Research Proposal

Mold and Fragrance: A Determination of the Fungistatic and Fungicidal Effect of Linalool on Textiles and an Evaluation of Any Deleterious Effects

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Introduction

Linalool (3,7-dimethyl-1,6-octadien-3-ol) is a terpene alcohol frequently found in plants and essential oils such as lavender, tea tree oil, and mints (fig. 1). It is one of the most commons fragrances in a variety of household items including: cleaning products, beauty supplies, insecticides, and carbonated beverages (Rastogi et al. 1998; Rastogi et al. 2002). The compound has been known to have fungistatic qualities, which makes it a tempting consideration for stewards of cultural heritage that have to combat high risks of mold in their collections. Some research has been completed on the use of linalool and essential oils for library collections that have found it to be fungistatic, but poses serious risks to the degradation of photographs, leather, and paper (Rakotonirainy and Lavédrine 2005; Rakotonirainy et al. 2007; Karbowska-Berent et al. 2018). The chemical is believed to readily oxidize and become a hydroperoxide, which is unsafe for artwork and is also a skin allergen (Sköld et al. 2002; Rakotonirainy et al. 2007). There are some recent publications within the field of cultural heritage continuing to evaluate the merits of fungistatic essential oils that contain this compound and even discuss the implementation of them in the treatments of objects (Borrego et al. 2012; Stupar et al. 2014; Noshyutta et al. 2016). It is clear that the effects of the volatile components found in essential oils on cultural heritage objects needs to be further studied, whether they are being used intentionally for their fungicidal properties, or if objects are peripherally exposed to them through the presence of fragrant products.

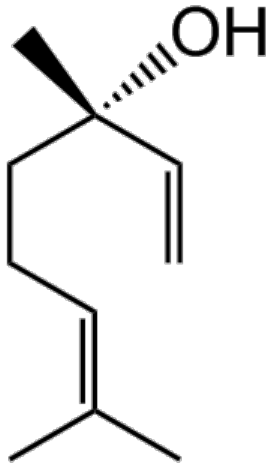

Fig. 1. Chemical structure of linalool

This study is designed to understand the fungistatic and fungicidal qualities of linalool on cotton textile collections, and to determine if linalool significantly contributes to the degradation of textile materials. Naturally aged protein-based and cellulosic-based textiles will be used for the study to see if any unique degradation to a specific category of organic polymer can be identified. The interest in doing this is based on the more negative results of exposing linalool to

protein-based materials such as gelatin photographs and leather as opposed to cellulosic materials in an earlier study (Rakotonirainy and Lavédrine 2005; Rakotonirainy et al. 2007). Since there is a foundation of this research with library-based materials, textiles were chosen as an unstudied category of materials present within that type of collection, but also common in a vast array of collection types. Also, due to the hygroscopicity and organic nature of many textile fibers, the risk for mold growth is high. It is the hope that research on this topic in a new category of material will serve as a means to present the important earlier research that has been completed on this topic to new audiences.

Literature Review – General Background

One of the biggest dangers to cultural heritage is the risk of mold growth. This is especially true when there are difficulties with controlling the temperature and relative humidity of the space, and when dust is permitted to accumulate. The conidium, (asexual chlamydospores), hyphal fragments, and sexual spores of fungal species are everywhere (Florian 2002, 2). These microscopic non-motile fungal spores have the ability to remain dormant for up to 20 years, awaiting the proper environment to activate and grow (Florian 2002, 30). When an outbreak takes place, it can be disastrous for the objects and can pose a health risk for those involved in responding to the disaster (Wiszniewska et al. 2018).

Airflow and controlled relative humidity and temperature is the current recommended method to reduce the risk of mold outbreaks (Ferreira et al. 2018). However, using an HVAC is not environmentally sustainable and may not be possible for collections housed in historic buildings, within individual's homes, or in areas of the world where controlling the climate of a store space is difficult. In these instances there is a real need to come up with alternative ways to prevent outbreaks, whether it is through novel ways to passively control the relative humidity, or through the introduction of fungistatic materials that are safe for the objects, the environment, and humans.

