

# Chapter 2

## Initial and Bulk Extraction of Natural Products Isolation

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### Abstract

Currently, there is a growing interest in the study of natural products, especially as part of drug discovery programs. Secondary metabolites can be extracted from a variety of natural sources, including plants, microbes, marine animals, insects, and amphibians. This chapter focuses principally on laboratory-scale processes of initial and bulk extraction from plant and microbial sources. With regard to plant natural products, the steps required for the preparation of the material prior to extraction, including aspects concerning plant selection, collection, identification, drying, and grinding, are detailed. The various extraction methods available (maceration, ultrasound-assisted solvent extraction, percolation, Soxhlet extraction, pressurized solvent extraction, extraction under reflux, steam distillation, and acid/alkaline extraction) are reviewed. Regarding microbial natural products, this chapter covers issues relating to the isolation and culture of microorganisms and presents the extraction methods available for the recovery of microbial metabolites. Methods of minimizing compound degradation, artifacts formation, extract contamination with external impurities, and enrichment of extracts with desired metabolites are also examined.

**Key words:** Acid–base extraction, Bulk extraction, Extraction under reflux, Initial extraction, Maceration, Percolation, Pressurized solvent extraction, Solid–liquid extraction, Soxhlet extraction, Steam distillation, Ultrasonication

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### 1. Introduction

Currently, there is an increasing interest in the study of natural products, especially as part of drug discovery programs, as they represent a formidable reservoir of potentially useful leads for new medicines. The natural products of interest here are small organic molecules (mol. wt. <2,000 amu approx.), which are also frequently called secondary metabolites and are produced by various living organisms, e.g., plants, microbes, marine organisms, insects, and amphibians (1).

Plants and microorganisms produce complex mixtures of natural products, and the selection of the best protocol for an efficient extraction of these substances is not a simple task. “Classic” solvent-based procedures, e.g., maceration, percolation, Soxhlet extraction, extraction under reflux, steam distillation, and acid–base extraction, are still applied widely in phytochemistry despite the fact that they lack reproducibility and are both time- and solvent-consuming. This is principally because they only require basic glassware and are convenient to use for both initial and bulk extraction. Accelerated solvent extraction is a newer instrumental technique. While it offers some advantages over conventional methods (mainly efficiency and reproducibility), it is best suited for initial rather than bulk extraction. To date, it has found a wider application in industry (where large numbers of extracts have to be produced in an efficient and reproducible way) rather than in academia.

### ***1.1. The Concept of Initial and Bulk Extraction***

Prior to any isolation and purification work, natural products have to be extracted (or released) from the natural material. An initial extraction is performed typically on a small amount of material to obtain a primary extract. This can be as part of a pharmacological study or to gain preliminary knowledge on the chemistry of metabolites present in the material. Once specific metabolites have been identified in the initial extract, it may then become desirable to perform a bulk extraction to isolate larger quantities of metabolites.

### ***1.2. Solid–Liquid Extraction***

Here, we describe the solvent extraction methods, mainly based on the principle of solid–liquid extraction, available for the initial and bulk laboratory-scale extraction of secondary metabolites from plants and microorganisms. Theoretical and practical aspects related to liquid–liquid extraction are covered in Chapter 9. Various other natural product extraction methods are discussed in Chapters 3–5, 11 and 13–18.

The process of solid–liquid extraction can be simplified by dividing it into different steps. A suitable solvent (see Note 1) is used to first diffuse into cells, then solubilize the metabolites, and finally diffuse out of the cells enriched in the extracted metabolites. The ideal solvent should have a low toxicity, a low flammability, a low risk of explosion, and a low potential for artifact formation. It should also be economical and environment-friendly (see Note 2). These issues are particularly important in the case of bulk extraction, where large volumes of solvents are employed. The ideal extraction method should be exhaustive, i.e., extract as much of the desired metabolites or as many compounds as possible. It should be fast, simple, economical, environment-friendly, and reproducible if it is to be performed repeatedly.

