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Reversed-Phase HPLC Determination of Citral in Locally Grown Lemon Grass Harvested at Different Season.

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by

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ABSTRACT

Reversed-Phase HPLC Determination of Citral in Locally Grown Lemon Grass Harvested at Different Seasons

By

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A simple HPLC procedure for the quantitative determination of citral, the major fragrant component in the lemon grass, has been developed. The procedure involves a C-8 stationary phase using a 90:10 methanol: water pH 5 mobile phase containing 0.25% 1-octanesulfonic acid and an UV detector (set at 233 nm). The lemon grass leaves were harvested fresh at different times of the year and were soaked in methanol for 48 hours without any mechanical assistance to extract the citral and other methanol soluble components. The method showed good reproducibility with relative standard deviation of 2.8% and 10.8% for two different sets of samples. The method showed linearity in the range of 0.89 - 35.52 μg/mL. The average recovery was 104.8 %. The amount of citral found as a percentage of the dried leaves are, 0.093, 0.27, 0.10, 0.13, 0.16, and 0.066 for fall, winter, three summer, and 1 commercial store samples, respectively.

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CHAPTER 1

INTRODUCTION

Herbs have been with us since the early civilizations. Herbs have been used to flavor foods and treat illnesses, and most of the people in the world still continue to use herbs that are beneficial to their health because they are safe and extremely reliable with few side effects. Steam distillation of herbs transforms herbs into essential oils. For example, lemon grass essential oil is distilled from the leaves of the lemon grass plant (cymbopogon citrates). Citral, the primary constituent of the lemon grass essential oil, provides a strong and refreshing scent that is popular in household products like detergents and room sprays (*1*).

History of Herbs

The word herb derives from the Latin "herba", meaning grass or, by extension, green crop (*2*). The word originally applied to a wide range of leafy vegetables. Herbs are a seed plant that does not produce a woody stem like a tree and that will live long enough to develop flowers and seeds (*2*). For thousands of years, herbs have been used as scents, foods, flavorings, medicines, disinfectants, and even as currency. Early cultures probably recognized that certain herbs had healing powers; therefore, some herbs were thought to have magical properties (*2*).

In ancient civilizations, people began to associate less magic with the treatment of diseases. They understood that illness was natural and not supernatural and medicine should be given without magic. Chinese herbalism is widely regarded as the oldest because it has the longest unbroken recorded history. They have practiced herbal use for 5000 years. They are noted for their knowledge of herbs and the use of ginseng which

they believe prolongs life (*3*). Ancient Egyptians also were highly skilled with herbs. Records of Egyptian culture mention the common uses of many herbs such as garlic, indigo, mint, and opium for food and medicine (*3*). The medical inheritance of ancient Egypt passed on to Greece and Rome (*3*). In Greek culture the physician Hippocrates used diet and herbs as the basis of treatment (*3*). Romans were noted for their use of mandrake herb, native to southern Europe, as an anesthetic (*3*). They also used herbs in cosmetics, in magical and religious ceremonies, and in cooking. In North America, Native Americans used ginseng, black cohosh, and cat's claw as medicinal plants. Also, early European settlers brought herbs to America for use as medication for illnesses and flavoring. Some herbs were used to improve the taste of meats in the days before other preservation techniques were developed. Other herbs were used to dye homespun fabrics (*4*).

Many people have used herbs to treat their illnesses because they are safe and reliable, with few side effects. In many regions where they grow they are still valued for their medicinal properties. Herbalists today seek to help people promote good health with natural sources. With the realization that synthetic chemical medicines are not always "magic bullets" and sometimes carry serious side effects, herbalism and ancient medicines are making a comeback.