Many have looked into various fumigation techniques to act as fungicides. While effective, ethylene oxide, thymol, and formaldehyde have been found to be carcinogenic and irritating to human skin when oxidized (Rakotonirainy et al. 2007; Sequeira, Cabrita, and Macedo 2012). If one has a mold outbreak, the Canadian Conservation Institute recommends to isolate the objects and either freeze or dry them (or a combination of both) and clean any remaining conidia and hyphal fragments to prevent regrowth. Another common technique for disinfection is to use a 70% solution of ethanol in water, however, this technique is only viable for objects that can withstand those solvents (Florian 2002).

Within the field of cultural heritage preservation, there is continued research to find fungistatic remedies for when humidity control is not viable. The potential use of essential oils for fungicidal and fungistatic qualities has been researched in many fields, including: medicine, cosmetics chemistry, and food science (Pattnaik, Kole, and Vemulpad 1997; Pattnaik et al. 1997; Guynot et al. 2003; Rasooli and Owlia 2005). There are entire scientific journals devoted to research in essential oils such as the *Journal of Essential Oil Research*. In recent years, with an increased interest in the use of natural products, there appears to be an epidemic in home recipes for essential oil cleaning solutions.

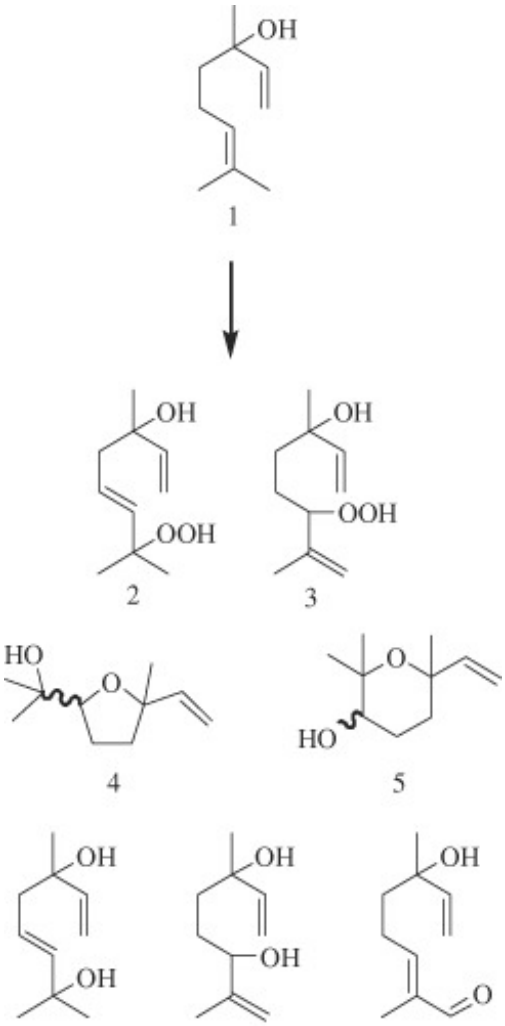

Fig. 2. Identified oxidation products formed by autoxidation of linalool (7, 8). 1, Linalool (3,7-dimethyl-1,6-octadien-3-ol); 2, 7-hydroperoxy-3,7-dimethylocta-1,5-diene-3-ol (major hydroperoxide); 3, 6-hydroperoxy-3,7-dimethylocta-1,7-diene-3-ol (minor hydroperoxide); 4, 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-ol; 5, 2,2,6-trimethyl-6-vinyltetrahydro-2H-pyran-3- ol; 6, 2,6-dimethylocta-3,7-diene-2,6-diol; 7, 2,6-dimethylocta-1, 7-diene-3,6-diol; 8, 6-hydroxy-2,6-dimethylocta-2,7-dienal. (Christensson et al. 2001, 248)

Essential oils are secondary metabolites formed by plants and in nature and they play an important role in protecting organisms from insects, bacteria, and fungi (Bakkali et al. 2008, 447). They are derived from plants and are mainly composed of terpenoid compounds (Sequeira et al. 2002, 75). These oils can vary in composition depending on the part of the plant it was extracted from, the mode of extraction, where the plant was grown, the soil it grew in, and the season it was harvested (Bakkali et al. 2008, 447). Because of the great variance in chemical composition of essential oils, their high cost, and the high industry demand for the fragrance, many have looked into the fungicidal and fungistatic qualities of the easily synthesized terpenoids, such as linalool, within plant extracts (Pattnaik, Kole, and Vemulpad 1997).

