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Extraction and Analysis of Terpenes/Terpenoids

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Abstract

Terpenes/terpenoids constitute one of the largest classes of natural products, this is due to the incredible chemical diversity that can arise from the biochemical transformations of the relatively simple prenyl diphosphate starter units. All terpenes/terpenoids comprise a hydrocarbon backbone that is generated from the various length prenyl diphosphates (a polymer chain of prenyl units). Upon ionization (removal) of the diphosphate group, the remaining allylic carbocation intermediates can be coaxed down complex chemical cascades leading to diverse linear and cyclized hydrocarbon backbones, which can then be further modified with a wide range of functional groups (*e.g.* alcohol, ketones, *etc.*) and substituent additions (*e.g.* sugars, fatty acids). Because of this chemical diversity, terpenes/terpenoids have great industrial uses as flavors, fragrances, high grade lubricants, biofuels, agricultural chemicals and medicines. The protocols presented here focus on the extraction of terpenes/terpenoids from various plant sources and have been divided into extraction methods for terpenes/terpenoids with various levels of chemical decoration, from the relative small, nonpolar, volatile hydrocarbons to substantially large molecules with greater physical complexity due to their chemical modifications.

Keywords

Terpene; terpenoid; isoprenoid; hydrocarbons; substituent groups; volatile; polarity

INTRODUCTION

Terpenes and isoprenoids in general have garnered much attention because of their important physiological roles (*i.e.* hormones, aliphatic membrane anchors, maintaining membrane structure), ecological roles (*i.e.* defense compounds, insect/animal attractants) (Kempinski et al., 2015), and their wide uses in pharmaceutical and industrial applications ranging from flavors and fragrances (Schwab et al., 2008) to medicines (Dewick, 2009; Niehaus et al., 2011; Shelar 2011). The biosynthesis of all isoprenoids starts from the two universal five carbon (C5) precursors: isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These initial prenyl units can be used directly to form hemiterpenes or polymerized in increments of five carbon units through the successive addition of IPP to generate prenyl diphosphates of varying chain lengths. These prenyl diphosphates are the universal precursors to all the primary terpenes found in plants, such as monoterpenes (comprised of 10 carbon atoms, C10), sesquiterpenes (C15), diterpenes (C20), triterpenes

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(C30), carotenoids (C40) and polyprenols (>45) (Figure 1). For the sake of this discussion, each class of terpenes can be further distinguished based on the protocol necessary to extract and measure the various forms: 1) ionization of the prenyl precursors leads to linear and cyclized hydrocarbon scaffolds (see the compounds highlighted in green in Figure 1 which will fall mostly under protocol 1); 2) Primary decorations can include the addition of methyl or hydroxyl groups yielding largely non-polar compounds (red highlighting in Figure 1, compounds subject to protocol 2); and 3) Further modifications including acylation, aroylation, glycosylation and other substituent groups may change the physical nature of the resulting terpenoids dramatically, thus necessitating the use of other extraction protocols (see the compounds highlighted in blue in Figure 1, which most likely require protocols 2 and 3 for their extraction and measurement). The infinite array of scaffold forms and modifications has made the extraction and measurement of terpenes/terpenoids one of the most challenging and rewarding areas of chemical analysis for well over 300 years and the foundation of more than 20 Nobel prizes (beginning with Otto Wallach in 1910 and Leopold Ruzicka in 1939), which promises to be developed, unabated, in the future.

Due to the incredible range of structures that terpenes can comprise, the methods for their purification will vary case-by-case, depending on the chemical properties of the target or suspected terpene, the physical properties and amounts of the starting plant material, and the availability of tools and reagents. Because the optimal extraction of a particular terpene will depend on its properties, in this unit we will focus on some standard methods for terpene extraction from plant tissue where the compounds may be present in as little as nanogram quantities, using techniques that will be suitable for most laboratories. In general, the methods include the following steps: 1) breaking the plant cells to release their chemical constituents; 2) extracting the sample using a suitable solvent (or through distillation or the trapping of compounds); 3) separating the desired terpene from other undesired contents of the extracts that confound analysis and quantification; and 4) use an appropriate method of analysis (*e.g.* thin layer chromatography [TLC], gas chromatography [GC], or liquid chromatography [LC]). Usually, the last three steps will vary depending on the polarity and sizes of the target terpenoid. Basic Protocol 1 will describe a method that can be used for extraction of most primary non-polar terpenes. Basic Protocol 2 will focus on another method that is applicable to terpenoids with low polarity (but higher than that of a pure hydrocarbon), and Basic Protocol 3 will describe a method suitable for more polar terpenoids. It is important to emphasize that combinations of these basic protocols can be used to optimize extraction of the desired terpenoid, and that mixing and matching specific steps from each of the protocols may be necessary and consequently may require empirical validation.