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## 2. Materials

### 2.1. Plant Material

The plant material can be selected in a variety of ways. For example, when biologically active secondary metabolites are sought or a specific pharmacological activity is investigated, a common approach is to collect plant material on the basis of specific traditional ethnomedical uses. The use of literature databases (see Note 3, and see Chapters 1 and 13) can provide some valuable preliminary information.

1. Collect only healthy plant specimens, as signs of contamination (fungal, bacterial, or viral) may be linked to a change in the profile of metabolites present.
2. Collect the whole plant or a particular plant part depending on where the metabolites of interest (if they are known) accumulate.
3. Record the name of the plant, the identity of the part(s) collected, the place, and date of collection (see Note 4).
4. Deposit a voucher (a dried specimen pressed between sheets of paper) in a herbarium for future reference. More details on this particular aspect can be found in Chapter 13.

### 2.2. Material of Microbial Origin

A wide variety of environments can be explored to isolate material of microbial origin. For many years, soil samples have been the traditional source of microorganisms. Other natural sources, such as plants and the marine habitat, have been investigated more recently (2, 3). We describe here the isolation of microorganisms from soil samples and from plant roots, i.e., endophytes.

#### 2.2.1. Soil Microorganisms

1. Collect some soil sample (see Note 5) and suspend it in distilled water.
2. Prepare appropriate dilutions of the supernatant and plate on a solid (agar) medium (see Note 6).
3. Place the plates in an incubator (see Note 7).
4. Subculture individual colonies obtained several times onto the selected solid medium until colonies display purity (morphologically and microscopically).
5. Store pure strains in liquid nitrogen or freeze-dry in the presence of a cryoprotective agent (see Note 8).

#### 2.2.2. Root Endophytes

1. Collect root isolates from healthy plants.
2. Clean roots with distilled water to remove soil and other debris.

3. Cut root into small fragments (5–10 mm) under a dissecting microscope, wash fragments gently with distilled water, retaining them on a flamed-sterilized tea strainer.
4. Sonicate fragments ( $2 \times 15$  s), and gently shake with 1% Tween 80 (1 min), then rinse with sterile distilled water. Shake in 30%  $\text{H}_2\text{O}_2$  for ca. 15 s, and then perform a final rinse (three times) in sterile distilled water.
5. Blot root fragments on a sterile filter paper in a Petri dish and aseptically cut them into smaller fragments (1 mm).
6. Transfer these smaller fragments aseptically onto agar plates (see Note 9).
7. Place plates in an incubator (see Note 10) and check daily for contamination.
8. Once cultures are obtained, subculture as necessary on agar medium.
9. Store pure strains in liquid nitrogen or freeze-dry in the presence of a cryoprotective agent.

### 2.3. Solvents

Aliphatic and chlorinated hydrocarbons, esters, and lower alcohols are commonly used for extraction (Table 1). They include analytical- or HPLC-grade *n*-hexane, petroleum ether, dichloromethane (DCM), ethyl acetate (EtOAc), *n*-butanol, ethanol (EtOH), methanol

**Table 1**  
**Physicochemical properties of some common solvents used in natural products extraction**

Solvent	Polarity index	Boiling point (°C)	Viscosity (cPoise)	Solubility in water (% w/w)
<i>n</i> -Hexane	0.0	69	0.33	0.001
Dichloromethane	3.1	41	0.44	1.6
<i>n</i> -Butanol	3.9	118	2.98	7.81
<i>iso</i> -Propanol	3.9	82	2.30	100
<i>n</i> -Propanol	4.0	92	2.27	100
Chloroform	4.1	61	0.57	0.815
Ethyl acetate	4.4	77	0.45	8.7
Acetone	5.1	56	0.32	100
Methanol	5.1	65	0.60	100
Ethanol	5.2	78	1.20	100
Water	9.0	100	1.00	100

(MeOH), and water (Fisher Scientific UK, and VWR International). Store all solvents at room temperature (unless indicated otherwise) and keep chlorinated solvents in amber glass bottles (see Note 11).

