Extraction and Analysis of Eugenol from Cloves

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Abstract: This paper describes a laboratory procedure for the extraction and identification of eugenol from cloves (*Syzygium aromaticum L.*). The purpose is to instruct students in the isolation and identification of a medicinally relevant compound from a plant via simple solvent extraction. The analytical tools employed to identify eugenol and other naturally occurring chemical components in the cloves extract include color tests, thin layer chromatography (TLC), infrared spectrophotometry (IR), and gas chromatography coupled to mass spectrometry (GC-MS). In addition to eugenol, the cloves extract contains acetyl eugenol which can be distinguished from eugenol by several test methods. Triethylamine is used as a reagent at two different stages of testing. In the TLC analysis, triethylamine is used to basify the mobile phase which facilitates the separation of eugenol from acetyl eugenol. The student will learn the concept of method development by optimizing separation parameters in the TLC experiments. Additionally, the student will learn concepts such as differential migration, interparticle forces, pK_a, and surface basicity. In the GC-MS analysis, triethylamine is used with acetic anhydride to promote the quantitative conversion of eugenol into acetyl eugenol by removing acetic acid from the product side of the chemical equation. This provides an opportunity for instruction of concepts such as drug derivatization, chemical equilibria, and Le Chatelier's principle. Several other terpenes common to plant extracts can also be identified by GC-MS. The laboratory-based pedagogy is designed to be progressively complex to accommodate various educational levels from high school to post-secondary.

Keywords: cloves, drug chemistry, eugenol, medicinal plants, GC-MS

Introduction

The medicinal benefits of cloves have been recognized for thousands of years (1,2). The main constituent, eugenol, has been used as a topical anesthetic, adjunct component in dental applications, and recently studied for numerous other medicinal qualities (3-10). Traditionally, eugenol is isolated in the essential oil of cloves which is produced via steam distillation of the dried flowering bud of the tree *Syzygium aromaticum L.*, which is in the evergreen family. The analytical profile of the essential oil reveals a secondary constituent of similar chemical structure with purported medical benefits, acetyl eugenol, also known as eugenol acetate (11-13), along with numerous other minor components such as terpenes, which are common to many essential oils derived from plants (14, see **Figure 1**).

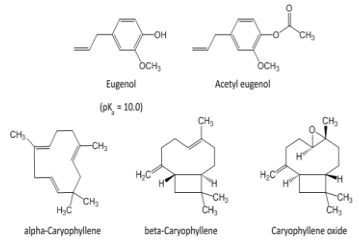

FIGURE 1 *Chemical structures for eugenol, acetyl eugenol, alpha-caryophyllene, beta-caryophyllene, and caryophyllene oxide*

The steam distillation method for producing the essential oil from cloves prescribed in many organic chemistry laboratory textbooks is fairly involved and requires specialized glassware and heating devices (15, 16). Several recent and novel alternatives to steam distillation include use of a microwave oven (17) and an espresso machine (18). In the same spirit of efficiency, this new procedure employs a simple solvent extraction to isolate eugenol followed by a multi-modal chemical analysis. The presence of acetyl eugenol in the extract gives the investigator an opportunity to separate and

identify the two main components by use of TLC, FT-IR, and GC-MS. This allows the student to learn chemical concepts such as acid/base chemistry, pK_a, derivatization, and Le Chatelier's principle of chemical equilibrium.

A unique feature of this experiment is the dual use of triethylamine (TEA). In the TLC analysis, TEA is used to basify the mobile phase and thus affect the separation of eugenol from acetyl eugenol. In the GC-MS analysis, TEA is used to promote the quantitative conversion of eugenol into acetyl eugenol with acetic anhydride by removing acetic acid from the product side of the chemical equation (**FIGURE 2-3**). In the absence of TEA, the conversion of eugenol to acetyl eugenol is incomplete. The extent of conversion is governed by Le Chatelier's principle of chemical equilibrium.

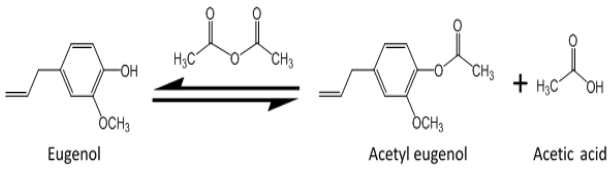

FIGURE 2 *Partial conversion of eugenol to acetyl eugenol via derivatization with acetic anhydride (an equilibrium process)*

However, upon addition of TEA, the conversion from eugenol to acetyl eugenol proceeds rapidly and quantitatively. This is due to the acid-base reaction of the by-product of esterification, acetic acid, with TEA.