BASIC PROTOCOL 1

EXTRACTION AND ANALYSIS OF A NON-POLAR TERPENE

Most of the primary (linear terpenes and cyclized terpenes without decoration) are exclusively composed of hydrocarbons, so these molecules are very non-polar. The terpenes with 15 carbons or less may be volatile due to their small size and low polarity which allow emission to the atmosphere. Extraction and analytical methods for volatile terpenes will be

discussed in alternative protocol 1. Non-volatile terpenes can be extracted using a very nonpolar organic solvent such as hexane. Using silica as a stationary phase in chromatography is another perfect tool to separate these terpenes from other compounds in the extract. Usually, terpenes with more carbon will elute more slowly than lower molecular weight compounds, but cyclized terpenes can elute faster than the corresponding non-cyclized terpene with the same carbon number because of their more compact size. In this protocol, we will describe how to extract the linear hydrocarbon molecule, squalene, from plants. This compound has 30 carbons, and is the linear precursor to all triterpenes, including tetracyclic sterols and pentacyclic saponins.

Materials—Scale capable of accurately weighing ± 0.1 mg

Glass wool

Glass serological pipettes

Glass Pasteur pipettes (Fisherbrand catalog number: 13-678-20A)

Glass vials with PTFE sealed lids (Kimble-Chase catalog number: 60940A24)

Liquid nitrogen

Gaseous nitrogen

Silica gel, pore size 60 Å, 230–400 mesh (Sigma-Aldrich catalog number: 227196), 500 mg prepared in a glass Pasteur pipette plugged with glass wool (see Figure 2)

GC-MS (*e.g.* Agilent 6890a GC equipped with a HP5-MS column [30 m $\times$ 0.250 mm $\times$ 0.25 μ m] coupled to an Agilent 5975C Mass Spectral Detector or Flame Ionization Detector (FID))

Extraction solvent (hexane:ethyl acetate, 85:15 [v/v])

HPLC (*e.g.* Waters 2695 equipped with a Nomura Chemical Develosil 60-3 250 $\times$ 20 mm column and a Waters 2696 photodiode array detector)

(-)- α -cedrene (Sigma-Aldrich, catalog number: 22133)

Hexadecane (Sigma-Aldrich, catalog number: 442679)

Extraction of plant materials with an organic solvent

- 1 Measure out between 100 mg and 1 g of plant material.

The amount of plant material you use depends on the purpose of your extraction. The squalene level in wildtype tobacco plants is usually between 0.5 μ g/g Fresh Weight (FW) to 10 μ g/g FW. Thus, at least 50 mg of tobacco leaf material should be used for suitable downstream detection.

- 2 Grind plant material into a powder in liquid nitrogen in a glass vial or using a mortar and pestle. Ensure that sample is in a glass container prior to adding solvent as plastic containers will leach components into the organic solvent that can confound analytic analyses and damage GC/HPLC columns and equipment.
- 3 Add the extraction solvent (50:1 mg/mL, plant material/solvent) of a hexane:ethyl acetate mixture (85:15 [v/v]) immediately after the material has been ground. The crude extract should be transferred to a glass vial or flasks for shaking for at least 3–4 hours or overnight.

The ratio of hexane: ethyl acetate can be changed depending on the polarity of the terpene sought. Usually a higher proportion of hexane will extract more non-polar terpenes, whereas, higher proportion of ethyl acetate will extract more polar terpenes. It is advisable to include an internal standard in the extraction solvent such as α -cedrene or hexadecane for quantification and calculations of recovery. Ideally, the internal standard will be of similar chemical composition to the target terpene. The amount of internal standard used will depend on the final concentration desired. Generally, for GC analysis, a final concentration of 50 ng/ μ L is appropriate. Thus, if the final volume is to be 100 μ L, add 5 μ L of a 1 μ g/ μ L standard.