Herbs and Essential Oils

Medicinal herbs are widely used as diet supplements and in the treatment of illness; for example, aloe vera, ginseng, alfalfa, peppermint, and black cohosh (*4*). Culinary herbs are used for flavors, preservatives, and color enhancers in food preparation and food products (*5*). Common examples are parsley, basil, rosemary, and

cinnamon (*5*). In addition, oil from aromatic herbs can be used to produce perfumes and various scents. Steam distillation, the most common method of extraction from herbs, transforms the spicy scent into essential oils (*6, 7*). Essential oils are used by the fragrance industry, natural products industries, and in aromatherapy which is the treatment of ailments using scents (*7*). The main requirements to produce essential oils are the correct plant material, good horticultural soils, irrigation, good shelter, harvest equipment, and distillation equipment (*7*).

Processes to Obtain Essential Oils

As mentioned above, essential oils are natural products and can be extracted from herbs and plants. They are responsible for the odors found in various herbs and plants. When they are exposed to air at ordinary temperature, they evaporate and are, therefore, called volatile oils, ethereal oils, or essential oils (*8*). The last term is applied because the oils represent the essence or odor constituent of the plants. The oils are usually colorless when they are fresh, but with age, they maybe oxidized and become darker in appearance. Therefore, they should be kept in tightly stopped glass containers in a cool, dry place (*8*).

Essential oils are concentrated plant extracts, and the oils are commonly extracted by steam distillation. They are highly volatile with characteristic odors. Most of the essential oils are insoluble and less dense than water. They are usually soluble in alcohol, ether, and other organic solvents (*8, 9*). They have many common physical properties: boiling points vary from 160 to 240 ^{0}C, densities are from 0.759 to 1.096 g/mL, high refractive indices, and most are optically active (*8*). Many essential oils are terpeniods, and few are benzene derivatives (*8*). Table 1 shows some of the more

common essential oils (*10*).

Essential oils can be divided into two broad categories:

- Large volumes oils that are usually distilled from the leaf such as lemon grass, citronella, and cinnamon leaves (*10*).
- Small volume oils that are usually distilled from fruits, seeds, buds, and flowers, e.g. cloves, nutmeg, and coriander (*10*).

Table 1. Essential Oils from Various Plants

Name	**Part of Plant used**	**Botanical name**	**Important constituents**	**Uses**
Lemon grass and Citronella	Leaf	Cymbopogon	**Citral** Citronella Terpenes	Perfumery Disinfectant
Eucalyptus	Leaf	Eucalyptus globulus Eucalyptus citriodora Eucalyptus dives	Cineala Citronella Terpenes	Perfumery
Cinnamon leaf	Leaf	Cinnamomum zeylanicum	Eugenol	Used to make artificial vanilla
Clove	Bud	Eugenia caryophyllus	Eugenol	Dentistry flavoring
Lavender	Flower	Lavendula intermedia	Linalol	Perfumery
Sandalwood	Wood	Santalum album	Sanatols	Perfumery
Nutmeg	Nut	Myristica fragrans	Myristicin	Flavoring
Almond	Nut	Prunis communis	Benzaldehyde	Flavoring
Coriander	Seed	Coriandrum sativum	Linalol Terpenes	Flavoring

To obtain essential oils, the harvesting of the herb is very important. If the plant is harvested at the wrong time, the oil yield can be severely reduced. The oil is usually contained in oil glands, veins that are often very fragile. Improper handling can break these structures and release the oils. This is the reason that the strong smell is given off when these plants are handled. Therefore, these plants should be handled very carefully to prevent oils from being lost. For lemon grass, harvest can take place 6-9 months after planting. Then the grass can be harvested up to four times a year. If the plant is allowed to grow too large, the oil yield is reduced. For lemon grass it should be 1.2 m high. The grass should be harvested early in the morning as long as it is not raining, and harvesting can be done with simple knives (*10*).

Drying the leaves increases the yield of the oil, and it should be dried in the sun not in the shade or partial shade. Then, dried leaves can be distilled, and it should be distilled as soon as possible. If it does have to be stored, it should be stored in the shade and should not be allowed to get wet (*10*).