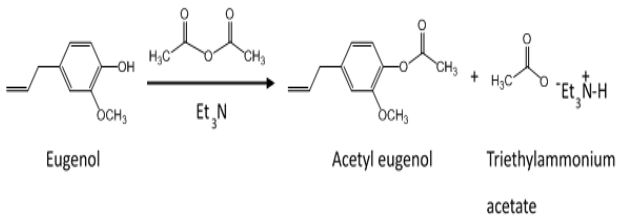

FIGURE 3 *Quantitative conversion of eugenol to acetyl eugenol via derivatization with acetic anhydride and triethylamine*

Methods

Materials: Cloves were purchased from a local supermarket as the dried flowering bud and ground to a 20-mesh powder with a coffee bean grinder to maximize surface area and increase extraction efficiency (**FIGURE 4).** Isopropanol, ethyl acetate, hexane, and chloroform were purchased from EMD Millipore.

FIGURE 4 *Images of dried clove bud and ground cloves*

Extraction: 0.20 to 0.60g of ground cloves (20 mesh) and 3.0 mL of IPA were placed in a 6 mL glass culture tube and the tube was inverted several times for adequate mixing. A clear supernatant liquid was obtained by filtering the extract through a tightly packed cotton-plug in a Pasteur pipet. Alternatively, a clear supernatant liquid can be obtained by decanting the extract into a disposable syringe and filtering through a 0.45-micron syringe filter or via centrifugation of the extract followed by decantation of the clear liquid. The same procedure was followed when extracting with chloroform.

Alcoholic solutions of eugenol and acetyl eugenol standards were prepared for color testing, TLC, and GC-MS. A concentration of 100 mg/mL was used for color testing and TLC analysis, whereas the concentration was reduced to 1 to 10 mg/mL for analysis by GC-MS. The standards are more convenient to work with in diluted form for several reasons. Color testing responses of the pure liquids are too intense and not representative of the lower concentrations of analytes found in most plant extracts. Spotting of the analytes on TLC plates using standard solutions gives a more appropriate analyte loading than the pure liquids, which would surely overload the capacity of the TLC plate. Likewise, analysis by GC-MS requires analytes in a highly diluted form to avoid overloading of the GC column. Eugenol and acetyl eugenol standards were purchased from TCI Chemicals.

TLC: Flexible, plastic-backed TLC plates were purchased from EMD Millipore (20 x 20 cm, silica gel, 200 mcm thickness, 60-angstrom pore size, F254). Spots were visualized under a 254 nm UV light. Plates are also available with a thin aluminum backing. These flexible plates are convenient for teaching and research purposes due to their ability to be cut to any size with a sharp scissors, or preferably a paper trimmer, and stored in a laboratory notebook. The analyte solutions were spotted with micropipettes that were prepared by heating and stretching glass capillary tubes on a Bunsen burner and cutting each to a useful length. The smaller diameter of micropipettes allows for a controlled loading of solution onto the TLC plate, which produces small, concentrated spots at the origin. This is important since longitudinal

diffusion causes spots to enlarge as they travel up the TLC plate via capillary action in the mobile phase.

Color Test Reagents: Color test reagents were prepared from sulfuric acid, formaldehyde, selenious acid, and ferric chloride, all purchased from Fisher Chemicals. Marquis reagent was prepared by mixing 10 mL of formaldehyde (40% by volume) in 90 mL of concentrated sulfuric acid (19). Mecke reagent was prepared by mixing 1 g of selenious acid in 100 mL of concentrated sulfuric acid (20). Ferric Chloride reagent was prepared by mixing 10 g of anhydrous ferric chloride (which can be substituted with 16.5 g of the hexahydrate) in 100 mL of DI water (19). Alternatively, color test reagents can be purchased as kits. Color testing was performed by placing a few drops of the test reagent into a white porcelain test well, followed by addition of one drop of sample solution into the well. In this order of addition, placing the test reagent in the well first is important to ensure that an initial negative response is obtained prior to addition of the sample, which indicates an uncontaminated well. The practice serves as a negative control for the experiment. Chemical supplies can be sourced from various vendors; however, it should be noted that analytical grade items produce the best results.

IR: Infrared spectra were acquired on a Perkin-Elmer Spectrum 100 series Fourier Transform Infrared Spectrophotometer fitted with a Universal Attenuated Total Reflectance Sampling accessory containing a ZnSe crystal. Spectra were recorded in % transmittance and scanned from 4000 to 650 cm^{-1} for four scans per spectrum at a resolution of 2 cm^{-1}. Spectra of eugenol and acetyl eugenol standards were obtained neat, whereas the spectrum for the alcoholic cloves extract was obtained for the residue by placing several drops of the extract onto the ATR window and allowing the solvent to evaporate.