- 4 A) For extraction from small amounts of tissue: concentrate the hexane extracts to ~500 μ L under a nitrogen stream without drying the samples. Then, load the concentrated sample onto a small silica column (Figure 2). Allow the sample to be absorbed into the column, then add 1–3 mL of hexane and collect the column eluate. Dry the eluate under a nitrogen stream and resuspend in 100–200 μ L hexane. Transfer this to an appropriate vial for GC analysis. If using a GC set-up as described in the materials section above, then the sample can be analyzed using an injector temperature of 250°C with an oven temperature of 150°C for 1:00 minute, then 10°C/min to 280°C at, then 5°C/min to 310°C for 1:00 minute (assuring removal of all materials injected onto the column); 0.9 mL/min He flow rate. If coupled to an MS detector, the spectra can be detected in positive ionization mode, 70 eV, scanning 50–500 m/z . If the GC is coupled to a Flame Ionization Detector (FID), then one must run a verified standard to know the exact retention time of squalene.

If the terpene is volatile, then all concentration and drying steps under a nitrogen stream should be conducted with the sample vial sitting in ice. Also, the small chromatographic step may not be enough to separate and purify all compounds in the mixture, and the eluate will contain a mixture of compounds. However, this chromatographic step can exclude pigments (large size molecules) and other chemical constituents, which may dirty and reduce the performance of the injector port or the injector syringe of the analytical equipment.

B) For purifying large quantities of the target terpene from a larger amount of plant material: concentrate the extract to as small a volume as possible, but keeping enough volume so that all chemical components in the extract remain solubilized (including some leaf or tissue debris) and can be easily loaded into the column in the next step.

Larger scale silica chromatography purification

- 5 Prepare the silica column as shown in Figure 2 and load solvent onto the column using at least two times the volume of silica within the column, ensuring that the silica is completely saturated with solvent.

It is very convenient to fill half the volume of the column with silica, so that an equal amount of solvent can be added to the top without the need to measure each time.

- 6 Load the concentrated extracts onto the top of column. Then wait for the extract to fully enter the column.

Concentrating the extract to as small a volume as possible ensures that the sample will enter the column as a sharp band and will prevent spreading of the compound over more fractions than necessary.

- 7 Add a volume of hexane equal to the silica volume (half column volume in the set-up recommended above) and allow this to enter the column. Repeat multiple times.

Do not add the hexane until all the extract has fully entered the column, otherwise the introduced hexane will dilute the concentrated sample. How long the entire elution takes will depend on the affinity of target terpene molecules to the silica gel. Usually the molecules with less polarity and smaller size will take less time to elute. For example, squalene may require multiple column volumes to elute fully. It is best to check empirically using a purified standard if possible. If a standard cannot be obtained, a compound that is of similar chemical composition can substitute as a starting point.

- 8 Collect the entire eluate or equally sized fractions, for each round of addition of a column volume of hexane, in clean glass vials/flasks, being careful to keep track of the fraction or eluate order.

Make sure the volume of the eluate collection vial/flask is 2 times greater than the expected collection volume.

- 9 Transfer a 100–200 μ L aliquot from each fraction into a GC vial. Analyze the sample using a GC (equipped with a MSD or FID) (See step 4, above), to determine which fraction contains the squalene.

In this step, if the plant has a relatively high concentration of the desired terpene, Thin Layer Chromatography, TLC (see protocol 2) can be used to directly show which fraction has the highest amount of

the desired terpene. However, in the example presented here, the squalene levels in plants are usually low and TLC is not sensitive enough to detect it.

- 10 Combine all the fractions that contain squalene or the terpenoid of interest. Then concentrate the fractions under a nitrogen stream.
- 11 The concentrated sample should contain most of squalene. The quantity and purity of the squalene can be evaluated by analyzing a small fraction of the sample by Gas Chromatography (GC).

The sample may contain other molecules which co-elute with squalene and these will affect the purity of the sample. High Performance Liquid Chromatography (HPLC) can be used for further purification.