Water distillation, water and steam distillation, and steam distillation are three methods of distilling essential oil (*8, 10*). Water distillation is the simplest method and is usually the cheapest distillation method. The plant material is immersed in water and boiled to about 100^{o}C. Then, the steam and oil vapor is condensed and the oil is separated from the water. This method is suitable for flower blossoms and finely powdered plant material. Water and steam distillation are almost the same as water distillation, except that the plant material is not immersed in water but held above the boiling water on a grid. This is slightly more expensive than water distillation but better for herbs and leafy material. The plant material does not need to be finely chopped or

powdered and the distillation temperature should be about 100^{o}C and at atmospheric pressure. In the steam distillation method, fresh or dried plant materials are placed in the plant chamber of a sill or retort, and pressurized steam is introduced in a lower chamber of the still or retort and passed through the plant material to vaporize the volatile oils (*10*).

The steam and oil vapor mixture is then passed through a condenser. Finally, the essential oil is extracted from the water in the separator. The remaining water is called floral water, distillate, or hydrosol. It retains many of the beneficial properties of the plant, making it useful in skin care for facial mists. In certain situations, floral water may be preferable to pure essential oil, such as when treating a sensitive individual or child or when a more diluted treatment is required. The steam distillation method is more expensive than the other methods. However, it is especially well suited for plant materials with high boiling points. The rate of distillation and yield of oil is high and the quality of the oil is good (*9, 10*).

Lemon Grass

The lemon grass plant (Cymbopogon citratus) is a perennial herb in the tropics and is native to India and Sri Lanka (*1*). It has since been used in many cultures as shown in Table 2 (*11*). It is widely cultivated in the tropics and subtropics and it prefers humid climate in full sun. However, with some help, it will tolerate a range of climates. This plant goes inactive in the winter because it is a tender plant that suffers leaf damage from frosts and is killed back to the roots by hard freezes. Lemon grass should, therefore, be potted and brought indoors during the winter (*12*). This tropical grass grows fast in dense clumps that can reach 6 ft in height and about 4 ft in width. The leaves are half to an inch

Table 2. Some Common Names of Lemon Grass in Different Cultures

Language or Region	**Common Name**
English	Lemon grass, Citronella
Middle Eastern (Arabic)	حَشِيشَة اللَيْمُون (Hashisha al-limun)
Chinese	草薑(Chou geung) 風茅(Fung maauh) 草薑(Chou geung) 風茅(Fung maauh) 檸檬草(Nihng mung chou)
Malaysia	Serai, Serai dapur
Dutch	Citroengras, Sereh
French	Verveine des Indes
German	Zitronengras, Citronella, Lemongras
Greek	Λεμονόχορτο (Lemonochorto) Κιτρονέλλα (Kitronella) (*Cymbopogon nardus*)
Hebrew	עשב לימון (Essef limon) לימון גראס(Limonit rehanit)
India (Hindi)	Sera, Verveine
Italian	Cimbopogone
Russian	Лимонное сорго(Limonnoe sorgo) Лимонная трава(Limmonaya trava)
Spanish	Zacate de limón, Te de limón, Caña de Limón, Citronella, Hierba de Limón
Japanese	レモングラス(Remonso) レモンソウ(Remonguraso)
Turkish	Limon out
Vietnamese	Så chanh, Xả (Sa chanh, Xa)

wide; about 3 ft long and have gracefully drooping tips (*13*). The evergreen leaves are bright bluish-green (*13*). Lemongrass is perhaps best known for its appearance in Thai and Vietnamese cuisine. It also is used in herbal teas and other nonalcoholic beverages, in baked goods, and in confections. Oil from lemon grass is widely used as a fragrance in perfumes and cosmetics, such as soaps and creams. As a medicinal plant, lemon grass has long been used in traditional Indian medicine to fight fever and infection. It has been considered a carminative (relieves gas in the alimentary tract) and insect repellent (*14*). The essential oil of lemon grass is an antiseptic, antibacterial, and antiviral. It also has good deodorizing properties. In aromatherapy, lemon grass oil is used as an antidepressant, to soothe aches and pains, and to relieve stress (*14*).