Linalool is a colorless monoterpene volatile compound. It is an unsaturated hydrocarbon, which makes it vulnerable to oxidation when exposed to the air (Sköld et al. 2002, 1697). In the 2002 study by Sköld et al., the oxidation products were isolated using flash chromatography and preparative HPLC, and identified with nuclear magnetic resonance (NMR) and gas chromatography-mass spectroscopy (GC-MS) by comparing the results to synthesized reference compounds. The study identified two hydroperoxides and several different secondary oxidation products with allergen features (Fig. 2) (Sköld et al. 2002, 1697). In the study, "The Effect of Linalool Vapour on Silver-Gelatine Photographs and Bookbinding Leathers," the creation of hydroperoxides and skin allergens were discussed and were among the reasons why it was not advised to use the chemical in cultural heritage collections (Rakotonirainy et al. 2007).

Literature Review- the Use of Essential Oils for Cultural Heritage

Thymol vapors, a monoterpenoid phenol found in some essential oils, has been used by conservators for many years (Sequeira, Cabrita, and Macedo 2014, 76). However, the effectiveness of the compound as a fungicide has been questioned (Craig 1986; Valentin 1986). In the same year, a study was published that showed the yellowing of paper and health hazards associated with the chemical (Daniels and Boyd 1986). The use of Thymol fell out of favor when a follow-up study on the deleterious effect of thymol on paper and its health hazards was completed in 1997 (Isbell 1997).

The 2003 study, "Preliminary Research on the Use of the Essential Oil of 'Melaleuca alternifolia' (tea tree oil) in Museum Conservation," is an early reference on the implantation of essential oil use within the cultural heritage setting (Gatenby and Townley 2003). In this study, the researchers studied the effect of the commercial gas, Bactigas® (composed of 0.3% tea tree oil, 97% liquid carbon dioxide, and 2.7% ethanol), in an emptied store room that had exhibited a mold outbreak.

One of the most referenced articles pertaining to the use of essential oils in cultural heritage collections is the 2005 study, "Screening for Antifungal Activity of Essential Oils and Related Compounds to Control the Biocontamination in libraries and Archives Storage Areas" (Rakotonirainy and Lavédrine 2005). The study looked at nine essential oils and five related aromatic compounds and tested their effectiveness on nine fungal species using a modified microatmosphere method (Maruzzella and Sicurella, 1960). They then looked into the application of linalool as a fungicide for inoculated papers inserted into a book and determined that there were promising results for the use of linalool as a fungistatic measure. The authors researched adverse effects on the papers exposed to the linalool and while the pH did lower, they determined these effects to be minor (Rakotonirainy and Lavédrine 2005, 145).

The authors of the previous study followed-up with their research to see if the linalool would have any deleterious effects on other materials found within a library collection (Rakotonirainy et al. 2007). They did this by exposing silver gelatin photographs and bookbinding leather to linalool (both protein-based) and artificially aging some of the samples to determine if there are long term degradation processes of concern. The results were unacceptable, and further research done into linalool in the dermatological field, yielded results of the autoxidation of the compound and the formation of hydroperoxides and skin allergens (Sköld et al. 2002, 1697; Rakotonirainy et al. 2007, 96).

In 2012, researchers in Cuba and Argentina looked at other essential oils for their biocidal effect on both fungus and bacteria in documentary heritage (Borrego et al. 2012). The study yielded alternative essential oils to those tested in the 2005 Rakotonirainy and Lavédrine research, but did not test any of the oils on cultural heritage material. Similarly, another study in 2014, looked into the use of other essential oils and benzalkonium chloride as possible uses for cultural heritage (Stupar et al. 2014).