Purification of squalene using HPLC

- 12 Transfer the concentrated extract into an appropriate vial for HPLC separation.
- 13 Inject the entire sample, and run in isocratic mode (100% *n*-hexane) at 8 mL/min.
- 14 Squalene elution can be detected by following max UV absorbance at 200 to 215 nm.

HPLC coupled to a photo diode array is generally not a very sensitive method for the detection of terpenes. This is due to the lack of specific UV wavelengths that they absorb. However, in larger-scale purification where the concentration of the terpene will be present in large amounts, using a wavelength of 200 nm will suffice. Using wavelengths of 200 to 215 nm is not very specific for any chemical feature, so it can also be used to track impurities.

- 15 Collect the eluate fractions, concentrate under a stream of nitrogen and evaluate the individual fractions for those containing the highest amount of squalene. GC analysis is the preferred method for this, but TLC may also be used.
- 16 Repetitive chromatographic runs can be used to enhance and increase the purity of the desired terpenoid compound, in this case squalene.

ALTERNATE PROTOCOL 1

EXTRACTION AND ANALYSIS OF A VOLATILE TERPENE

Many of the monoterpenes and sesquiterpenes can be volatile, and while these serve important roles in plant interactions with their environments, they require special methods for their analysis. The volatile terpenes present in specific plant tissues can be studied directly through traditional extraction techniques (*e.g.* hydrodistillation, extraction by organic solvent) and more novel techniques, such as solid-phase micro extraction (for a

review see [Pawliszyn, 2012]) or microwave-assisted extraction (Chemat et al., 2012). However, the terpenes that are emitted to the atmosphere accumulate only temporarily in leaf aqueous and lipid phases, and thus are present in only small concentrations in tissue samples (Wu et al., 2006). Hence, studying the biosynthesis and emission of terpene emissions requires molecular trapping techniques. In this alternative protocol, we describe how to trap the volatile sesquiterpenes, like patchoulol, emitted from transgenic tobacco plants generated by Wu et al. (2006).

Materials—Gas tight, glass chamber with two ports capable of attaching tubing

Tenax resin 20–35 mesh, 150 mg (Sigma-Aldrich catalog number: 11049-U)

Compressed or house provided air (velocity: 300–500 mL/min)

Vacuum line (velocity: 300–500 mL/min)

Glass Pasteur pipettes (Fisherbrand catalog number: 13-678-20A)

Ethyl acetate

Hexane

Collecting the volatile terpenoids

1. Put the plant in a sealable chamber with two ports (see Figure 3). Connect one port to a compressed air tank (or in-house air source) and connect the other to a vacuum line with a matched gas flow velocity of approximately 300–500 mL/min. Set up a glass Pasteur pipette packed with 150 mg of the tenax resin (similar to the set up used for the silica column presented in Figure 2) and plumb in-line prior to the air entering the chamber. This will pre-filter the air entering the chamber. Use simple connectors to allow easy exchange of the traps.

Plumb the input gas line into the bottom of the chamber. Be sure to wrap the plant pot in plastic wrap, covering all the soil and wrapping around the plant stem. This should leave only the aerial portions of the plant exposed to the circulating air.
2. Ensure that a balance of air is pumped into and out of the chamber, because the small tubing used can create resistance.
3. Collect the volatile compounds by plumbing a glass Pasteur pipette packed with 150 mg tenax resin (the same as that filtering the input air entering the chamber) connected in-line with the output gas line via simple connectors to allow for easy exchange of the traps.
4. Exchange the traps every 1–4 hours, and elute the trapped terpenes with 2 or more washes of 0.5 ml of hexane or hexane:ethyl acetate (85:15 [v/v]). Examine the eluate using GC-MS or GC-FID as described in protocol 1.

BASIC PROTOCOL 2

EXTRACTION AND ANALYSIS OF A TERPENE WITH MODIFICATIONS

It is common for terpenes to be modified by the addition of substituent groups that increases their polarity, for instance by the addition of hydroxyl groups or oxidation of a methyl group to the corresponding carboxylic acid function. However, it is important to remember that the number and type of modifications can increase or decrease the polarity of the final terpene product. Furthermore, while the addition of a small number of polar modifications may allow for analysis by GC, addition of a large number of such modification or a very polar group (*e.g.* a mono- or sesquiterpene with an acidic group or glycosylation) will necessitate LC analysis rather than GC, or at least derivatization to assure the compound can be resolved by GC. This protocol will use the extraction of a sesquiterpenoid, capsidiol, from a tobacco cell culture as a demonstration of how to extract a moderately polar terpenoid. These compounds can be extracted from the cell culture medium and methanol extracts of cells using a chloroform partitioning method.