Chemical Composition of Lemon Grass Oil

The essential oil of lemon grass consists mainly of citral (*1, 15)*. The quality of lemon grass oil is generally determined by its citral content, the aldehyde responsible for the lemon odor (*15*). Citral is a mixture of two stereoisomeric monoterpene aldehydes. In lemon grass oil, the *trans* isomer geranial (40 to 62 %) predominates over the *cis* isomer neral, (25 to 38%) (*16*). Figure 1 shows the structures of the two isomers (*16*).

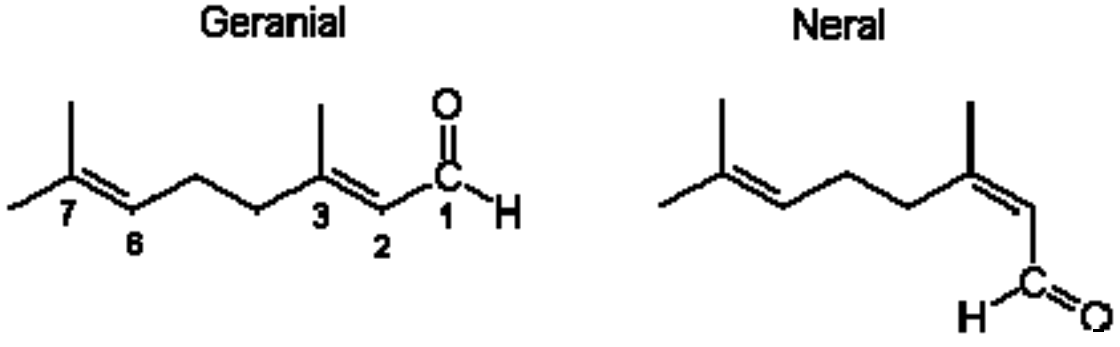


Figure 1. The Trans and Cis Forms of Citral in Lemon Grass

In addition, citral is an example of monoterpenes. Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Many terpenes are hydrocarbons. Their structures may be derived from isoprene, $CH_2=C(CH_3)-CH=CH_2$ (*20*). Terpenes are classified according to the number of isoprene units. Monoterpenes have two isoprene units. Other terpenes in lemon grass oil are nerol, limonene, linalool, β-caryphyllene, and a low amount of myrcene (*1, 16*).

The aldehyde, citral, was first separated form the oil of *backhousia citriodora* by Bertram (*17*), who gave it the correct empirical formula, $C_{10}H_{16}O$. In 1890, Semmler made the important observation that geraniol on gentle oxidation produced an aldehyde called geranial, $C_{10}H_{16}O$ shown in Figure 2 (*18*).

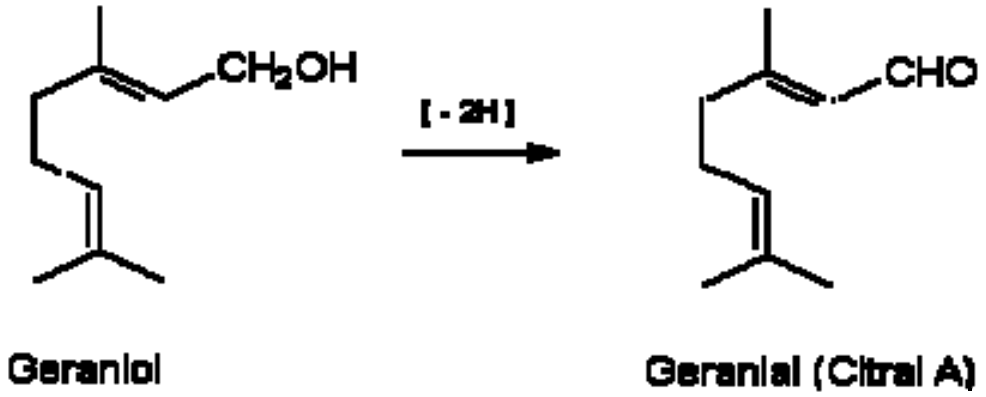


Figure 2. The Oxidation of Alcohol, Geraniol, to Produce Geranial

Semmler recognized that this aldehyde, geranial, was identical with the aldehyde, citral, that had been obtained previously from some essential oils. In the same year, Dodge separated an aldehyde, which he called citral, from lemon grass oil, and he showed it reacted abnormally with sodium bisulphate solution (*19*). In the following year, Semmler showed geranial and citral to be identical compounds (*20*). The important discovery about citral was made by Barbier and Bouveault who showed that the citral