In 2016, a paper was published about the actual implementation of tea tree oil in the treatment of a coptic manuscript (Noshyutta, Osman, and Mansour 2016). The authors evaluated the fungicidal and fungistatic effect of different essential oils and then performed tests on the effect of tea tree oil on a comparable paper to the paper within a 19th century Coptic manuscript with significant mold damage and contamination. Feeling confident that the tea tree oil would have minimal effect on the paper, they described a treatment that involved the blending of tea tree oil with paper pulp to create paper fills for some of the losses in the folios of the book. The tea tree oil that was used did contain linalool, but the authors did not reference the 2007 research completed by Rakotonirainy et al. (Noshyutta, Osman, and Mansour 2016).

Most recently, an early 2018 study was published in the journal, *International Biodeterioration & Biodegradation,* entitled, "The Initial Disinfection of Paper-Based Historic Items –

Observations on Some Simple Suggested Methods" (Karbowska-Berent et al. 2018). The article was looking into several fungicidal treatments and evaluated their contribution to the deterioration of the paper-based materials. The researchers found the results of the use of tea tree oil to be unacceptable and proposed the use of ethanol and water vapor chambers as a possible alternative worthwhile of further research (Karbowska-Berent et al. 2018).

Hypotheses

This study is composed of two portions. The first component is to determine the fungistatic and fungicidal effect of linalool on a cotton textile inoculated with a known species of fungi within the same genus as those shown to be sensitive to linalool in 2005 study by Rakotonirainy and Lavédrine. Due to these positive results of linalool on certain genera, similar results are expected to be seen on a cotton textile. A variation in results could suggest synergistic effects with the textile. The in vitro method with serial dilution can better elucidate the effect of the linalool over the course of time.

The second portion of the study is developed to determine if there are any deleterious effects of exposing cellulose and protein-based textiles to linalool. It is expected that the linalool will autoxidize and have an oxidizing effect on the materials. The hydroxyl groups present in the polymers may also form esters with the alcohol groups in the linalool products. In turn, this should cause the materials to become more hydrophobic. It is anticipated that yellowing will occur since it was evidenced in earlier studies (Rakotonirainy and Lavédrine 2005; Rakotonirainy et al. 2007). Also based on previous research, the pH of the samples is expected to decrease (Rakotonirainy and Lavédrine 2005; Rakotonirainy et al. 2007). Tensile strength is expected to decrease with aging due to polymer degradation and chain scission within the cellulose of the cotton due to an decrease in pH.

Materials

For this experiment, the sample materials to be tested are antique textiles estimated to be from the early part of the 20th century. One textile is a plain weave white cotton (weft: single ply z twist, 88 yarns per inch, warp: singly ply z twist, 96 yarns per inch) and the other is a seafoam green silk fabric (weft: fiber bundle with 74 yarns per inch, warp: fiber bundle with 186 yarns per inch). The textiles were chosen because they represent proteinaceous and cellulosic-based textiles. Both textiles were examined with x-ray fluorescence spectroscopy (Bruker Tracer 3) and the calcium, iron, copper, and zinc that were identified likely originate from surface contamination (fig. 3) (Grayburn, pers. comm.).

A culture will be completed from sampling the cotton textile species will be isolated. It is hoped that one of the following species will be identified: *A. fumigatus*, *A. repens (teleomorph Eurotium repens)*, *Cladosporium herbarum*, *Penicillium frequentans*, *Trichoderma viride, Chaetomium globosum*, and *Stachybotrys atra,* since linalool was shown to have a fungistatic effect on them (Rakotonirainy and Lavédrine 2005).