Materials—Pear shaped, collection flasks

Separatory funnel

Glass serological pipettes

Glass Pasteur pipettes (Fisherbrand catalog number: 13-678-20A)

Rotovaporator

TLC sheets, Silica gel 60 F₂₅₄ (Millipore catalog number: 105735)

TLC chromatography tank

Developing solvent: cyclohexane:acetone (1:1)

Glass atomizer (must be connected to compressed air or nitrogen)

Spray box (to catch excess TLC indicator reagent)

Chloroform

Hexane

Cyclohexane

Acetone

Vanillin indicator reagent (see recipe)

Extraction of terpenoid from cell culture and liquid media

1. Rinse collection flasks and separatory funnels with chloroform.

2. Pour entire media sample (10 mL) into separatory funnel with closed stopcock.
3. Add 20 mL chloroform, secure glass stopper, invert funnel, open stopcock, and gently shake for 15 sec.
4. Close stopcock, invert funnel, remove glass stopper and let stand for 1 to 2 min, or until the phases separate and sharp interface forms.
5. Drain the lower CHCl_3 phase into a collection flask.
6. Repeat extraction steps 3–5 and collect the extract into same flask as the first extraction.
7. Secure the collection flask onto the rotoevaporator, start the flask rotation (50 to 100 rpm), immerse the collection flask into a water bath (40°C–50°C) and evaporate to dryness.

The rotoevaporator temperature should be adjusted given the relative heat lability of your target terpene.
8. Resuspend the dried extract in 100–200 μL hexane and transfer to a GC vial.

TLC analysis of extracted capsidiol

1. Mark a TLC plate with pencil (1.5 cm up from bottom, 1.5 cm spacing).
2. Spot 20 μL aliquots of samples and allow samples to dry.
3. Develop TLC plate using cyclohexane:acetone (1:1) until solvent has moved 7 cm past application zone. Remove plate and dry.
4. Spray plate with vanillin indicator reagent, then heat (hottest setting of a conventional hair dryer, or incubate at 100°C) for color development.
5. Terpenoids can be seen clearly on the TLC plate as bright blue-green bands.

One of the advantages of using the vanillin indicator reagent is that it will produce a specific colored band depending on the terpene it interacts with, thus it adds a layer of specificity to determining the presence of your compound. Other indicator dyes are possible as well.

ALTERNATE PROTOCOL 2

EXTRACTION AND ANALYSIS OF TERPENES DECORATED WITH LARGE GROUPS

Analysis of terpenes conjugated to fatty acids or other acyl derivatives using an ester bond can be broken using saponification. These ester bonds leave a hydroxyl group present on the terpenoid backbone which can be silylated using a derivatization agent to allow for better volatility and analysis using GC. The protocol presented below demonstrates how to extract and analyze phytosterols. It is important to note that saponification is not required to analyze

free sterols, as they will partition into the organic phase using methods such as those described in protocol 1. Derivatization at the end can still be used even if saponification is not, as it will allow for better sensitivity in detection using GC. Saponification will allow extraction and analysis of the total phytosterol population of the analyzed tissue. This protocol includes elements modified from that presented by Du and Ahn (2002).

Materials—Plant tissue (20–100 mg)

Scale capable of accurately weighing ± 0.1 mg

Heat block set at 50°C

Glass vials, 14 mL capacity with PTFE cap (*e.g.* Kimble Chase, catalog number: 60940A24)

Glass serological pipettes

Glass Pasteur pipettes (Fisherbrand catalog number: 13-678-20A)

GC vials

Glass GC vial inserts

Deionized (DI) water

Ethanol

Potassium hydroxide (KOH) 33% dissolved in water

Hexane

Pyridine

MSTFA + 1% TMCS (Thermo Scientific catalog number: TS-48915)

GC-MS (*e.g.* Agilent 6890a GC equipped with a HP5-MS column [30 m $\times$ 0.250 mm $\times$ 0.25 μ m] coupled to an Agilent 5975C Mass Spectral Detector)

