

RESEARCH ARTICLE

An Undergrad Experiment for the at-home Study of Fluorescence: Extraction of Quinine and Chlorophyll from Cinchona Tree Bark

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Abstract

Herein we describe a method for the effective extraction and qualitative detection of quinine and chlorophyll. Both compounds can be successfully extracted from Cinchona tree bark (*Cinchona pubescens* Vahl) using nothing but household materials, usually cheap and easily acquired items available in any hardware store. During a first step, solid-liquid extraction with reflux, using an organic solvent both compounds are retained. Afterward, the compounds are partitioned between water and paint solvent. The organic phase containing the chlorophyll is saved, and the aqueous layer is acidified. Finally, an ultraviolet (UV) light is used to provoke fluorescence in both compounds.

Keywords

chlorophyll, fluorescence, liquid-liquid and solid-liquid extraction, quinine, reflux

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Background

Cinchona pubescens (a Rubiaceae, usually called red Cinchona or quina) is a native tree from Central and South America (Aymard, 2019). Cinchona bark contains quinoline alkaloids (ca. 6.5 g/100 g) including quinine (representing 70 to 90 g/100 g alkaloids), quinidine (a quinine isomer, 1 g/100 g alkaloids), cinchonine, and cinchonidine (the 6-dimethoxy analogs), among others, as major components (Noriega et al., 2015; Murauer & Ganzera, 2018). Quinine demand has increased due to their extensive uses, especially as a treatment for malaria (Gachelin et al., 2017; Gupta et al., 2017; Singh & Sharma, 2019). Besides, Aryl-amino alcohols have been reported to possess

State of the literature

- Quinine has historically been extracted from Cinchona bark tree to exploit its therapeutic value.
- Overall, few educational experiments focus on solid-liquid extraction.
- Some liquid-liquid extraction experiments are available, and a small number have been recently published for developing at-home.
- Several experiments regarding fluorescence (including those based on the extraction of active compounds from plants) are available for which presence in a laboratory is required.
- Only one experiment regarding quinine fluorescence was encountered, while no laboratory experience regarding chlorophyll fluorescence from Cinchona was found.

Contribution of this paper to the literature

- Improving knowledge in natural products as quinine (an alkaloid) and chlorophyll (a photosynthetic pigment) are two compounds extracted from trees from the genus *Cinchona*.
- Using a single experiment to show the student two different extraction techniques (solid-liquid and liquid-liquid extraction).
- Teaching principles of fluorescence with two different fluorescent compounds extracted from the same source.
- The experiment will contribute to the gamut of hand-on homeschooling experiments available; few are available regarding fluorescence.
- Meanwhile the experiment could easily be transferred in full, on laboratory experience if so desired.
- This experiment is versatile enough to be used to highlight concepts from several undergrad courses, including General and Organic Chemistry.

antiviral activity (D'Alessandro et al., 2020). In fact, quinine was recently explored as a possible treatment against SARS-CoV-2 infection (Große et al., 2020). Quinine has also been proposed to inhibit muscular acetylcholine evoked disruptions (Gisselmann et al., 2018). In commercial non-alcoholic tonic beverages (e.g., tonic water and related soft drinks), quinine is added as a flavoring agent (as it imparts a bitter taste and a refreshing sensory stimulation) (Liu et al., 2020).

Historically, *C. pubescens* bark has been used to extract pharmacoactive compounds (Canales et al., 2020). Traditional alkaloids are extracted using quicklime and an aromatic solvent (Boratyński et al., 2019). As a lignocellulosic material, *C. pubescens* is also expected to contain considerable quantities of chlorophylls as described for other plant species (e.g., *Eucalyptus saligna*, Johnstone et al., 2012; Johnstone et al., 2014).

Separation techniques are still current and essential procedures to understand and practice for any undergrad chemistry student. As such, both extraction methods are commonly found as part of chemistry students' curricular activities. Several laboratory approaches have been developed to teach them in the lab, especially those such as solid-liquid (Naviglio et al., 2015; Ciaccio & Hassan, 2020) and liquid-liquid extraction (see, for example, Turner, 1994; Radford et al., 2013; Usher et al., 2015; Celius et al., 2018; Sampaio et al., 2020). As quinine and chlorophyll's fluorescence is quite evident, we expect to introduce students to fluorescence processes and, hopefully, down the road, they can relate this observed behavior with modern chemical analyses.

