a very powerful and large liquid helium-cooled superconducting magnet. Second, *r*adio *f*requency (RF) pulses are used to perturb the alignment in order to obtain a nuclear magnetic resonance response, also called a *f*ree *i*nduction *d*ecay (FID). Following the pulse, the population nuclei in the sample are excited and flip orientations. This flipping of energy states produces a very weak voltage, which is detectable in sensitive radio receivers surrounding the sample tube. This signal is then digitized and Fourier transformed to yield a frequency spectrum of the NMR signal, or the NMR spectrum.

This nuclear magnetic resonance phenomenon is commonly used to study ^{1}H and ^{13}C atoms in a compound in order to determine each of their environments and subsequently elucidate the compound's structure. These isotopes, respectively present in 99.98% and 1.1% abundance, are studied because they exhibit nuclear spin and are thus detectable by NMR. The general workflow for determining the structure of a natural product often begins by obtaining a proton (^{1}H) spectrum and carbon-13 (^{13}C) spectrum. For organic compounds, the ^{1}H NMR spectrum is characterized by peaks representing at least one proton each, each having a unique chemical shift in the range +14 to −4 ppm, indicating local electronegativity. Peaks containing multiple peaks represent the spin-spin coupling between protons, and the integration curve for each peak relative to the others reflects the number of protons represented by that peak. The ^{13}C spectrum is analogous to ^{1}H spectrum in that it allows the identification of carbon atoms, though ^{13}C chemical shifts fall along a much larger range than for ^{1}H. This 1D data is then combined with additional data from 2D experiments, obtained from spectra such as the ^{1}H-^{1}H correlation (COSY) spectrum and the one-bond ^{13}C-^{1}H correlation (e.g., HMQC) spectrum, to determine the structures of fragments of a compound. These fragments are then tied together using information from the long-range ^{13}C-^{1}H correlation (HMBC) spectrum. Many different pulse sequences for 2D NMR have been developed to obtain various correlations. It is necessary to combine a variety of NMR methods with calculations of the energies of different conformations, and techniques for doing so have been thoroughly reviewed [77]. Here, we give an overview of the most commonly employed 2D NMR techniques.

COSY (*Co*rrelation *S*pectroscop*y*) provides correlation data for all coupled protons (^{1}H-^{1}H correlation). This data gives unequivocal proof of proton assignments. DEPT (*D*istortionless *E*nhancement of *P*olarization *T*ransfer) determines the number of hydrogen atoms bonded to each carbon in the molecule, and thus differentiates between primary, secondary, and tertiary carbon atoms.

HETCOR (*Het*eronuclear *Cor*relation spectroscopy) reveals all coupling of protons and attached carbons (^{1}H-^{13}C correlation), complementing DEPT, but is less frequently used. HMQC (*H*eteronuclear *M*ultiple *Q*uantum *C*oherence) gives the same results as HETCOR, but is much more efficient in terms of sensitivity and speed and has almost completely replaced the technique. HMBC (*H*eteronuclear *M*ultiple *B*ond *C*orrelation) allows long-range correlation of protons and attached carbons (^{1}H-^{13}C correlation) over 2–3 bonds. It is important to note that all the aforementioned 2D techniques examine through-bond correlations. NOESY (*N*uclear *O*verhauser *E*ffect *S*pectroscop*y*) is unique in that, instead of determining through-bond relationships, the technique determines through-space NOE relationships. The NOE is the transfer of energy from one nucleus to another (usually protons) when the first nucleus relaxes from an excited nuclear spin state to its ground nuclear spin state. The NOESY spectrum reveals which protons are close enough to each other to transfer energy this way. NOE is not observed beyond roughly 5 Å, and is usually observed within 3 Å.

In addition to NMR's utility in the determination of chemical structures, it can also be employed for spectral fingerprinting of complex mixtures, such as a natural product extract or fraction. Due to the NMR's inherent reproducibility the instrument can provide rapid analysis of an extract. This data can be compared to a previously prepared data set to determine if the material is the same as other batches or lots of the same material. It can also be employed to determine if a sample is indeed authentic or adulterated. The limitation of these analyses is based on the diversity and depth of the samples used for the training set to create the model of what is an "authentic sample." Nevertheless, given a robust enough set of authentic samples NMR fingerprinting is an extremely powerful tool for determining batch-to-batch consistency or adulteration of raw materials. Several reviews have discussed the details of these techniques [78, 79].