from lemon grass oil was not homogenous (*21*). This was fully confirmed by Tiemann and his co-workers (*22*), and methods were devised for the separation of the isomeric aldehyde that was designated citral A, geranial, and citral B, neral. Citral A, which forms the major part of the aldeyhde fraction of lemon grass oil, is most readily separated from citral B by the sodium bisulphate method suggested by Tiemann (*23*). His method yields a semicarbozone of melting point 164^{o}C. For further support, Zeitshel (*24*) showed that citral A is obtained almost free from citral B by the oxidation of geraniol. Also, citral B is obtained free from citral A by taking advantage of the greater reactivity of citral A with cyanoacetic acid (*24*).

CHAPTER 2

TECHNQIUES FOR DETERMNATION AND ANALYTISS OF CITRAL

Lemon grass is generally recognized as safe for human consumption as a plant extract/essential oil; therefore, little research has been done on developing new methods for the determination and analysis of citral in lemon grass or analysis of the essential oil in general. There are some methods that have been developed, such as near –infrared spectroscopy (NIRS) and titration analysis. The most common method is chromatography that includes gas chromatography and high performance liquid chromatography.

Near-Infrared Spectroscopy Technique

Lemon grass oils and lemon oils that contain the carbonyl compound citral were both studied. Lemon grass oil contains citral at levels of approximately 65- 85% w/w (*25*), and lemon oil contains citral at a concentration of approximately 2-5 % w/w (*26*).

In 2002, Wilson and his co-workers (*31*) developed a method to determine the content of citral in lemon grass and lemon oils by near –infrared spectroscopy. The reference method for both types of oils was the British Pharmacopoeia (BP) titration assay for citral content of lemon oil and lemon grass. In the method approved by the British Pharmacopoeia, 25 samples of pure lemon oil and 14 pure lemon grass oil samples were obtained. Each oil sample of lemon oil and lemon grass oil was mixed with absolute ethanol. Then, hydroxylamine hydrochloride solution and bromophenol blue solution were added. Hydrochloric acid is librated upon the reaction of hydroxylamine with the citral in both oils. The librated hydrochloric acid was then titrated slowly against a standardized solution of ethanolic potassium hydroxide until the

color changed from yellow to olive–green. For lemon grass oil samples, the range of citral content was found to be 69.89-76.95 % (w/w), and for lemon oil samples 2.24 – 3.70 % (w/w) (*27*).

For their proposed NIR spectroscopy method, 26 samples of lemon grass oil and 35 samples of lemon oil were scanned over the wave length range of 1100 – 2500 nm on a Foss NIR- System 6500 Rapid Content Sampler using a reflectance vessel as sample presentation method. Three spectra were obtained for each sample (each spectrum was the average of 32 scans). The spectra were then averaged using the NSAS Software version 3.52 obtained from Foss-NIR System Company. For each mean spectrum of citral obtained for each lemon and lemon grass sample, a reference value (BP method of % w/w citral content) was assigned. The mean percentage accuracy (relative difference to the BP method as reference) for lemon grass oil samples was 1.00 %, and for lemon oil samples it was 4.28 % (*27*).

For the determination of the reproducibility (precision) of the NIR method, a single sample of lemon grass and lemon oil was assayed for citral content six times using the BP method as a reference. Six NIR spectra for the single sample of lemon grass oil and lemon oil were obtained on a single day. Using the BP method for the lemon grass oil samples, the mean of citral content was 75.55 % (w/w) with coefficient of variation of 0.36 %. For lemon grass oil samples, the mean of citral content was 4.29 % (w/w) with coefficient of variation of 0.75 %. Using the NIR method, the mean of citral found in lemon grass oil sample of six spectra was 75.77 % (w/w) with coefficient of variation of 0.23 %. For lemon oil, the mean of citral content found was 4.25 % with coefficient of variation of 2.44 % (*27*).