Saponification and extraction of phytosterols

1. Collect and weigh the plant materials.
2. Homogenize the samples in liquid nitrogen in a glass vial.
3. Add 5 mL ethanol:33% KOH (94:6 [v/v]) to the sample.
Use glass pipettes for transferring all liquids to prevent leaching of plasticizers into solution.
4. Add 10 μ L 5- α -cholestane (1 μ g/ μ L) to the sample, mix and incubate at 50°C for 1 hour, then cool on the ice for 10 min.
5. Add 7.5 mL deionized (DI) water and 7.5 mL hexane.

6. Vigorously shake the mixture for minimally 30 seconds, then allow all the phases to separate.
7. Transfer the upper hexane phase to a clean vial. Repeat the extraction of the water phase with another 7.5 mL hexane, and combine this hexane extract with the first. Concentrate the hexane extracts under a stream of nitrogen to ~100 μ L and move to a GC vial (with appropriately sized insert vial if necessary). Dry sample under a stream of nitrogen.
8. Add 100 μ L pyridine + 100 μ L MSTFA + 1% TMCS for derivatization at room temperature overnight or at 50°C for 1 hour with occasional vortexing.

Pyridine and MSTFA + 1% TMCS should only be used in a fume hood wearing correct personal protective equipment. Pyridine has a foul odor and MSTFA + 1% TMCS is toxic, causing chemical burns if contacted with skin and potentially fatal if inhaled. The final volume of the pyridine: MSTFA + 1% TMCS is not as important as keeping them in a proper 1:1 ratio. It is possible to scale this down to 50 μ L of each. It is only important the derivatization agent be kept in excess, otherwise all of the compounds will not be silylated. This will be apparent in the GC-MS trace as underivatized peaks will be present (in conjunction with the corresponding TMS-ether peak).

9. Analyze 1 μ L using GC-MS (if using HP-5MS column as indicated above, utilize an injector temperature of 250°C, with an initial oven temperature of 200°C for 0.5 min, followed by a ramp to 270°C at 10°C/min, then to 320°C at 3°C/min and hold for 10 min).

BASIC PROTOCOL 3

EXTRACTION AND ANALYSIS OF POLAR TERPENOIDS

In nature, many interesting terpenoids will be decorated with one or multiple polar groups or molecules, which increase significantly the size and polarity of the terpenoid molecule. Extraction using a non-polar solvent (*i.e.* hexane) and analysis by GC-MS cannot be used for these types of terpenoids because of its general inability to partition into the organic phase as well as the inability of the compound to be efficiently volatilized for analysis using GC. Therefore, a more polar solvent should be used for extraction (*e.g.* methanol) and a LC-MS system will be more suitable for analysis. In this protocol, we will briefly describe a simplified method for extraction and analysis of artemisinin, which is one of the most important medicinal terpenoids. Lapkin et al. (2006) evaluated different extraction methods for artemisinin from *Artemisia annua* examining such parameters as operating costs, toxicity, risk and safety, greenhouse gas emissions, and capital costs. These authors deduced that some emerging technologies (*e.g.* ionic liquid extraction) will be able to compete with traditional extractions (*e.g.* hexane), but in terms of the considered parameters, hexane is better than ethanol. However, the protocol presented below is a reproducible method using

HPLC electrospray (ESI) quadrupole time of flight tandem mass spectrometry (Q-TOF MS/MS) for the extraction of artemisinin from plant tissue, taken from Van Nieuwerburgh et al. (2006), and is appropriate for a lab-scale extraction where high throughput and high recovery takes precedence over commercial/industrial scale considerations.