4.3 Hyphenated Techniques

When two or more different analytical techniques are coupled via a proper interface, a hyphenated technique has been set [7]. Most commonly, chromatographic techniques are coupled to spectroscopic techniques, with the benefit of rapid identification of natural products directly from plant and marine extracts or fractions without the necessity of isolation. For example, HPLC is often coupled to MS to make HPLC-MS. In this case, the compounds that comprise a given sample are separated over time by HPLC and then introduced into an MS, yielding mass data of the HPLC eluate over time. This way, separation and detection are performed via one technique. The most common separation techniques that see hyphenated use are HPLC and GC, whereas the most common spectroscopy techniques are MS and NMR. UV detection is built into most automated chromatographic techniques and is not

considered a standalone spectroscopic technique here. The ability to rapidly identify compounds allows for dereplication of natural products, metabolomics studies, chemotaxonomic studies, chemical fingerprinting, quality control of extracts, and many other studies. We will discuss dereplication here and metabolomics in the next section.

Dereplication is the process of identifying compounds for which the structure is already known [80]. It is an integral part of natural products discovery, as it ensures that time and resources are not wasted toward elucidating structures of compounds that are already known. Most commonly, LC-MS is utilized to analyze crude extracts and fractions thereof to identify the masses of all constituents. These mass values are then screened against databases in order to identify putative chemical formulae and structures for the compounds. In bioassay-guided fraction studies, bioactive fractions are analyzed as such to determine whether active fractions contain previously identified compounds with the known therapeutic activity of interest. Some of the most commonly consulted databases include SciFinder [81], Dictionary of Natural Products [82], NAPRALERT [83], Global Natural Products Social Molecular Network [84], and MarinLit [85]. In microbial dereplication specifically, it is highly desirable to identify microbial strains capable of producing novel chemistries. To this extent, LC-MS data is often analyzed by principle component analysis or other multivariate methods to identify strains that synthesize molecules most different from where the rest of the strains cluster. There has also been interest in the field of natural product peptides to develop informatics tools to predict fragmentation data of known structures. This knowledge would help find matches with experimental fragmentation data, further aiding in dereplication [86–89].

While LC-MS enjoys an important role in natural products discovery, LC-NMR has proved less useful [77]. Whereas MS is capable of detecting the masses of LC eluates almost instantaneously during the course of continuous flow, NMR is capable of no more than obtaining ^{1}H spectra, which takes several seconds. Additionally, LC-NMR requires either the use of costly deuterated solvents or the use of solvent peak suppression, which could result in loss of sample signals. To circumvent the first limitation, either (1) a stopped flow mode is employed where a valve stops elution when analyte reaches the flow cell volume of the rf coil, or (2) without interrupting the chromatographic run, a loop-storage mode is employed, where fractions are stored in individual capillary loops for later NMR analysis [90]. In both cases, analyte can be subjected to NMR longer to achieve adequate data acquisition times for less abundant material and for more time consuming sequences such as ^{13}C NMR. To further improve NMR detection after LC, fractions can be prepared for NMR by solid-phase extraction (SPE), concentrating them into an appropriate deuterated solvent prior to

analysis. Indeed, numerous laboratories have had success with automated LC-SPE-NMR for the identification of natural products [91, 92]. MS can be coupled to this hyphenated technique via a splitter that performs a typically 95:5 split of the flow to NMR versus MS [93].

4.4 Metabolomics

Recent advances in computing power and analytical instrumentation has allowed previously unthinkable analyses to be performed. These advances, combined with techniques developed from genomics, have allowed the use of larger data sets to produce proteomics and metabolomics analyses. The term proteomics, as well as proteome, was coined in 1995 [94] and can be narrowly defined as analyzing the expressed levels of proteins in a cell, thus providing insight into the current physiological state. From proteomics it was a short transition to the transcriptome, proteome, and finally analyzing the metabolome, the small molecules and metabolic products produced by an organism. Metabolomics in its most general definition involves completely analyzing all the known and/or unknown metabolites in a given biological sample [95].

Metabolomic analysis involves three major parts: sample preparation, data acquisition, and data analysis or chemometrics. The data acquisition can make use of several analytical instruments; NMR, GC-MS, or LC-MS to perform a targeted or non-targeted analysis. The preferred instrument in MS-based metabolomics is a high accuracy MS such as TOF, FT, or Orbitrap. Metabolomics analyses can be grouped based on approach: targeted and untargeted. In a targeted metabolomics study, the levels of a known set of compounds are quantified. The study can be quantitative or semiquantitative by using appropriate chemical standards in the study design [96]. One of the advantages of a targeted study is that since the compounds of interest are defined initially, the sample preparation and analytical methodologies can be optimized for those compounds, thus potentially reducing signal interference from other compounds. An untargeted metabolomics study is the analysis of all the measurable compounds in a sample, both the chemical knowns and unknowns [96]. Due to the nature of the study, careful sample preparations must be followed with a focus on minimizing any compound loss, degradation, or unintentionally enriching one group of compounds. The analytical method used to generate the metabolomics data must be broad spectrum and may require using multiple separations in tandem, such as HILIC and RP-C18 LC-MS methods. An untargeted study may generate a large amount of data and therefore is best analyzed with one of several chemometric data platforms such as MetaboAnalyst [97], XCMS [98], or others [99].