There are several examples of natural fluorescence (Darken, 1961; Marshall & Johnsen, 2017; Taboada et al., 2017), so some familiarization with the phenomenon is expected. Finally, some laboratory experiments have already been described that help the student relate and to understand fluorescence (Clarke & Oprysa, 2004; Rivera-Figueroa et al., 2004; MacCormac et al., 2010), including those pertaining to fluorescent compounds from trees (Muyskens, 2006; Muyskens & Vitz, 2006; Acuña, 2007; Wharton et al., 2018). Additionally, we refer the reader toward experimental discussions concerning principles in fluorescence (see for example, Tausch et al., 2017) and photosynthesis in biological systems (Atici & Atici, 2012).

Due to the current health crisis due to the COVID-19 outbreak, classes have suffered interruptions as lockdowns have been implemented worldwide. Though educational strategies have been implemented to ameliorate this issue (Mahmood, 2020), chemistry laboratories are especially challenging to migrate to an online approach. Some efforts have been made to translate laboratory some of these experiences to other media (see, for example, Buchberger et al., 2020; McKnelly et al., 2020); still, hands-on experiments as a means for the student to acquire the needed manual ability and skill necessary in chemistry is a must. Performing experiments at home using readily and commercially available substances and even commonly found in households in an alternative. In some cases, other colleagues have “converted” the kitchen into a provisional laboratory (see, for example, Shultz et al., 2020; Al-Soufi et al., 2020).

Herein, we describe an extraction method of quinine and chlorophyll from dried Cinchona tree bark. The student will explore the principles of two different partitioning methods, solid/liquid-liquid extraction. Additionally, qualitative phenomena related to fluorescence for both compounds extracted are also studied.

Experimental Procedure

Part I. Solid-liquid extraction of chlorophyll and quinine from tree bark

Measure $\frac{1}{4}$ cup (ca. 20 g) of the dried and ground bark sample (Figure 2a) into a 150 mL beaker or a similar opened-mouth container. In this first step, alkaloid recovery must be considered. Then, assuming 5 g alkaloids/100 g bark, 20 g of bark will theoretically render 1 g alkaloids. A considerable amount since quinine must be below 83 mg kg⁻¹ in drinks (according to the FDA, Feás et al., 2009) and still will provide notable fluorescent lighting. After that, $\frac{1}{2}$ cup (ca. 100 mL) of 95-96 mL/100 mL ethanol was added to the sample.

In another container, a pellet or a flake of sodium or potassium hydroxide is dissolved. Both these substances can be found in commercially available drain openers (e.g., Pure Lye!, Crystal lye, or Crystal Drãno, Figure 2b). If sodium hydroxide is used, the flake can be dissolved in the least amount of water possible and then added to the extraction solvent. If potassium hydroxide is used, it can be dissolved directly in the solvent. The base will favor the QS form of the quinine (Figure 1).

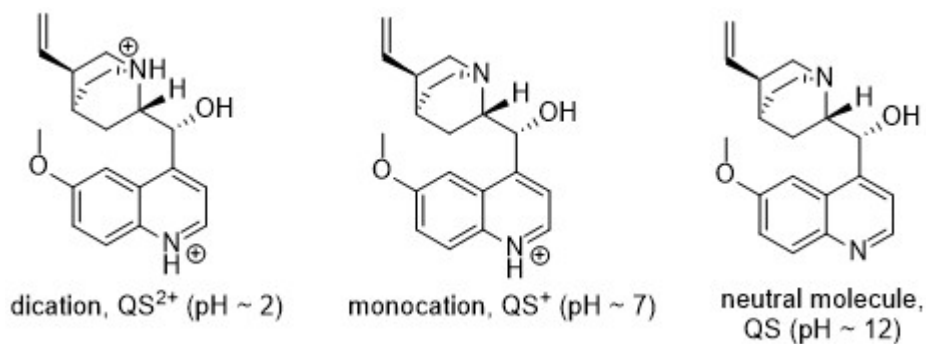


Figure 1. Structure of the quinine molecule various species at different pH values (modified from Joshi & Pant, 2015).