GC-MS: Total ion chromatograms and mass spectra were obtained on an Agilent 7890A/5975C fitted with a HP-5 column (30 m long x 0.320 mm diameter x 0.250 mcm film thickness), a helium carrier gas flow of 1.3 mL/min (constant flow mode), inlet temperature of 275 deg C, MS transfer line temperature of 280 deg C, oven program of 60-320 deg C at a 30 deg/min ramp with a 2 minute hold and solvent delay at 60 deg C and a 3 minute hold at 320 deg C. The inlet split was 50:1. The quadrupole was set to scan 40-550 Daltons. The MS source temperature was 230 deg C and the MS quadrupole temperature was 150 deg C.

Derivatization: 0.20 mg of ground cloves was extracted with 4.0 mL of chloroform and filtered as described earlier. The derivation was conducted in two stages. First, 1-2 drops of acetic anhydride was added to each of two - 1 mL aliquots of the extract. Next, 1-2 drops of triethylamine was added to only one aliquot to facilitate quantitative conversion of eugenol into acetyl eugenol. Acetic anhydride and triethylamine were purchased from Aldrich Chemicals and chloroform was purchased from BDH. Dichloromethane can be substituted for chloroform.

Hazards and Safety Precautions

Several of the color test reagents contain strong acids, hence appropriate personal protective equipment is required. All organic solvents and reagents should be handled in a fume hood. These materials should be stored, used, and disposed of in an appropriate manner.

Results

Color Tests: The observed color changes of the alcoholic cloves extract, eugenol, and acetyl eugenol solutions are noted in **TABLE 1**. Marquis and Mecke are reagents in the "sulfuric acid series" of color tests. In the Marquis test, all three turned a red color. In the Mecke test, all three turned an initial and brief green that immediately turned to black. It is not surprising that the three samples respond similarly in the sulfuric acid tests. The strongly acidic conditions unmask reactive groups to give a common and responsive product that contains a substituted catechol moiety. A similar effect is seen in the ecstasy family of drugs in which the unmasking of a 3,4-methylenedioxy group produces a catechol. The Ferric Chloride test proved to be more discriminating. In the Ferric Chloride test, the cloves extract and eugenol standard turned light green, whereas acetyl eugenol standard showed no response. The positive response for the cloves extract was due to the presence of eugenol. This ability of Ferric Chloride to distinguish a phenol from an aryl ether or ester is found in the opiate class of drugs, namely in the differentiation of morphine from heroin. Morphine and heroin are indistinguishable in any of the sulfuric acid series of tests. However, in the Ferric Chloride test, morphine responds with a color change (blue), whereas heroin has no response. In both the eugenol/acetyl eugenol and morphine/heroin examples, Ferric Chloride is a more discriminating test due to the milder chemical conditions.

TABLE 1 *Color test results for alcoholic cloves extract, eugenol, and acetyl eugenol*

Sample	***Marquis***	***Mecke***	***$FeCl_3$***
Cloves extract	red	green to black	light green
Eugenol	red	green to black	light green
Acetyl eugenol	red	green to black	NR

TLC: The TLC results show an increasing, yet identical response factor (R_f) for the single spot observed in the three samples (cloves extract, eugenol, and acetyl

eugenol) as the proportion of ethyl acetate in the mobile phase is increased.

R_f = distance traveled by spot/
distance traveled by mobile phase (1)

However, when the mobile phase was modified with 10% triethylamine (by volume), two spots were resolved from the cloves extract. A comparison of the R_f values of the spots indicates a positive identification of eugenol and acetyl eugenol as components in the cloves extract (**FIGURE 5 and TABLE 2).** Further confirmation of the component identifications was performed by combining TLC separation with color testing. This was done by dropping a small amount of color test reagent onto the resolved spots of the TLC plate and observing the color change.

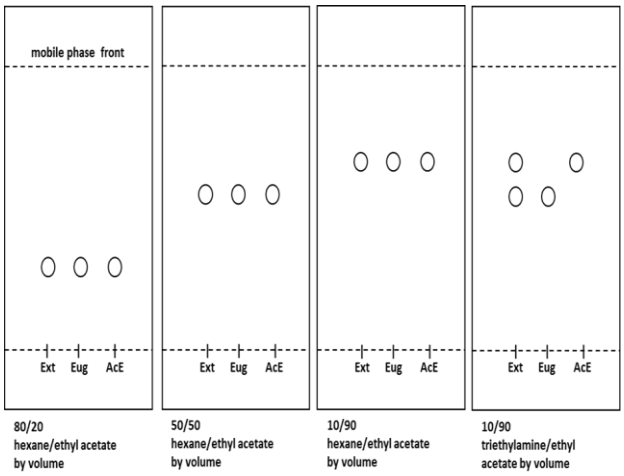

FIGURE 5 *TLC plates developed with hexane, ethyl acetate, and trimethylamine (Ext = alcoholic cloves extract, Eug = eugenol, AcE = acetyl eugenol).*

It is interesting to note that throughout the TLC experiment, lighter intensity spots that appear at lower R_f values in the cloves extract give color test responses that do not match the standards. (Caution: TLC plates exposed to corrosive agents such as strong acids should be discarded in a responsible manner following a color change observation and not stored in a laboratory notebook.)