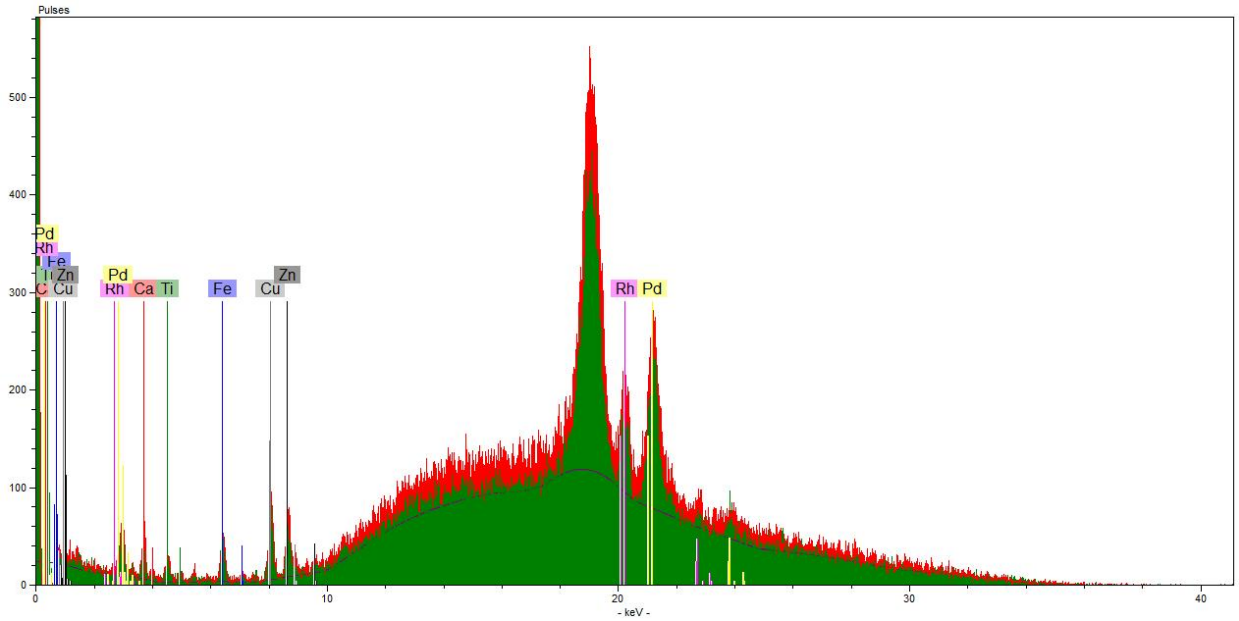

Fig. 3. XRF data from cotton (red) and silk (green) samples

As a backup, a freeze-dried isolated culture of *Cladosporium herbarum* can be purchased. This species was chosen because it is exceedingly common in textiles and is prevalent in cultural heritage collections. It is a particular risk for cellulose degradation with its production of the cellulase enzyme (Raschle 1989, 239). Linalool has also been shown to be effective against this fungal strain on paper materials (Rakotonirainy and Lavédrine 2005, 145; Michaelson et al. 2013).

Methodology- Fungicidal and Fungistatic Effect of Linalool on Textiles

The methodologies for the fungicidal/fungistatic treatments were developed to replicate portions of the 2005 Rakotonirainy and Lavédrine study, *Screening for antifungal activity of essential oils and related compounds to control the biocontamination in libraries and storage areas.* The even dispersal of fungal spores on the samples will be achieved by a suspension with a known concentration of cells (Michaelson et al. 2013). The white cotton sample was chosen because of the greater risk of biodeterioration from cellulolytic enzymes produced by specific fungal species. Silk protein fibers consist of fibroin and sericin that make them less of a risk to proteolytic enzymes produced by fungal species (Gutarowska et al. 2017, 2389). The high humidity was chosen due to the need for extreme moisture for the development of mold in textiles (Gutarowska et al. 2017).

- ***Preliminary Research***
    1. Create sterile solution of potato glucose agar and pour into sterile petri dishes and allow to cool.
    2. Swab cotton textile sample with sterile swab and streak over potato glucose agar plates. Store in a dark area in room temperature and allow to grow.
    3. Isolate species of mold by transferring colonies to separate plates.
    4. Identify species through known characteristics and UV epi fluorescence microscopy.
    5. Prepare cotton textile samples

i. Cut each sample to 24 mm x 12 mm
ii. Sew basting thread through groups of fabric samples so they can be hung with pressure from the lid of the double-glass container and with enough length in between the samples to ultimately separate and use remaining thread to hang with thread in individual test tubes for exposure to linalool
iii. Sterilize thread and cotton samples in autoclave
iv. Store in sterile air-permeable envelopes