## **2.4. Other Materials and Specific Equipment**

### **2.4.1. Drying and Grinding of Plant Material**

1. Pulverisette 15 Cutting Mill (Fisher Scientific, UK).
2. Basic S2 Analytical Mill (VWR International).
3. Fan-assisted oven.

### **2.4.2. Isolation and Culture of Material of Microbial Origin**

4. Liquid and solid nutrient media.
5. Petri dishes.
6. Conical flasks.
7. Shaking incubators.
8. Small fermenters.

### **2.4.3. Extraction of Plant and Microbial Material**

1. Ultrasonic bath.
2. Accelerated solvent extractor; extraction cartridges; cellulose filters for ASE cartridges (Dionex, UK); sea-sand (50–70 mesh) (Sigma-Aldrich, UK), nitrogen gas (oxygen-free) supply.
3. Percolator (cylindrical or conical container with a tap at the bottom).
4. Soxhlet extractor connected to a round bottom flask and a reflux condenser; cellulose thimbles for Soxhlet extraction (VWR International); cotton wool; and antibumping granules.
5. Rotary evaporator.
6. Steam distillation apparatus.
7. Freeze-dryer.
8. BioPrepNylon® cloth (VWR International).

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## **3. Methods**

### **3.1. Drying and Grinding of Plant Material**

1. Wash the plant material under cold running tap water, and gently brush to remove soil and other debris.
2. For plants containing volatile or thermolabile compounds, snap-freeze the material in liquid nitrogen as soon as possible after collection and store at  $-20^{\circ}\text{C}$  or freeze-dry (see Note 12). When ready to extract, grind samples in a mortar with liquid nitrogen and extract the pulverized residue immediately or store at  $-20^{\circ}\text{C}$  to prevent any changes in the profile of metabolites (4, 5).

3. For other plants, slice the collected material into small pieces and distribute the material evenly on trays at ambient temperature in a room with adequate ventilation to facilitate homogeneous drying (see Note 13).
4. Store the dried plant material in sealed containers in a dry and cool place. Avoid storage for prolonged periods, as some constituents may decompose.
5. Grind the dried plant material using either an analytical mill (for initial extraction) or a cutting mill (for bulk extraction) to improve the subsequent extraction by rendering the sample more homogenous, increasing the surface area, and facilitating the penetration of solvent into the cells (see Note 14).

### **3.2. Extraction of Plant Material**

Plants are complex matrices, producing a range of secondary metabolites with different functional groups and polarities. Although water is used as an extractant in many traditional protocols (see Note 15), organic solvents of varying polarities are generally selected in modern methods of extraction to exploit the various solubilities of plant constituents (see Note 16). Solvent extraction procedures applied to plant metabolites include maceration, ultrasound-assisted solvent extraction, percolation, Soxhlet extraction, pressurized solvent extraction, extraction under reflux, steam distillation, and acid-base extraction. The selection of a method is governed by the nature and amount of material to be extracted. If large amounts are to be extracted, the ease of transfer from initial to bulk scale should be considered. In all cases, dried crude extracts are obtained following evaporation of the organic solvents or freeze-drying (of aqueous solutions) (see Note 17).

#### **3.2.1. Maceration**

1. In a closed glass container, leave the pulverized plant material to soak in a suitable solvent (see Note 18).
2. After some time in contact, separate the residual plant material (marc) from the solvent. This involves a rough clarification by decanting, which is usually followed by a filtration step using Whatman # 1 filter paper (VWR International) (see Note 19).
3. Add some fresh solvent to the marc to ensure exhaustive extraction (see Note 20).
4. Pool all filtrates together.

Maceration is suitable for both initial and bulk extraction of plant material. However, exhaustive bulk extractions can be quite time-consuming, taking from a few hours up to several weeks, and consume large volumes of solvent (6).