TABLE 2 *R_f values for cloves extract, eugenol, and acetyl eugenol in various TLC systems containing hexane, ethyl acetate, and triethylamine measured as volume ratios.*

	Hexane/Ethyl Acetate (v/v)				*TEA/Ethyl Acetate (v/v)*
	(80/20)	*(50/50)*	*(10/90)*	*(0/100)*	*(10/90)*
Cloves extract	0.32	0.58	0.66	0.64	0.58, 0.67
Eugenol	0.32	0.58	0.66	0.64	0.58
Acetyl eugenol	0.32	0.58	0.66	0.64	0.67

IR: IR spectra of the alcoholic cloves extract residue and neat standards of eugenol and acetyl eugenol are displayed in **FIGURE 6**. The cloves residue appears to be an additive spectrum of the two main components, eugenol and acetyl eugenol. This confirms the TLC result that the alcoholic cloves extract contains mainly eugenol and acetyl eugenol, from an IR spectroscopic perspective. The most distinguishing feature in the spectrum of the extract is the band at 1766 cm^{-1}, which corresponds to the acetate ester moiety in acetyl eugenol (absent in eugenol). It should be noted that there is a slight red shift for this carbonyl band of about four wavenumbers in the extract compared to the acetyl eugenol standard (1762 cm^{-1} in acetyl eugenol). This is most likely due to some hydrogen bonding from eugenol present in the extract. In controlled testing, this assertion was confirmed by observing a similar three-wavenumber red shift of the band in a series of binary eugenol/acetyl eugenol mixtures from zero to 100 % by weight (unpublished results from a separate manuscript in preparation).

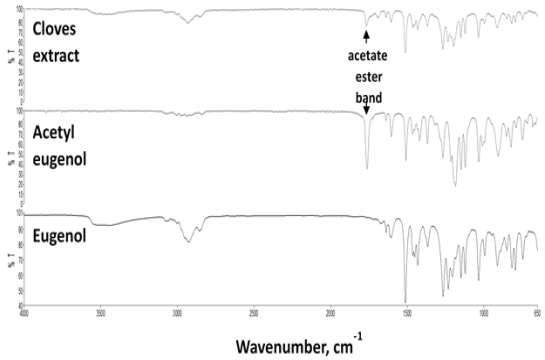

FIGURE 6 *IR Spectra of cloves extract (residue), acetyl eugenol, and eugenol*

GC-MS: A total ion chromatogram of the alcoholic cloves extract is shown in **FIGURE 7** and mass spectra are shown in **FIGURE 8-12**. All five of the main chemical components are resolved on a HP-5 phase column. Similar chromatographic results were obtained on a lower polarity HP-1 column (unpublished results, not shown).