Both NIR and BP methods were comparable in precision although the NIR method may be less accurate in comparison with the BP titration method for lemon oil samples. The NIR method has advantages over the BP method in that it was simpler to carry out, no sample preparation was required, and it was more rapid (*27*).

Gas Chromatography Technique

Gas Chromatography (GC) with its low cost and great versatility has been a method of choice to analyze essential oils in natural materials and products. GC in combination with another technique, such as mass spectroscopy (MS), has been used widely for separation, identification, and quantification of several volatile compounds.

Radix Bupleuri is an example of medical herb grown widely across China (*28*). It is used as an anticancer (*29*), anti-inflammatory (*30*), and plasma cholesterol–lowering herb (*31*), and the essential oil has one of the important active components in Radix Bupleuri. There was no specific quantification method suitable for use in controlling the quality of the essential oils of this medical herb in Chinese pharmacopoeia. It, therefore, was important to develop a method for the analysis of the essential oil from Radix Bupleuri. Xiuqin and his co-workers (*28*) employed gas chromatography –mass spectroscopy (GC-MS) to identify the components of the essential oil. Their results showed that E-2 heptenal, 2-pentyl furan, and E-2 nonenal were some of the main compounds of the oil, and their contents control the quality of the essential oil from Radix Bupleuri (*28*).

The steam distillation method was chosen to extract the essential oils from Radix Bupleuri using hexane as the extraction solvent. Then, the hexane layer was collected and decane was added to the solution sample as the internal standard. Stock standard

solutions of E-2 heptenal, 2-pentyl furan, and E-2 nonenal were prepared by dissolving each compound in hexane at an appropriate concentration. The stock standard solutions of each compound were diluted with hexane to make standard solutions of each compound at appropriate concentrations. Decane was used as the internal standard and it also was diluted with hexane (*28*).

Calibration curves were obtained by plotting the peak area ratios of anlayte to internal standard against analyte concentration. The concentrations and the corresponding peak area ratios were found to be linear. The precision of the method was tested and relative standard deviations (RSDs) were found to be 2.7%, 0.7%, and 3.1% for E -2 heptenal, 2-pentyl furan, and E-2 nonenal, respectively. The RSD values of the repeatability of six samples for E-2 heptenal, 2-pentyl furan, and E-2 nonenal were 1.6%, 0.7%, and 2.6%, respectively. The average recovery was 100.3% (RSD 4.2%) for E-2 heptenal, 102.8% (RSD 5.2%) for 2-penyle furan, and 97% (RSD 3.8%) for E-2 noneral. These results showed that the GC method exhibited good recovery and repeatability (*28*).

The combined GC-MS system has become more familiar to many analysts. The combination gives high specificity to the analysis in which two or more mass fragment ions are monitored in the MS system (*32*). Paul and his co-workers (*33*) employed the same method, GC-MS, to identify and quantify volatile compounds of the essential oil of Ageratina adenophora (snakeroot) growing in the Canary Islands, Spain. Ageratina adenophora is an herb, native to Mexico, and grows mainly in the warmer regions of America. A total of 78 volatile compounds were identified, and *p*-cymene (11.6 %) was the major component in the oil (*33*). The identification was confirmed using online National Institute of Standard and Technology (NIST) and Wiley library spectra (*34-39*).

The relative component concentrations were obtained directly from the GC peak areas. This GC-MS method allowed analysis of about 78 volatile compounds in the essential oil, which is a great deal better than any other method (*33*).

High Performance Liquid Chromatography (HPLC) Technique

High performance liquid chromatography is one of the fastest growing analytical method techniques in many research and industrial laboratories. It is the method of choice in the analysis of the less volatile constituents of essential oils.