#### **3.2.2. Ultrasound-Assisted Solvent Extraction**

1. Place the powdered material in a glass container in contact with the extraction solvent.
2. Put the container in an ultrasonic bath (high-frequency pulses, 20 kHz) (see Note 21).

Ultrasound-assisted solvent extraction is rarely applied to large-scale extraction; it is used mostly for the initial extraction of a small amount of material. It is commonly applied to facilitate the extraction of intracellular metabolites from plant cell cultures (7).

### 3.2.3. Percolation

1. In a percolator, place the powdered plant material to soak in contact with the extraction solvent (see Note 22).
2. Pour some additional solvent on top of the plant material and allow the extract to percolate slowly (drop-wise) out of the bottom of the percolator (see Note 23).
3. Perform successive percolations to extract the plant material exhaustively by refilling the percolator with fresh solvent.
4. Pool all extracts together.

Percolation is adequate for both initial and large-scale extraction (8). The main disadvantages of bulk percolation, however, are that large volumes of solvents are required and the process can be time-consuming. To ensure that the extraction is complete, the percolate can be tested for the presence of metabolites with specific reagents (see Chapter 6).

### 3.2.4. Soxhlet Extraction

1. Put the powdered plant material in a cellulose thimble and cover with cotton wool.
2. Place the thimble inside the Soxhlet extraction chamber.
3. Assemble the Soxhlet extraction chamber on top of a collecting round-bottom flask containing some antibumping granules.
4. Add a suitable solvent to the Soxhlet chamber (see Note 24). When a certain level of solvent has accumulated in the thimble, it is siphoned into the flask beneath.
5. Connect a reflux condenser to the Soxhlet chamber.
6. Place the collecting flask in a heating mantle and heat the set-up under reflux.

Soxhlet extraction is widely used for both initial and bulk extraction (see Note 25) (9, 10). Its main advantage is that the material is extracted continuously, i.e., the solvent saturated in solubilized metabolites empties into the flask, fresh recondensed solvent then re-extracts the material in the thimble. This method is less time- and solvent-consuming than maceration or percolation. Its main disadvantage, however, is that the extract is constantly heated at the boiling point of the solvent used, which can damage thermolabile compounds and/or initiate the formation of artifacts.

### 3.2.5. Pressurized Solvent Extraction

Also called accelerated solvent extraction (see Chapter 4), this method uses high pressure to maintain the solvent in a liquid state

at high temperatures (see Note 26). We have employed pressurized solvent extraction for the initial extraction of plant material as follows (11).

1. Mix the powdered material with some sea-sand in a 4:1 ratio.
2. Load the plant-sand mixture (ca. 40 g) into a 100 mL ASE® extraction cartridge.
3. Place the extraction cartridge in the ASE® 100 extractor.
4. Fill in the reservoir bottles with a suitable extraction solvent.
5. Program the ASE® 100 system to extract at a pressure of 1,500 psi and temperature of 100°C in four static cycles (static time of 8 min/cycle) with a flush volume of 60% and a purge time with nitrogen gas of 150 s.
6. Collect the extract, which is automatically filtered, in the receiving flask.

With low solvent requirements, pressurized solvent extraction offers a more economical and environment-friendly alternative to conventional approaches (12). The other advantages are that high temperatures and pressures improve metabolite solubilization, thus enhancing extraction speed and yield (13); the procedure is entirely programmable, and offers good reproducibility. This method is best suited for the rapid and reproducible initial extraction of a high number of samples.

### 3.2.6. Extraction Under Reflux and Steam Distillation

1. In a round-bottomed flask, immerse the plant material in a suitable solvent, and connect the flask directly to a reflux condenser (see Note 27).
2. Place the set-up in a heating mantle. When the solvent reaches its boiling point, the vapor is condensed and the solvent is recycled to the flask (see Note 28).

The main disadvantage of extraction under reflux and steam distillation is that thermolabile components risk being degraded. Steam distillation is commonly applied to the extraction of plant essential oils.