Materials—Chloroform

Methanol

Ammonium acetate, 1 mM, pH 5.5

β -artemether

HPLC (*e.g.* Waters Alliance 2695 equipped with two pumps [pump 1: 1 mM ammonium acetate buffer, pH 5.5 set with acetic acid; pump 2: methanol] and an Waters XTerra MS C₁₈ 5 μ m guard column (10 mm $\times$ 2.1 mm) coupled to an Alltech Ultrasphere C₁₈ IP 5 μ m column (150 mm $\times$ 2.1 mm) with a LC Packings ACUrate ICP-04-20 post-column splitter sending a quarter of the column eluate into the LC-MS)

ESI Q-TOF MS/MS (*e.g.* Ultima MS with ESI source operating in positive mode with a capillary voltage of 2.7 kV, a source temperature of 130°C and a desolvation temperature of 300°C; nitrogen as the desolvation gas with a flow rate of 500 L/h; MS/MS was conducted using argon (0.9 bar) for collision; cone voltage set for 40 V and a 7 eV collision energy is optimal for artemisinin)

Extraction and analysis of artemisinin

1. Immerse one gram of leaf material in 6 mL of chloroform for 1 min. Remove a 10 μ L aliquot to 1 mL of methanol:1 mM ammonium acetate buffer, pH 5.5 adjusted with acetic acid (1:1 [v/v]).

The methanol:ammonium acetate solution can contain 0.4 mg/mL of an internal standard, β -artemether. This brief extraction is believed to be possible due to the fact that the majority of artemisinin in *A. annua* resides in subcuticular glands which are readily accessible to solvent. Note that tissue homogenization before extraction will probably be needed for most other extraction methods.

2. Inject 100 μ L of the sample onto the HPLC system and separate the components using a gradient elution flow of 0.2 mL/min. The initial composition of 1 mM ammonium acetate (1 mM, adjusted to pH 5.5 with acetic acid):methanol was 50:50 and maintained for 1 min. The methanol concentration was increased linearly to 80% over 6 min and maintained for 18 min. The column was allowed to equilibrate for 10 min between samples.

3. Analyze the artemisinin content using the MS/MS signal as the sum of the intensities of m/z peaks of 219, 229, 247, and 265 fragmented from the parent m/z of 283.

REAGENTS AND SOLUTIONS

Vanillin indicator reagent for TLC

1.4 g vanillin
40 mL methanol
250 μ L H₂SO₄
Store at 4°C

COMMENTARY

Background Information

Terpenoids comprise a large group of distinct natural metabolites, many of which were discovered in plants. Terpenes discovered and isolated from plants have been utilized widely in foods, cosmetics, pharmaceuticals and in various biotechnological applications. The ability to isolate and purify these valuable molecules from plants is key to elucidating their potential applications. For example, the anti-cancer drug, paclitaxel, which was initially extracted from bark from the Pacific yew tree, continues to have efforts developed to improve its extraction efficiency from plant cell cultures (Theodoridis et al., 1998; Kawamura et al., 1999; Oh et al., 2012; Taura et al., 2013; Kim and Kim, 2015). This reiterates the points made in the introduction, that the more complex the target terpene becomes, the more complex the extraction procedure can be to optimize recovery, fully.

In nature, a terpenoid may contain multiple cyclized structures and various types of groups (e.g. hydroxyl groups, fatty acids, sugars, benzyl rings). These decorations will increase significantly the polarity of the molecule. The polarity of the molecule is the most important feature to consider when determining how to purify the desired terpenoid. In addition, volatility and size are critical factors, which will dictate important aspects of the method as well as the type of analytical equipment which should be used. The methods described in basic protocols 1 and 2 will be suitable for most non-polar terpenoids, whereas the methods described in basic protocol 3 and alternative protocol 2 may be used for polar terpenoids. Again, Figure 1 may be used as a general guide—you can see which terpene most closely matches with your (anticipated) structure, and the shaded region in which it falls should serve as a general guide as to which protocol can be used for extraction: molecules best extracted and analyzed using a protocol similar to those presented in 1, 2, or 3 are highlighted in green, red, or blue, respectively.

However, extraction of any one terpenoid efficiently and economically usually requires a more specific and optimized method. Due to the length restrictions of this report and the prospect that an optimized extraction procedure could be developed for each and every terpene compound based on its properties and progenitor tissue, this protocol should be

considered only as an initial starting point for the extraction of any specific terpene/terpenoid compound. One would also be well served to consult other reference materials like those describing specific extraction protocols for carotenoids (Rodriguez, 2001; Taylor et al., 2006), terpenoid lactones (such as those used for *Ginkgo biloba*; Ding et al., 2004; Sun et al., 2005; Croom et al., 2008) and terpene glycosides (Kodama et al., 1981). Many current primary research articles continue to report refinements in extraction methods that allow significant advantages over conventional methods (*e.g.* reduction in use of organic solvents, avoidance of sample degradation, and elimination of additional steps in sample clean-up and concentration before chromatographic analysis.)