- ***Determination of Dilution Needed for Proper CFU Count***
    1. Grow fungal species (*Cladosporium herbarum or identified species from textile*) on potato glucose agar (2%) to obtain cultures with mature fruiting bodies or reproductive structures.
    2. Serial dilution of mold species to determine proper dilution for 20-40 CFU (colony forming units) using a spectrophotometer to measure a corresponding optical density

Control 85%RH T_0	Control 85%RH (no linalool) T_1	Control 85%RH (no linalool) T_2	Control 85%RH (no linalool) T_3	Control 85%RH (no linalool) T_4
1	1	1	1	1
2	2	2	2	2
3	3	3	3	3
	Linalool 85% T_1	Linalool 85% T_2	Linalool 85% T_3	Linalool 85% T_4
	1	1	1	1
	2	2	2	2
	3	3	3	3

- ***Fungicidal/Fungistatic Treatment with Linalool***
    1. Harvest conidia with a sterile loop from growing culture of mold species and dilute in sterile water with Tween 80 (0.01%, Sigma, Italia) to obtain solutions with a standard concentration of about 5000 spores/ml. Perform further dilution with a Czapek broth to obtain 50 spores μl^{-1} (Michaelson et al. 2013).
    2. Dip vapor sterilized textile into sterile water and inoculate each sample with a measured volume of broth
    3. Place samples in sterile double-bottom glass containers and maintain 97% RH with saturated salts (potassium chromate or potassium sulfate) and keep the temperature for 30 °C until there is an even covering of mold on the samples (Greenspan 1977).
    4. Separate samples by cutting thread.
    5. Dry samples and/or store them in sterile air-permeable envelopes
    6. Perform serial dilution on the control samples to determine initial amount of cells
    7. Place sterilized saturated salt (created with water and potassium chromate or potassium sulfate – 95%RH) in small sterile glass vial the within larger sterile test tube. Add known concentration of linalool to the bottom of half the glass vials, drape moldy samples over the lid and quickly enclose with silicone stopper, wrap with Teflon tape, and Parafilm to prevent loss of linalool vapors

(fig. 4). Do this for all the samples one at a time with no linalool added to the controls.

8. Place vials in oven (30°C)
9. In 7 days remove T_1 samples and perform serial dilution to determine the cell count. Complete this step every 7 days until you reach T_4 samples.
10. Analyze data by examining and charting increases in (no fungicidal effect), decreases in (fungicidal effect), and stable (fungistatic effect) numbers of cells in both the controls and linalool samples.

Methodology- Determining any Deleterious Effects of Linalool on Textiles

The second portion of the study was developed from the 2016 study by Karbowska-Berent et al., the 2007 Rakotonirainy et al. study, and the 2016 study by Noshyutta, Osman, and Mansour. The exposure to linalool portion of the experiment varies from others in that the linalool is directly applied to the glass vial instead of placing it on a piece of paper. This is to mimic the possible residue of cleaning supplies on surfaces within the proximity of objects and to avoid any interaction between the compound and the paper. All of these studies were measuring the degradation of paper, photographs, and leather. Research was completed to look into ways to evaluate the deterioration of textiles (Abdel-Kreem and El-Nagar 2005, 4; Karbowska-Berent et al. 2016; Bílková 2012; Olaru et al. 2013; Noshyutta, Osman, and Mansour 2016). The experiments will take place in the Dark Room at the Winterthur Museum Garden & Library research building that maintains a relatively stable temperature of 21°C. The two humidity levels are controlled with saturated salts, potassium bromide (81.67% RH), and magnesium nitrate (54.38% RH) (Greenspan 1977). The different humidity levels were chosen to represent the multiple situations where linalool may get exposed to a collection as a fungistat/fungicide in uncontrolled environments, and when collections in controlled environments are exposed to fragrant products containing linalool.

- ***Exposing the Textiles to Linalool***
    1. Prepare test samples by cutting to 12 mm x 24 mm and sew basting stich through the top of each sample. Then sew one end to a paper label with the designated sample number.
    2. Create saturated salt solution to be pipetted into each of the small vials that will be placed in each test tube.
    3. Suspend half of fabric samples with string above drop of known concentration of linalool (42.5 microliters to 100 ml ratio with linalool to the volume of the test tube respectively) and the other half within vial with no linalool.