### 3.2.7. Acid–Base Extraction

In this method, the pH of the extracting aqueous phase is altered to solubilize selectively groups of metabolites (such as acids or bases), which are subsequently extracted into a water-immiscible organic solvent, i.e., liquid–liquid extraction. Acid–base extraction is usually employed in the extraction of alkaloids (14, 15) and anthocyanins (16). One drawback of the acid–base treatment is that it can produce some artifacts and/or lead to the degradation of compounds (17).



### 3.3. Culture of Microorganisms

The culture of microorganisms is carried out in a nutrient broth initially dispensed in flasks. This allows a relatively good set-up to monitor both the growth rate of the biomass and the production of metabolites. It also provides a means of carrying out initial studies to optimize culture conditions and increase metabolite production. The process can be scaled up once the effects of important parameters have been optimized, e.g., aeration, temperature, pH, and the absence of external contamination have been ascertained.

1. Initiate liquid flask cultures by inoculating a couple of 2–3 mm<sup>2</sup> agar blocks of inoculum from the growing edge of a colony into a nutrient solution (100 mL) in sterile conical flasks that are sealed with a cotton plug (see Note 29).
2. Incubate flasks at the required temperature (see Note 30) and inspect regularly for possible contaminants.
3. For bulk extraction, transfer the strain to small fermenters (stainless steel closed vessels) (see Note 31).

### 3.4. Extraction of Material of Microbial Origin

A variety of approaches are employed in the recovery of microbial metabolites depending on whether the compounds of interest are completely or partially excreted by the cells into the medium or are present within the cells. Microbial metabolites are often produced in low yields, and one strain can yield a complex mixture of compounds. As for plant material, water-miscible and immiscible organic solvents, e.g., EtOAc, DCM, *n*-butanol, MeOH, and so on, are used for the extraction of microbial metabolites (Table 1). Evaporation of the organic solvents or freeze-drying (of aqueous solutions) yields dried crude extracts (see Note 17). Further details on the purification and characterization of microbial metabolites are provided in Chapter 15.

#### 3.4.1. Extraction of Whole Microbial Culture

1. Freeze-dry the whole culture.
2. Extract both intra- and extracellular microbial metabolites by maceration or sonication using a suitable solvent (18).

#### 3.4.2. Extraction of Microbial Biomass

1. Separate the microbial biomass from media constituents and other contaminants (see Note 32).
2. Extract intracellular microbial metabolites by maceration or sonication using a suitable solvent (11).

#### 3.4.3. Extraction of Liquid Medium

1. Remove any physical “impurities,” e.g., cell debris, insoluble medium components (see Note 32).
2. Partition the liquid medium (aqueous phase) between a water-immiscible organic solvent by changing the pH of the aqueous phase and/or selecting a solvent into which the desired metabolites partition to extract metabolites selectively depending on their  $pK_a$  and/or partition coefficients (11, 19).