A simple reflux system is prepared as follows: a glass containing ice and water is placed on top of the beaker, has more ice available to keep the temperature shock constant throughout the process, and an aluminum foil is placed at the junction between the beaker and the glass with ice.

The reflux system is placed in a water bath (this setup can very well be a kitchen pan with a small portion of water set in the stove, Figure 2d), and it is heated slowly until the mixture starts to boil and constant condensation of the solvent can be perceived at the surface of the aluminum foil. From that point on, the same temperature is kept for an additional 30 min (a temperature that should be near the ethanol boiling point of ca. 78 °C or 173 °F, if available, this can be verified using a thermometer, Figure 2c).

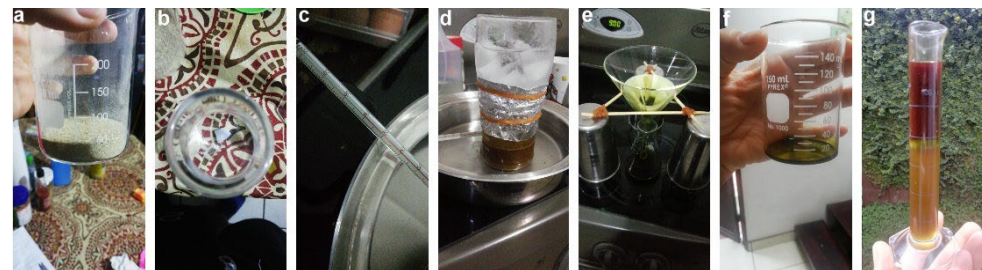


Figure 2. Extraction steps during quinine and chlorophyll from Cinchona

Solid-liquid extraction is a common laboratory technique and noteworthy. It has been used in teaching laboratories to extract polyflavonoids from lignocellulosic biomass (i.e., tree bark; Parajó et al., 2008).

In this case, ethanol was selected as an adequate solvent as both quinine and chlorophylls are readily soluble. However, it should be noted that a variety of components have been identified from cinchona bark [e.g., dimers and trimers proanthocyanidols tannins, catechin tannins, flavonoids, catechin, kaempferol, apigenin and quercetin, glycosides, organic acids (quinotanic acid, cinconic red), monoglycosides such as quinic acid (3β -hydroxyurea-hydroxybenzoic acid) and terpene compounds (Noriega et al., 2015)] that can be coextracted. However, it has been reported that alkaline ethanol media can favor the precipitation of some tannins (Gong et al., 2014).

The mixture is then removed from the water bath, it is allowed to cool to room temperature, and the glass with ice is retired. The mixture is sifted through a coffee filter, and the filtrate is saved (Figure 2e). The filtrate container is then set in the stove and placed under mild heating and gentle stirring (avoiding any splashing), and kept in these conditions until dry (Figure 2f).

Part II. Chlorophyll and quinine separation by liquid-liquid extraction.

The resulting viscous extract from *part I* was dissolved completely using 1 tablespoon (ca. 10 mL) of preheated water at 60 °C (140 °F), followed by another 10 mL of paint thinner (turpentine or mineral/white spirits will do fine). The mixture is transferred to a 25 mL graduated cylinder with a stopper (a tall glass container with a cap will do) and shook. After a few resting moments, two distinguishable phases should be apparent (the denser phase will be the aqueous layer) (Figure 2g). A small fraction of ethanol is added to the mixture; this will rupture any potential emulsions and increase quinine solubility in water. Fluorescence of quinine has been reported not to be quenched by ethanol (Linnewiel & Visser, 1962). A common practice in troubleshooting emulsions formed during extraction is the addition of NaCl to increase the aqueous layer polarity. However, Cl⁻ ion has already demonstrated a quencher quinine fluorescence (Gutow, 2005). Using a dropper or an injectable syringe, the organic phase was separated and transferred to another container (Figure 3a-c).