Critical Parameters and Troubleshooting

The protocols presented in this unit are based on standard procedures that have been thoroughly tested within our laboratory or derived from peer-reviewed published literature. However, optimal extraction of any specific desired terpene/terpenoid will require specific changes and fine-tuning of these protocols. Other important considerations that should be taken into account during extraction are the integrity and purity of the target compound, which may be degraded by oxidation due to exposure to the atmosphere or degradation due to harsh treatments used during the extraction or analytical processes (*i.e.* lyophilizing, heating during saponification or high temperatures utilized during GC analysis). As stated in the first protocol, the best indicator to understand and estimate any losses is inclusion of an external standard which has similar chemical properties to the target compound prior to extraction. Ideally, one would like to use a purified standard having similar chemical features to the target compound, which can then be used to determine overall extraction efficiency. This would then provide a valuable guide to assessing actual percentage recovery, which may be diminished by poor partitioning between extraction solvents, degradation, and other mitigating factors.

Anticipated Results

Following the protocols described above as guidelines should allow one to detect their desired terpenes using the suggested analytical method. It is critical to understand the detection limits for the desired analytical method and recovery efficiencies of the methods to determine the amount of tissue necessary for the initial extractions. Once this has been verified, the protocol volumes can easily be scaled to suit one's needs. As an example, a minimum of ~20 mg FW tissue would be needed in alternate protocol 2 with a 30–60% recovery of the external standard (5- α -cholestane), in order to reliably detect and quantify major phytosterol components (*i.e.* sitosterol, campesterol, and stigmasterol).

Time Considerations

The complete extraction process will take 1 to 2 days depending on the extraction protocol chosen. Extraction with organic solvents generally takes one-half to one full day, while inclusion of purification adds, minimally, one or two more days.

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Figure 1.

A schematic depiction of terpene metabolism emphasizing the biosynthesis of the different classes of compounds and their physical properties (volatility and polarity). DMAPP and IPP are the basic building blocks used to generate the allylic diphosphate precursors specific to each terpene class: GPP for monoterpenes; FPP for sesquiterpenes and triterpenes; and GGPP for diterpenes and tetraterpenes. Ionization of the phosphorylated precursors yields linear hydrocarbon forms, while the coupled ionization/cyclization reactions catalyzed by synthases/cyclases yield an incredibly rich array of cyclized hydrocarbons. These linear and cyclized hydrocarbon scaffolds are generally nonpolar or mono-hydroxylated, and their volatility is correlated with their molecular mass. The smaller the compound, the more volatile they will be. But all these terpene scaffolds are also subject to additional layers of modification including hydroxylations, glycosylations, acylations, and aroylations, which alter the physical size and nature of the terpene molecule, and can increase their polarity. This figure is also color coded in reference to the protocols discussed here which might be the most efficient for extraction, quantitation and structural identification of the individual

terpene molecules. Protocol 1 is designed for largely nonpolar compounds and is highlighted in green; Protocol 2 is for those molecules having a more polar nature (red); and those terpenes having the greatest polarity are probably best extracted, quantified and qualified by Protocol 3 (blue).

Figure 2. Silica chromatography columns prepared for isolating of terpenes and terpenoids on a small scale (left column, ng to μg quantities) and a large scale (right column, μg to mg quantities). Glass wool is used to plug the columns and the columns can be overlaid with a small amount of sodium sulfate (Na_2SO_4) to absorb any water contamination that can confound the chromatographic separations.

Figure 3.

Head space gas analysis for volatile terpenes. To efficiently collect the volatile terpenes emitted from the aerial portions of a plant, the pot and soil portion of the plant are tightly wrapped in cellophane and placed in a gas tight, glass chamber like a desiccation chamber. A gas exchange system is plumbed into the chamber to facilitate efficient air exchange (300 to 500 ml/min) with a tenax trap plumbed onto the output line to collect volatile compounds. The tenax trapped compounds are subsequently eluted from the resin using appropriate organic solvents and concentrated as need prior to GC analysis.