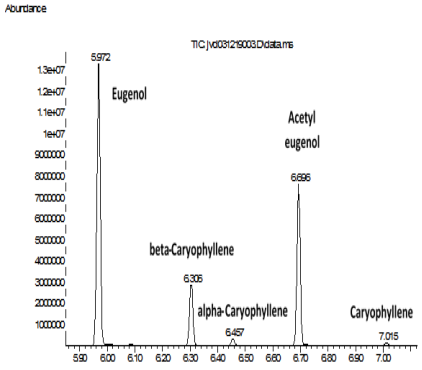

FIGURE 7 *GC-MS total ion chromatogram of alcohol extract on HP-5 phase column*

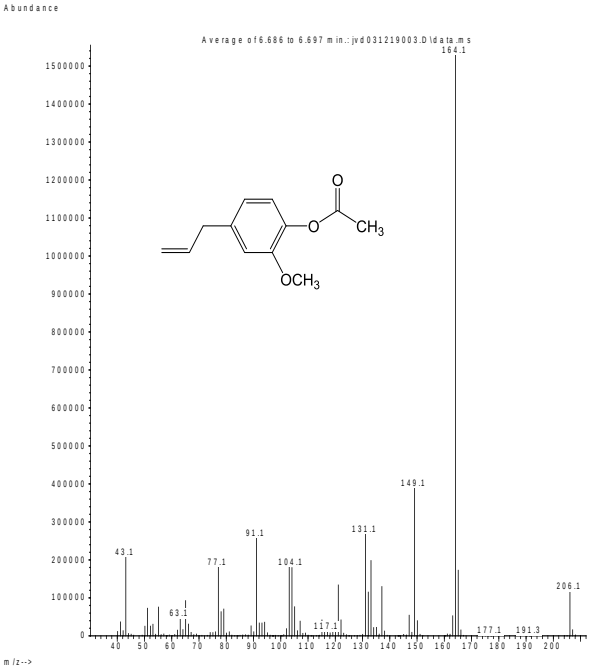

FIGURE 8 *Mass spectrum of eugenol in alcohol extract (RT=5.972 min)*

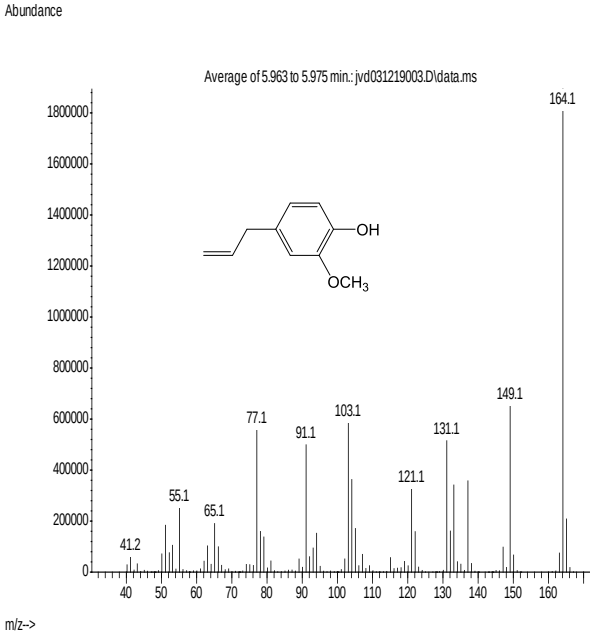

FIGURE 9 *Mass spectrum of acetyl eugenol in alcohol extract (RT = 6.696 min)*

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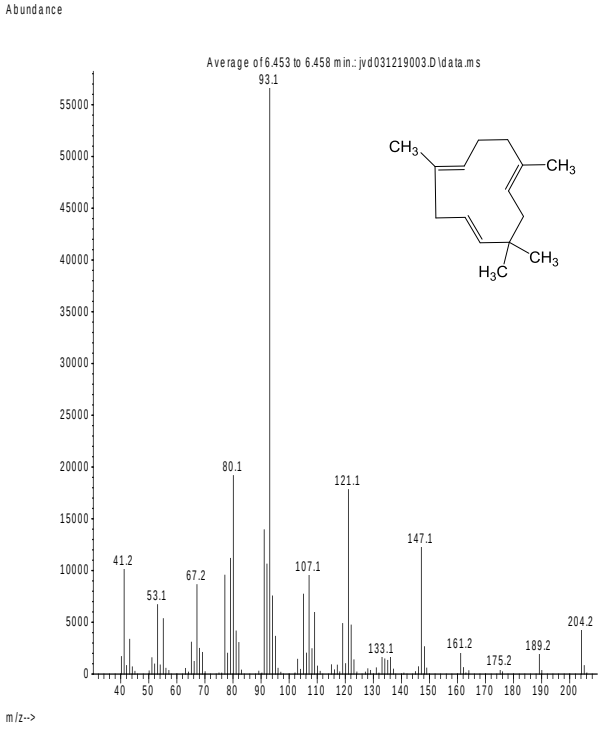

FIGURE 10 *Mass spectrum of beta-caryophyllene in alcohol extract (RT = 6.306 min)*

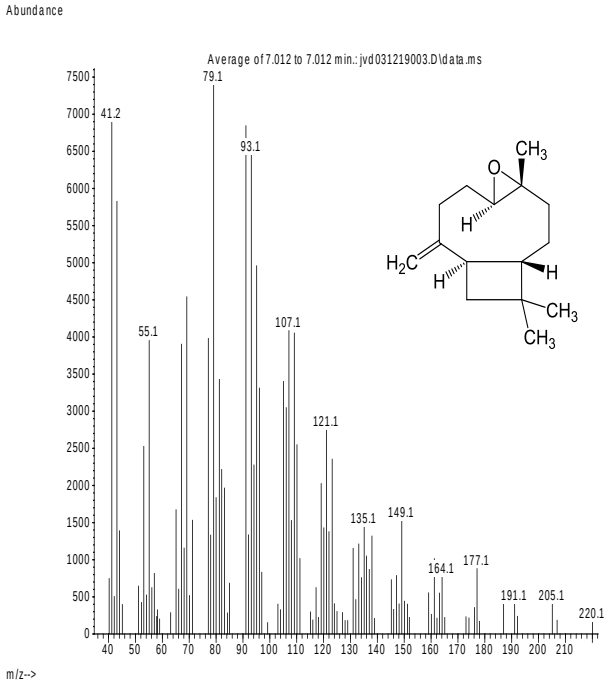

FIGURE 11 *Mass spectrum of alpha-caryophyllene in alcohol extract (RT = 6.457 min)*