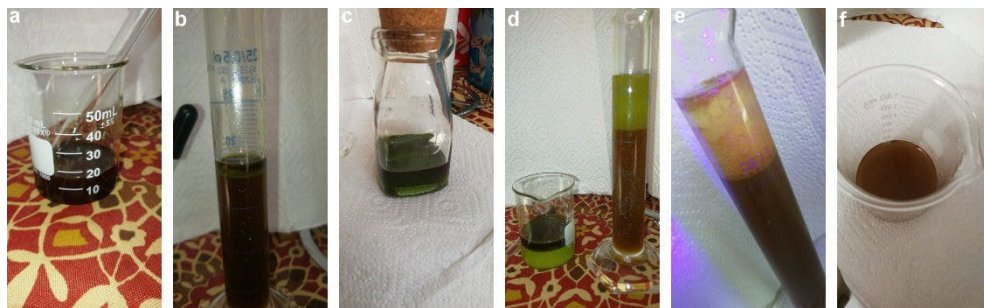


Figure 3. Liquid-liquid extraction of quinine and chlorophyll and recovery steps

At this point, the student should keep in mind several keynotes. When a soluble material is subjected to a system with two immiscible solvents, A and B, the substance will distribute itself among them (Jones & Champion, 1978). After extraction, the system will return to equilibrium, which means $\text{Solute}_A \rightleftharpoons \text{Solute}_B$. For such a system, the equilibrium constant can be written as K_D (partition coefficient) = $[\text{Solute}]_B / [\text{Solute}]_A$ (Jones & Champion, 1978).

Then, the rate of mass exchange between the liquid phases can be expressed by the fundamental equation of the mass exchange: $dM = K \Delta dF d\tau$, where dM is the amount of substance passing from one phase to the other, dF is the surface of contact of the phases, $d\tau$ is the time of contact, Δ is the driving force of the diffusion process (ΔX or ΔY), and K is the mass-exchange coefficient (Plyashkevich & Zamyshlyeva, 1970).

In this case, two segregations co-occur; chlorophyll will mostly part toward the paint thinner and quinine to the aqueous layer. Then for the systems in this experiment, the equilibrium is chlorophyll (in water) \rightleftharpoons chlorophyll (in thinner), and quinine (in water) \rightleftharpoons quinine (in thinner). And their respective equilibrium constants $K_D = [\text{Chlorophyll}]_{\text{thinner}} / [\text{Chlorophyll}]_{\text{water}}$ and $K_D = [\text{Quinine}]_{\text{thinner}} / [\text{Quinine}]_{\text{water}}$.

Chlorophyll molecules are conjugated tetrapyrroles joint with two cyclopentanone rings, linked together by methylene bridges, with magnesium as a central atom (the hydrophile). These molecules contain a propionic acid chain esterified with the phytol, a diterpene alcohol, as well (the hydrophobe, Schoefs, 2002). For example, the molecular formula for chlorophyll a is $\text{C}_{55}\text{H}_{72}\text{MgN}_4\text{O}_5$, a compound that reflects green light (500-570 nm wavelength), imparting plants with their characteristic color. Hence, chlorophyll is water-insoluble, but is readily dissolved in organic solvents such as ethanol, acetone, ether, and chloroform (Schryver, 1909).

Meanwhile, quinine (with a formula $\text{C}_{20}\text{H}_{24}\text{N}_2\text{O}_2$) is a moderately lipophilic compound and has a log p value of 3.4 (K_{ow} , octanol–water partition coefficient) and has an aqueous solubility of 0.5 mg mL⁻¹ at 15 °C (Strauch et al., 2011). On another hand, quinine sulfate is sparingly soluble in water (i.e., one part in 500 parts of water at 100 °C) (Strauch et al., 2011; Kacprzak, 2013).

An additional 10 mL of diluent was added to the remaining aqueous phase; the cylinder covered, shook, and settled as described above (Figure 3e-f). This procedure is repeated yet another time, for a total of 3 extractions. The organic extract was placed under UV light, and the solution's reddish hue characteristic of chlorophyll was observed.

Part III. Generation of quinine sulfate in the aqueous phase.