## 4. Notes

1. A solvent of an appropriate polarity is used to extract metabolites following the principle of “like dissolves like.” The material can either be extracted sequentially with solvents of increasing polarity (selective extractions) or a total extraction can be performed using an alcoholic solvent which has the ability to increase cell wall permeability, thus facilitating the efficient extraction of large amounts of polar and medium- to low polarity constituents. When a solvent mixture is necessary, a binary mixture (two miscible solvents) is usually employed.
2. Toxic solvents and those detrimental to the environment, e.g., benzene, toluene, and carbon tetrachloride, must not be used. Diethyl ether should be avoided as it is highly flammable and can lead to the formation of explosive peroxides. Aliphatic hydrocarbons are highly flammable. DCM is preferred to chloroform ( $\text{CHCl}_3$ ), the latter being more toxic. Acetone may give rise to artifacts under acidic conditions. MeOH, DCM, and  $\text{CHCl}_3$  may also produce artifacts (see Chapter 13).
3. Several databases and journals (such as the *Journal of Ethnopharmacology*) can be consulted on the Internet. These include the following links:  
<http://ukcrop.net/perl/ace/search/PhytochemDB>.  
<http://ukcrop.net/perl/ace/search/MPNADB>.  
<http://ukcrop.net/perl/ace/search/EthnobotDB>.  
<http://www.ars-grin.gov/duke/>.  
<http://www.leffingwell.com/plants.htm>.
4. Geographical considerations should be taken into account for recollection purposes to ensure a reproducible profile of metabolites.
5. For the selective isolation of actinomycetes, e.g., *Streptomyces*, soil samples are air-dried for a few days to reduce contamination with nonsporulating bacteria (especially Gram-negative organisms) and then heated at 100–120°C for 1 h. For the selective isolation of Gram-positive *Bacillus* strains, soil samples are heated at 70°C or suspended for 1 h in 50% EtOH.
6. Placing a membrane filter on the agar plate and inoculating its surface with the soil suspension will favor the exclusive growth of the filamentous hyphae of actinomycetes. A selective isolation medium with peptone and other amino acids will favor the germination of *Bacillus* spores. A selective isolation medium with an antifungal agent will inhibit the growth of molds and fungi.
7. The optimum incubation temperatures for the preferential isolation of bacteria and fungi are 37 and 25°C, respectively.

Thermophilic strains will require incubation temperatures at  $\geq 50^{\circ}\text{C}$ .

8. A stock is usually prepared by storing aliquots, e.g., 1 mL, of the culture at  $-80^{\circ}\text{C}$ . Glycerol (20%) is used as a cryoprotective agent.
9. We have used solid media, such as modified Melin Norkrans (MNN) (per liter):  $\text{KH}_2\text{PO}_4$  (0.5 g),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.15 g),  $\text{CaCl}_2$  (50 mg),  $\text{NaCl}$  (25 mg), thiamine (100  $\mu\text{g}$ ),  $(\text{NH}_4)_2\text{HPO}_4$  (0.25 g),  $\text{FeCl}_3$  (1% solution) (1.2 mL), agar (10 g), supplemented with glucose (10 g/L), or malt extract (3 g/L); Potato Dextrose and Malt Extract agar (Oxoid, UK) at full or 1/2 strength formulation, supplemented with streptomycin and penicillin G (both 30 mg/L) (Sigma-Aldrich, UK), with or without the fungicidal agent benomyl (2 mg/L) (Sigma-Aldrich, UK), for the selective isolation of fungal endophytes from root samples.
10. Incubation at  $15\text{--}20^{\circ}\text{C}$  has been used to isolate fungal root endophytes.
11. General purpose grade solvents (available commercially in plastic containers or in plastic-stoppered Winchester bottles) frequently contain plasticizers (additives used in the manufacture of plastic). Minimizing contamination with plasticizers is especially important when bulk extraction is carried out and large volumes of solvent are used. Dioctyl phthalate ester is the most common contaminant. It can be detected by the presence of an intense purple-pink spot on a silica gel TLC plate ( $R_f$  value = 0.4 in petroleum ether-EtOAc, 95:5) following detection with anisaldehyde-sulfuric acid reagent and incubation for 5 min at  $110^{\circ}\text{C}$ . Spectroscopic data: UV  $\lambda_{\text{max}}$  275 nm ( $\log \epsilon$  3.17), 282 nm (sh);  $^1\text{H}$  NMR ( $\delta$ ,  $\text{CDCl}_3$ ) 7.70 (2H, dd, H-3 and H-6 aromatic protons), 7.52 (2H, dd, H-4 and H-5 aromatic protons), 4.20 (4H, dd, H-1' and H-1'' 2-ethylhexyl moiety), 1.2–1.8 (14H, m, CH and  $\text{CH}_2$ s of 2-ethylhexyl moiety), 0.90 (12H,  $\text{CH}_3$  groups); EIMS ( $m/z$ ) 279, 167, and 149 (100%). Distilling general purpose grade solvents prior to extraction and using glass containers for storage can eliminate contamination with plasticizers. Another possible contaminant is high-vacuum grease (a silicone-based lubricant) used as a seal and to prevent glassware joints from seizing. Mass spectrometry can be used to detect contamination; silicone grease presents a typical mass fragmentation pattern ( $m/z$  429, 355, 281, 207, and 133) which differs from aliphatic hydrocarbon greases (fragmentation every 14 mass units intervals).
12. If fresh plant material is used, enzymes can be denatured (or deactivated) by soaking the material in an organic solvent, e.g., MeOH or EtOH.