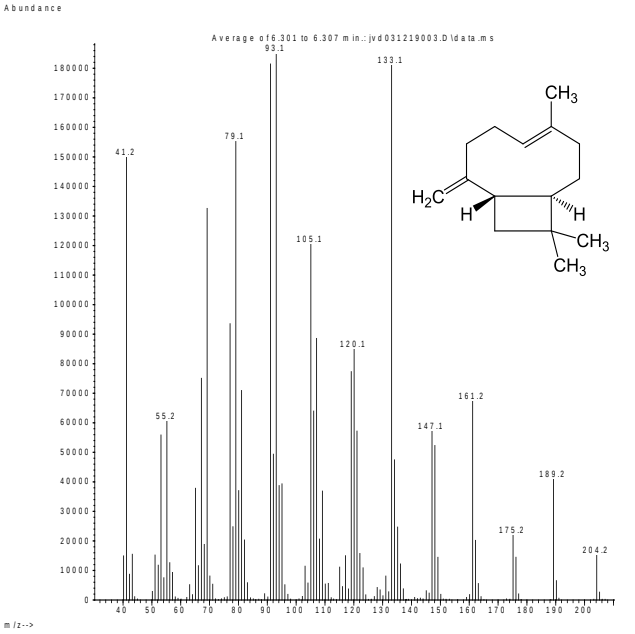

FIGURE 12 *Mass spectrum of caryophyllene oxide in alcohol extract (RT = 7.012 min).*

However, when the analysis was conducted on a more polar HP-17 column, resolution of the five components degraded (**FIGURE 13**). Caryophyllene oxide was only marginally resolved as a shoulder on the acetyl eugenol peak and alpha-caryophyllene disappeared completely. Upon closer inspection of the eugenol peak purity, several fragments appeared that belong to alpha-caryophyllene and not eugenol, namely m/z 93, 147, and 204. Subsequent testing of the extract by evaporation of the solvent followed by re-extraction of the resulting residue with hexane, then back washing of the hexane solution with several volumes of 1N NaOH to remove eugenol revealed a pure mass spectrum for alpha-caryophyllene at the same retention time as eugenol. Obviously, the preferred GC column phases for this analysis are HP-1 and HP-5 (0% and 5% phenyl, respectively), whereas the HP-17 column phase (50% phenyl) is too polar.

The effect of adding acetic anhydride to a chloroform extract of cloves over a 24-hour period is shown in **FIGURE 14**. In the absence of triethylamine, the peak ratio of eugenol/acetyl eugenol remains nearly unchanged. An extended time analysis showed moderate, but steady decline of this ratio after several days. This is indicative of the unfavorable kinetics of this conversion in the presence of only acetic anhydride. However, the addition of triethylamine to the mixture has an immediate and drastic impact on the ratio. After only 16 hours, the conversion of eugenol to acetyl eugenol was nearly complete.

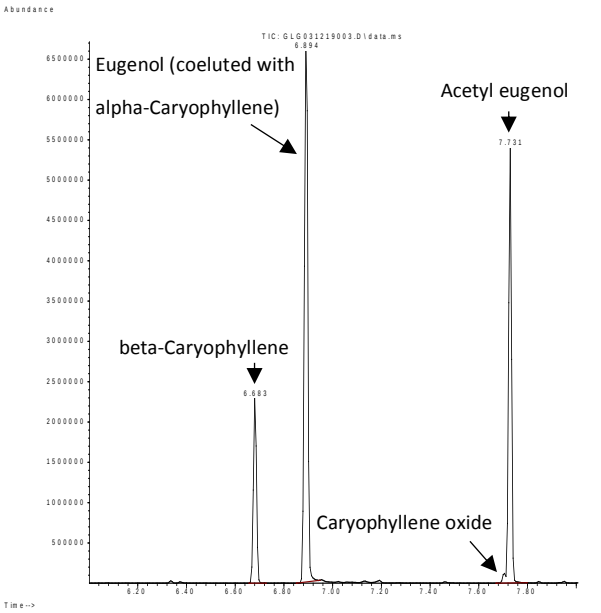

FIGURE 13 *GC-MS total ion chromatogram of alcoholic extract on HP-17 phase column*