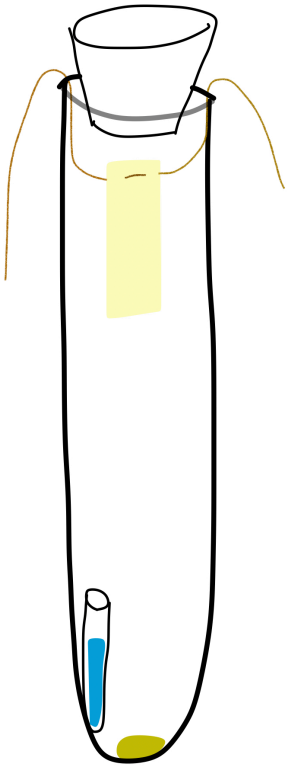

Fig. 4. Illustration of the test tube setup for both parts of the study, A drop of linalool and a small vial of saturated salts are at the bottom. The textile is suspended by a thread with a basting stitch that extends over the edges and locked in place with the silicone stopper. For the second part of the study, labels with the sample name should be placed on the side of one of the strings.

4. Once all of the components are placed inside the test tube (fig. 4), place all test tubes in room with stable temperature for 21 days
5. Remove lid from test tubes to allow evaporation of remaining volatile organic compounds for one day.
6. Remove samples from test tubes and place half in aging oven 80°C 65% RH for 21 days (Karbowska-Berent et al. 2016).

Cellulose (Linalool)					Cellulose (Control)			
Linalool (low RH) Unaged	Linalool (high RH) Unaged	Linalool (low RH) Aged	Linalool (high RH) Aged		Control (low RH) Unaged	Control (high RH) Unaged	Control (low RH) Aged	Control (high RH) Aged
1CL	**1CL**	**1CL**	**1CL**		**1CC**	**4CC**	**7CC**	**10CC**
2CL	**2CL**	**2CL**	**2CL**		**2CC**	**5CC**	**8CC**	**11CC**
3CL	**3CL**	**3CL**	**3CL**		**3CC**	**6CC**	**9CC**	**12CC**
Protein (Linalool)					Protein (Control)			
Linalool (low RH) Unaged	Linalool (high RH) Unaged	Linalool (low RH) Aged	Linalool (high RH) Aged		Control (low RH) Unaged	Control (high RH) Unaged	Control (low RH) Aged	Control (high RH) Aged
1PL	**4PL**	**7PL**	**10PL**		**1PC**	**4PC**	**7PC**	**10PC**
2PL	**5PL**	**8PL**	**11PL**		**2PC**	**5PC**	**8PC**	**11PC**
3PL	**6PL**	**9PL**	**12PL**		**3PC**	**6PC**	**9PC**	**12PC**

- ***Methodology- Measuring the Damaging Effect of Linalool***
    1. Stereomicroscope cross polarized microscope to identify fibers and determine if there are any visible differences (all samples).
    2. SEM to determine changes in surface morphology of threads (select samples) (Abdel-Kreem and El-Nagar 2005, 4; Noshyutta, Osman, and Mansour 2016)
    3. Standard Practice for Calculating Yellowness and Whiteness Indices from Instrumentally Measured Color Coordinates ASTM E313 – 15 (all samples) (Karbowska-Berent et al. 2016; Noshyutta, Osman, and Mansour 2016)
    4. Water angle test to see if polarity of textile has changed possibly due to the creation of esters (all samples) (Ni and Zhang 2017)
    5. FTIR-ATR to determine differences and if OH level has gone down (select samples) (Abdel-Kreem and El-Nagar 2005; Bílková 2012; Noshyutta, Osman, and Mansour 2016).
    6. Tensile Strength (DMA) ASTM D1445 (all samples) (Abdel-Kreem and El-Nagar 2005; Bílková 2012; Olaru et al. 2013; Noshyutta, Osman, and Mansour 2016)
    7. Py-GCMS used as evolved gas analysis to evaluate side chains on the polymers caused by interactions with the volatile compounds.
    8. pH of cold extract to get accurate reading of full depth of the material (select samples) (Hughes 2017)

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