Once the aqueous phase has been separated from the organic phase (Figure 4a), add a drop of sulfuric acid (car or motorcycle battery acid or drain opener/acid drain cleaner) to the aqueous phase. As the acid is poured with each drop, momentary cloudiness is to be expected, and turbidity will somewhat decrease. Battery acid will improve this effect, as lead ions in the solution will produce lead (II) tannates (Wall et al., 1969). As pH decreases by the addition of sulfuric acid,

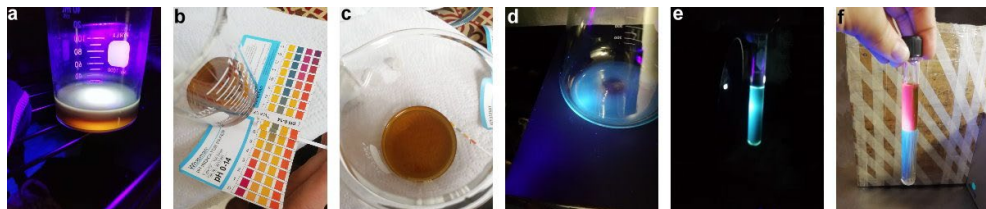


Figure 4. Neutralization and light absorbance steps for quinine and chlorophyll

alkaloid sulfates, and bisulfates are produced. As a diprotic weak base, quinine has a pK_{a1} and pK_{a2} of 4.34 and 8.43 at 20°C, respectively (Schulman et al., 1974; Strauch et al., 2011) appearing as a free base at high pHs and as one of its ionized forms (QS^+ or QS^{2+}) at lower pHs (Figure 1, Strauch et al., 2011).

The mixture is continuously stirred as drops of the acid are added. With each drop, pH should be measured using universal indicator paper (sold online by several e-commerce platforms). If paper not available, the water-soluble pigment present in red cabbage (anthocyanin) can be used as an indicator. Briefly, the cabbage is roughly chopped, boiled for some minutes, and strained (Fortman & Stubbs, 1992).

Repeat until you get a pH close to 4 (Figure 4b). In aqueous media, at a pH 4, the species QS^{2+} will predominate (Figure 1), a molecule which will render a stronger fluorescence by exhibiting higher values of extinction coefficient (ϵ) and a fluorescence quantum yields (Φ) of 4.33 and $5.40 \times 10^3 \text{ cm}^{-1} \text{ mol/L}^{-1}$ and 0.55, respectively (Schulman et al., 1974). If using red cabbage, a lilac color tone will indicate a pH near 4.

A solution with slight turbidity is still expected (Figure 4c). When the aqueous extract is placed under ultraviolet light, the solution should emit a faint blue light, characteristic of quinine (Figure 4d-e). A black-light or UV lightbulb (available in most hardware stores) will emanate sufficient radiation to match the absorption of quinine [excitation wavelength (λ_{ex} = 317/347 nm for QS^{2+} at pH 2 in aqueous media)] and achieve emission at its correspondent lower energy, longer wavelength (i.e., λ_{em} = 448 nm) (Schulman et al., 1974), which will render the characteristic bluish hue.

Suppose both phases are placed on the same container and let to rest, once under ultraviolet. In that case, the light, blue, and the red hue characteristic of both quinine and chlorophylls can be appreciated (Figure 4f). As part of the plants' photosystem I/II (protein complexes in the light-dependent reactions of oxygenic photosynthesis), chlorophyll *a* (e.g. λ_{ex} = 429 nm; λ_{em} = 661 nm in ethyl ether) and *b* (λ_{ex} = 453 nm; λ_{em} = 643 nm) are the most responsible for the fluorescence emission (Bauer et al., 1972; Pedrós et al., 2008; Kalaji et al., 2014; Kalaji et al., 2017; Taniguchi & Lindsey, 2021).

Hazards

Sodium hydroxide, potassium hydroxide, and sulfuric acid are caustic or corrosive. These reagents can cause severe skin burns and eye damage and are harmful to the environment, especially aquatic life; their residues should not be discarded until neutralized. You should avoid breathing dust or vapors. Preferably, perform this experiment under the kitchen extractor or in a well ventilated area. If the skin is exposed, it should be washed thoroughly. If contact with eyes, rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If inhaled, the person should be removed to fresh air and keep comfortable for breathing. The student should wear eye protection, face protection, protective clothing, and gloves during the experiment's duration.

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