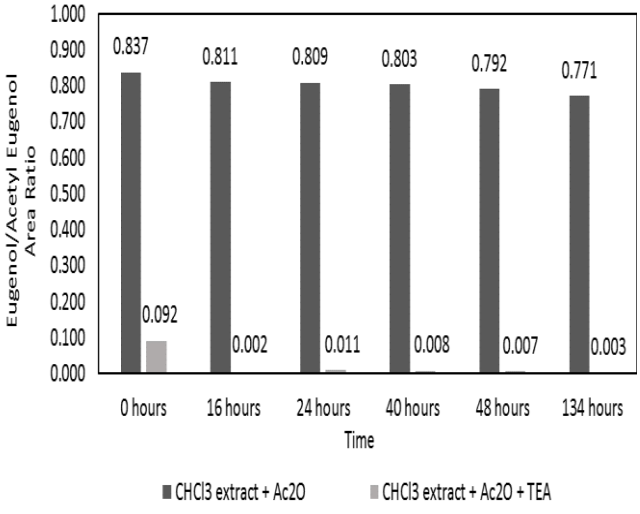

FIGURE 14 *Peak area ratios of eugenol to acetyl eugenol for chloroform extract + acetic anhydride and chloroform extract + acetic anhydride + triethylamine, over 134 hours*

Discussion and Conclusion

The experimental design described in this work provides an effective means to extract drug components from plant material followed by a chemical analysis that uses a progressively advancing series of analytical techniques. Basic tests such as color testing and TLC

allow for the experiment to be meaningful to modestly resourced laboratories, whereas more advanced techniques such as IR and GC-MS are appropriate for the college undergraduate and graduate levels.

The order in which the techniques are employed follows a pedagogy that dovetails various chemical concepts with analytical concepts. The use of triethylamine in the TLC analysis causes eugenol to separate from acetyl eugenol to the extent that each can be identified based on the R_f values (**TABLE 2**). The mechanism by which triethylamine affects separation is unclear. It is either by an increase in solvent polarity or by introduction of an acid-base interaction. The solvent polarity effect can be ruled out by replacing triethylamine with an even more polar solvent such as isopropanol. In a TLC experiment using a mobile phase of 10/90 isopropanol to ethyl acetate by volume, there is no separation of eugenol from acetyl eugenol in the cloves extract (R_f of 0.74 for both, unpublished results). As for the acid-base interaction, it can occur in several ways. Triethylamine can cause deprotonation of eugenol to form the phenoxide which bears a full negative charge and thus has a significantly stronger interaction with the siloxy sites on the silica gel surface than the phenol (eugenol) or the acetate ester (acetyl eugenol). It is also possible that triethylamine creates a more basic silica gel surface, which in turn creates a stronger interaction with the acidic phenol than the neutral acetate ester. The literature pK_a values of eugenol and triethylamine (actually, the ammonium salt of triethylamine) are 10.00 and 10.77 (21), respectively (**TABLE 3**). This slight difference of 0.77 pK_a units is enough to support a mechanism by which the majority of eugenol is deprotonated in the presence of triethylamine base.

The use of triethylamine in the GC analysis, in combination with acetic anhydride, causes a rapid conversion of eugenol to acetyl eugenol. The mechanism of this accelerated conversion can occur in two ways: either by removal of acetic acid from the product side of the acetylation reaction, thus tilting the equilibrium in favor of product formation in a classic Le Chatelier manner or by deprotonating eugenol to form eugenol phenoxide which is a more powerful nucleophile for attacking the electrophilic acetic anhydride. Triethylamine is a sufficiently strong base to produce the eugenol phenoxide and can act as a proton sponge for acetic acid. Controlled testing of the cloves extract with a base that is too weak to deprotonate eugenol, but strong enough to react with acetic acid, should resolve the issue. In fact, when triethylamine is replaced with pyridine (pK_a of pyridine HCl = 5.25) in a chloroform extract of cloves in the presence of acetic anhydride, the same accelerated conversion of eugenol to acetyl eugenol is observed (**TABLE 3, FIGURE 15**). If the mechanism proceeded via deprotonation of eugenol, pyridine should have no effect on the velocity of the acetylation reaction. Thus, these experiments support the mechanism by which a base such as triethylamine or pyridine accelerates the conversion of eugenol to acetyl eugenol by removing acetic acid from the product side of the equilibrium. Of course, the fact that both triethylamine and pyridine are tertiary bases is no coincidence. If a secondary or primary amine is used in place of a tertiary amine, the rate and extent of acetylation would be diminished due to the reactivity of these amines with acetic anhydride to form amides. Amide formation will out compete esterification from both a thermodynamic and kinetic perspective.