Rauber and his co-workers proposed (*40*) an analytical technique for the determination of citral in cymbopogon citrates (lemon grass) volatile oil. The method involved normal-phase high performance liquid chromatography (HPLC) with UV detection because of its selectivity and sensitivity. In this method, the mobile phase used consisted of 85:15 n-hexane: ethanol with flow rate of 0.3 mL/min, and the absorbance read at 233 nm. The analytical column used was normal phase CN column. The linearity of the method was determined using standard citral as a reference substance at five concentrations, and the slopes of three calibration curves were calculated by linear regression. The citral retention time was about 13.8 min. The calibration curves were constructed by plotting peak area versus concentrations and showed good linearity. The RSD of the slopes of the three calibration lines was 1.78 with excellent correlation coefficients (r = 0.9991) (*40*). The repeatability of the method was evaluated by eight repeated assays of the volatile oil of the same concentration, with triplicate injections on the same day and under the same experimental conditions. The relative standard deviation obtained was 1.37 %. The concentration of citral in volatile oil obtained in this assay was 75.2 %. Accuracy was determined by recovery in which known amounts of

standard citral were added to the volatile oil solution samples. The recovery study was performed at three concentration levels. The mean recovery was 99.06 % with RSD less than 2.0 % (*40*).

In this study, the concentration of citral in volatile oil obtained was 75.20 %. A gas chromatograph (GC) in combination with a flame ionization detector (FID) gave concentrations similar to that obtained by HPLC. It revealed a similar citral content of 76 %, which showed the suitability of the proposed HPLC method. The HPLC method developed in this study showed excellent performance and was found to be simple, linear, precise, and accurate (*40*).

Sacks and his co-workers (*41*) employed the steam distillation and HPLC method to separate and analyze citral isomers (geranial and neral) from lemon grass oil. Steam distillation was used to isolate the citral isomers from commercial or synthetic lemon grass oil. The crude citral mixture was then subjected to vacuum distillation and six fractions were collected and analyzed by HPLC. A chromatogram was obtained with mobile phase 60:40 methanol: water. The flow rate was 2.0 mL/min and the absorption wavelength was set at 254 nm. The early fractions showed considerable enrichment of the neral isomer, while the latter fractions showed an increase in the amount of geranial. The last fraction collected was relatively pure, > 95 % geranial. In this work, the HPLC method was shown to be fast, simple, precise, and presents an immediate indication of the purity of the samples (*41*).

Because HPLC is becoming a favored analytical method for analysis of natural material and products, McKone (*42*) used the same method above to analyze essential oils in six common commercial spices (Allspice, Clove, Anise, Fennel, Dill, and

Caraway). The essential oil was isolated by steam distillation for each spice. About 25 μL of the extracted oil from each spice was dissolved in 10 mL of methanol. Furthermore, the samples then were diluted and analyzed by HPLC and the results were compared to literature chromatograms of standard essential oil extracts. The mobile phase was 85:15 methanol: water. The analytical column used was reversed phase C-18 packing. The flow rate was 1.0 mL/min and the absorbance detector was set at 254 nm. Eugenol was the major absorbing essential oil of both allspice and clove. Anethole was the predominant compound in both anise and fennel. Carvone was the only detectable UV absorbing constituent of both dill and caraway oil. The literature chromatograms of standard essential oil extracts were in general agreement with the results of the essential oils analysis in this study. This HPLC method for the analysis of the distilled essential oils was relatively simple to carry out and applied to natural products analysis (*42*).

CHPTER 3

METHODOLOGY

High Performance Liquid Chromatography

High performance chromatography, usually abbreviated as HPLC, is a widespread and commonly accepted analytical method used today. This sensitive method is a form of column chromatography used to separate compounds that are dissolved in solution. An HPLC instrument consists of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Reversed phase HPLC and normal phase HPLC are the two main types of chromatography. Normal phase HPLC is based on the use of polar stationary phases containing groups such as cyano, diol amino, or dimethylamino bonded to silica, and a non-polar mobile phase such as hexane. Reversed phase HPLC uses a non polar stationary phase such as octadecyl (C-18) and n- octyl (C-8) in conjunction with a polar mobile phase such as water, methanol, or acetonitrile (*43*).