13. Dry conditions are essential to prevent microbial fermentation and subsequent degradation of metabolites. Additionally, protection from direct sunlight is advised to minimize chemical reactions (and the formation of artifacts) induced by ultraviolet rays. To accelerate the drying process (especially in countries with high relative humidity), the material can be dried in an oven. This can also minimize enzymatic reactions (e.g., hydrolysis of glycosides) that can occur as long as there is some residual moisture in the plant material. Temperature below 30°C is recommended to avoid the loss/degradation of thermolabile compounds, e.g., the volatile constituents of essential oils.
14. Some material, e.g., seeds and fruits rich in fats and volatile oils, however, may clog-up the sieves and that the heat generated may degrade thermolabile metabolites.
15. Traditional methods rely principally on the use of cold/hot water, sometimes alcoholic, and/or aqueous alcoholic mixtures to obtain preparations that are used externally or administered internally as teas.
16. Nonpolar solvents solubilize mostly lipophilic compounds, e.g., alkanes, fatty acids, pigments, waxes, sterols, some terpenoids, alkaloids, and coumarins. Medium-polarity solvents can extract compounds of intermediate polarity, e.g., some alkaloids, flavonoids, while more polar ones are used for more polar compounds, e.g., flavonoid glycosides, tannins, and some alkaloids. Water is not used often as an initial extractant, even if the aim is to extract water-soluble plant constituents, e.g., glycosides, quaternary alkaloids, and tannins.
17. Solvent removal should be done immediately after extraction to minimize the loss of compounds unstable in solution. Prolonged exposure to sunlight should also be avoided because of the potential for degradation. For organic solvents, the extract is concentrated by evaporation under reduced pressure (using a rotary evaporator) at a temperature below 40°C to minimize the degradation of thermolabile compounds. Precautions are required if extracts contain some metabolites that foam as these may spill into the solvent collecting flask. Small volumes of solvents (<5 mL) can be evaporated under a gentle stream of nitrogen gas. If an organic/aqueous mixture was used as the extractant, the sample is evaporated under reduced pressure and then freeze-dried. The freeze-dried extract is best stored in a sealed container in a freezer (−20°C) until required to minimize degradation at room temperature.
18. Maceration is often done at room temperature. Occasional or constant stirring of the preparation (using mechanical shakers or mixers to guarantee homogenous mixing) can increase the speed of the extraction.