For many ethnobotanically related questions, an untargeted metabolomic analysis is the preferred study design. Again, sample

preparation must be carefully designed and all samples processed using the same methods. The method of sample preparation needs to consider both the location and time the plant material is harvested, as well as if the plant tissue will be processed fresh, frozen or dried. The tissue is usually ground or homogenized to increase the extraction efficiency, and care must be taken to avoid sample cross contamination such as from the grinding equipment itself. The extraction of the plant material can be accomplished using various methods and solvents, many of which are discussed previously in this chapter. For metabolomics studies, a mid-polar solvent such as aqueous methanol or aqueous ethanol is commonly used. Depending on the extraction method, the crude extract can be directly analyzed, or it may need to be concentrated and prepared at a set concentration prior to the analysis. A 2009 review examined the aspects of plant sample preparation in detail [100].

The data acquisition portion of the analysis will depend on what instrumentation is available. A high accuracy LC-MS system is recommended; however, direct MS infusion of the sample is also possible [101]. The combination of HPLC and MS will provide an advantage of two dimensions of separation for the sample: the LC chromatographic separation as well as resolution in the mass domain. This allows for shorter chromatographic methods using smaller columns such as a 2.1 × 50 mm. Efforts should be made to develop a chromatographic system that resolves a wide range of compounds and is rapid. A 2017 review addresses the use of LC mobile phases and mobile phase additives to maximize ionization for plant metabolomics [102]. Since the chemicals of interest are not known in an untargeted metabolomics method, maximizing the number of chemicals that ionize for MS analysis is very important.

Once the data has been collected, it must be analyzed to identify individual features [103]. For LC-MS data, a feature is an ion with a unique *m/z* and retention time. Every detected metabolite will have at least one and often several features. As part of the data analysis, a preprocessing step should be incorporated in order to determine the method's noise floor and filter out "known unknowns" and identified contaminants. The remaining features may need to be filtered further, normalized, and scaled in order to yield the most information from the analytical analysis [104, 105]. Once the features are processed, they can be visualized and interpreted using a variety of algorithms and data visualization techniques, depending on the type of study being undertaken. One common method of analyzing the data is to use principle component analysis (PCA) [106].

Additionally, high-resolution mass spectral data can be used to dereplicate known compounds. One approach that has yielded success is searching chemical databases that can be filtered by botanical genus or species for compounds with exact masses corresponding to

the experimental ions and comparing empirical formulas and fragmentation patterns [36]. The Dictionary of Natural Products and Scifinder are both useful for this type of screening.

5 Final Considerations

In this chapter we summarize the chemistry workflow for an ethnobotanical approach to natural product isolation and drug discovery. This workflow begins with the plant collection itself, and is followed by plant material processing, extraction, chromatography, and spectroscopic analysis. The processing of plant material and their extraction essentially determines the portion of the chemical library contained in the plant that will be examined by all future studies. The chromatography of these extracts, especially when done in a bioassay-guided fraction framework, is perhaps the most time-consuming part of the workflow. Indeed, all fractions generated must be handled and stored with care. Although often only one fraction out of a set is proceeded with for further chromatographic separation, particularly in drug discovery, sister fractions should be preserved for potential future studies, including for studies of the originally pursued bioactivity. For example, it may very well be that a less active fraction, upon further fractionation, proves to be a source of highly bioactive compounds. To the extent that numerous fractions are generated through such studies, it is of the utmost importance to practice good note keeping and to maintain an updated database. This way, the origin and method of production of every fraction is known. If the same fractions are made on separate occasions or by different scientists, their identity can be compared to those of previous batches via spectroscopic methods such as analytical HPLC, ensuring reproducibility. With advances in spectroscopic technology in the past decade, now only very small quantities of fractions and single compounds are required for chemical analyses. The nature of ethnobotanical drug discovery is highly interdisciplinary, and collaborations are highly favored for combining areas of expertise of different laboratories, including botany, phytochemistry, in vitro and in vivo biological assays, and drug screening.

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