TABLE 3 *pK_a values for acetic acid, pyridine, eugenol, and triethylamine*

Acid	***Base***	***pK_a***
Acetic acid	Acetate (anion)	4.76
Pyridine-H^+	Pyridine base	5.25
Eugenol	Eugenol (anion)	10.00
Triethylamine-H^+	Triethylamine base	10.77

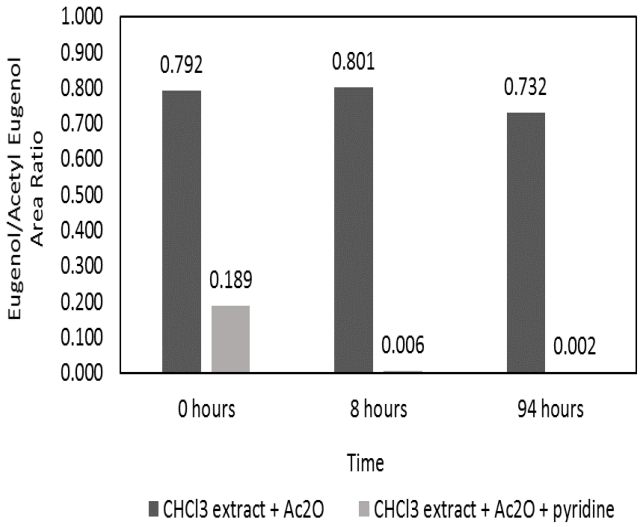

FIGURE 15 *Peak area ratios of eugenol to acetyl eugenol for chloroform extract + acetic anhydride and chloroform extract + acetic anhydride + pyridine, over 94 hours*

Compared to prior methods of producing essential oil of cloves via steam distillation, this new experiment presents a simpler extraction method that takes a more expansive approach to the chemical analysis. The experiment exploits the unique combination of chemical components in the extract to investigate chemical concepts of acidity, functional group transformation, and chemical kinetics. The analytical testing scheme provides a comprehensive

and orthogonal analysis of the main chemical components. The analytical methods used are appropriately selective and specific to distinguish eugenol from acetyl eugenol. Furthermore, the progressive experimental design presents an opportunity for collaboration between secondary, post-secondary, and postgraduate institutions. Despite the comprehensive scope of testing, the analytical approach is focused mainly on qualitative analysis. Future work will focus on quantitative analysis of the chemical components identified in this work by chromatographic and spectroscopic means.

Student Learning Outcomes

The analysis of chemical components in plants is fundamental to the field of forensic drug analysis. The list of controlled substances at the federal and state levels is replete with examples of drugs that are directly or indirectly derived from plants. The most widely abused drug in all of human history, ethanol, is the result of the action of microflora on macroflora, namely yeast on grain or other vegetative organic matter. Therefore, regardless of the legal status of an organic compound, chemical analysis is chemical analysis.

This paper is structured so that experimentation is conducted in a tiered fashion and at several cognitive levels. The laboratory-based pedagogy is designed to be progressively complex to accommodate various educational levels from high school to post-secondary. The sequence of experiments provides a pedagogical roadmap of discovery for the student that can be tailored to match the expertise of the student and educator, as well as the resources of the institution. For example, solvent extraction, color testing, and TLC are relatively affordable laboratory techniques that provide a great deal of chemical information, whereas instrumental analysis by IR and GC-MS requires advanced expertise and resourcing. Nonetheless, the importance of chemical concepts can be appreciated regardless of the resources available for experimentation. The student will learn concepts of method development, differential migration, interparticle forces, pK_a, and surface basicity in the TLC experiments. The use of triethylamine with acetic anhydride in the GC-MS analysis teaches the concepts of drug derivatization, chemical equilibria, and Le Chatelier's principle.

From an analytical perspective, the student will learn the concepts of selectivity and specificity and how to evaluate the merits and limitations of the various analytical tools of identification. Color changes produced in the Marquis, Mecke, and $FeCl_3$ tests are examples of analyte specificity. That is, a specific chemical structure must be present to produce the observed color change. Separation of compounds by TLC is an example of analyte selectivity. That is, differential migration of components observed in the chromatographic process produces a selective bias of the distance traveled of one compound compared to another that is based on the strength of interparticle forces. The progression from TLC to GC teaches the student that analyte selectivity can be enhanced by increasing the number of theoretical plates. The progression from a color test response to a mass spectrum or IR spectrum teaches the student how analyte specificity can be increased. That is, spectral data contain far more information about a certain chemical structure than a color test response. The progression of testing can be easily connected to the testing categories set forth in the SWGDRUG guidelines which are grouped by the level of selectivity (22).

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