19. The plant can be left to macerate within a muslin bag to facilitate further filtration and addition of fresh solvent. Centrifugation may be necessary if the powdered material is too fine to be filtered.
20. The extraction ultimately stops when an equilibrium is attained between the concentration of metabolites in the extract and that in the material.
21. Ultrasound is used to induce a mechanical stress on the cells through the production of cavitations in the sample. The cellular break down increases the solubilization of metabolites in the solvent and improves extraction yields. The efficiency of the extraction depends on the instrument frequency, and length and temperature of sonication.
22. A metallic container is recommended if hot solvent is used. Details of the apparatus can be found in a variety of pharmacopeia monographs. The extent to which the material is ground can influence extracts' yields. Hence, fine powders and materials, such as resins and plants that swell excessively, e.g., those containing mucilages, can clog the percolator. Furthermore, if the material is not distributed homogenously in the container, e.g., if it is packed too densely, the solvent may not reach all areas and the extraction will be incomplete. Only coarsely fragmented material that passes through a 3 mm sieve is adequate for percolation. Both the contact time between the solvent and the plant, i.e., the percolation rate, and the temperature of the solvent will influence extraction yields. The plant is usually left to soak initially for up to 24 h in the percolator. High temperatures improve extraction yields, but may lead to decomposition of thermolabile metabolites.
23. Additional filtration of the extract is not required because there is a filter at the outlet of the percolator.
24. It is preferable to use a single solvent simply because one of the solvents in the mixture may distil more rapidly than another. This may lead to a change in the solvent proportions in the Soxhlet extracting chamber.
25. Initial and bulk Soxhlet extraction can be performed for up to 72 h. An initial extraction uses 200–500 mL on average while a bulk extraction uses 2.5–5 L of solvent. For safety purposes, it is recommended the Soxhlet apparatus be in a walk-in type of fume cupboard.
26. Accelerated solvent extraction is a registered process (ASE®) developed by Dionex (Sunnyvale, CA, USA). Extractions are carried out under high pressure (100–200 bar) within 12–20 min, using 15–45 mL of solvent and at temperatures ranging from ambient to 200°C. Two systems are currently available. The ASE® 150 has a single loading cell (1–100 mL)

and is best suited for low-throughput initial extractions. High-throughput extractions can be achieved with the ASE<sup>®</sup> 350, which has a carousel that holds up to 24 cells (1–100 mL). Both systems can accommodate 1–100 g of sample.

27. In steam distillation, the plant material is immersed in distilled water. Care should be taken not to powder the material too finely. Coarsely fragmented or crushed material is preferable. Some glycerol can be added to the water to facilitate the extraction of tough material, e.g., barks, seeds, and roots.
28. In steam distillation, the vapors condense upon heating and the distillate is collected in a graduated tube connected to the condenser. Xylene may be added to the graduated receiver to trap the distillate produced. Both the description of the distillation apparatus and the steam distillation procedure are found in a variety of pharmacopeia monographs. Optimum extraction conditions, e.g., distillation rate, have to be determined depending on the nature of the material being extracted.
29. Typically, 50–100 mL of liquid medium is dispensed into flasks (500 mL capacity). The small volume of medium relative to the size of the flasks is necessary to increase aeration. Sterile cotton plugs are used to avoid external contamination and allow gaseous exchange. We have used liquid media, such as MNN supplemented with glucose (10 g/L), glucose/malt extract (5 and 3 g/L, respectively), or glucose/malt/yeast extract (5, 2, and 1 g/L, respectively), Hagem's containing (per liter):  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.5 g),  $\text{KH}_2\text{PO}_4$  (0.5 g),  $\text{NH}_4\text{Cl}$  (0.5 g),  $\text{FeCl}_3$  (1% solution) (0.5 mL), thiamine (50  $\mu\text{g}$ ), supplemented with glucose/malt/yeast extract at 5, 2, and 1 g/L, respectively; Malt Extract and Potato Dextrose broth (Oxoid, UK) for the culture of fungal endophytes from root samples.
30. Flasks (up to 50–100) may be placed in a temperature-controlled shaking incubator. We have cultured fungal root endophytes by incubating flasks at 15–20°C for 3–5 months.
31. Small fermenters have a 5–10 L capacity. Mini-fermenters (up to 2 L) can provide an intermediate stage in the scale-up process.
32. Separation is achieved by filtration or centrifugation depending on the broth's physical properties and the morphology and size of cells. Microbial cells include bacteria (1  $\times$  2  $\mu\text{m}$ ), yeasts (7  $\times$  10  $\mu\text{m}$ ), and fungal hyphae (1  $\times$  10  $\mu\text{m}$ ). The latter can grow as a filamentous mycelium. We have used a BioPrepNylon<sup>®</sup> cloth (VWR International, UK) to separate the mycelium of fungal endophytes from aqueous broths.

## References

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Natural Products Isolation

Sarker, S.D.; Nahar, L. (Eds.)

2012, XII, 552 p., Hardcover

ISBN: 978-1-61779-623-4

A product of Humana Press