Reversed- Phase HPLC

Reversed-phase chromatography is the most popular and widely used of liquid chromatography because of the wide range of analytes that can dissolve in the mobile phase. It is called 'reversed' because of the comparison with 'normal phase' that is called "normal" because it was invented first. As mentioned above, reversed phase HPLC uses a relatively nonpolar stationary phase and a polar mobile phase. The most common bonded stationary phases are n-octadecyl (C-18), n-octyl (C-8) and phenyl groups and they are hydrophobic. The more polar the analyte, the less it will be retained on the column. The proper selection of the mobile phase is the most important parameter in reversed-phase HPCL in the development of the separation method. Often, a gradient

from fairly polar solvents like methanol, acetonitrile, water, or mixtures of these solvents is used in the analysis to produce a mobile phase of a suitable solvent strength for a particular separation. In addition, the mobile phase must be able to keep the sample components in solution. The viscosity of the mobile phase is of concern because a less viscous solvent can be used at a higher flow rate without requiring a very high pump pressures. Purity of the mobile phase as well as its availability, cost, and ease of disposal or recycling are other important considerations in HPLC (*44*).

Detectors Used in HPLC

HPLC detectors can be classified as either bulk or property detectors. Bulk detectors respond to a mobile phase property such as refractive index, dielectric constant, conductivity, or density that is altered by the presence of the analyte. On the other hand, solute property detectors respond to properties of solute such as UV-Vis absorption, fluorescence, or electrical current that is not possessed by the mobile phase. There is no sensitive universal detector available for use in HPLC. The only really universal, bulk property HPLC detector is the refractive index detector. It is based on the presence of an analyte in a sample stream that will lead to a deflection of the light beam on the photodetector and a corresponding change in signal that is amplified and recorded. It requires excellent temperature control, and it is as much as 10^3 times less sensitive than other detectors. Therefore, the detectors most often used are those spectroscopic detectors such as UV-Vis absorption detectors or fluorescence detectors that respond to some property of the sample that is not exhibited by the mobile phase (*44*).

UV-Vis Absorption Detectors. UV-Vis detectors are the most commonly used detectors. They measure the ability of a sample to absorb light. The absorbance can be measured at one or several wavelengths. The simplest UV-Vis detectors are filter–based detectors. They are simple detectors that use Hg lamp (usually at 254 nm) in conjunction with filters. Absorption at 254 nm is extremely useful for detection of biological compounds such as proteins (amide bond absorption) or DNA (nucleotide absorption). They can also operate with D_2 or tungsten lamps in conjunction with interference filters to allow operation at other wavelengths where aromatic groups absorb (*44*).

Grating monchromators can be used for UV-Vis detection. This type of detector usually operates in either single or dual wavelength mode. It can measure absorption at one wavelength at a time but can detect over a wide range of wavelengths. Photodiode array detectors are now the most common and useful UV-Vis absorbance detectors that measure absorbance across a broad spectrum of wavelengths simultaneously. This type of detector can obtain the whole spectrum in one second and it provides good sensitivity. It is a useful diagnostic tool to identify compounds (*44*).

Fluorescence Detectors. A fluorescent detector measures the ability of a compound to absorb then re-emit light at given wavelengths. Each compound has a characteristic fluorescence. Fluorescence occurs when a compound is excited by shorter wavelength energy and then emits it at higher wavelength. Therefore, the fluorescence detector consists of a source and two monochromators or filters to isolate the excitation and emission wavelength (*44*).

Fluorescence detectors are the most sensitive of all HPLC detectors and also very selective. Simple fluorescence detectors use a mercury (Hg) lamp in conjunction with

filters, while more sophisticated systems use Xe arc lamps and monochromators. Fluorescence detectors are useful for analysis of pharmaceuticals, natural products, petroleum products, and clinical samples (*44*).

Electrochemical Detectors. Electrochemical detectors are based on amperometry, polarography, coulometry, or conductivity. They are based on the measurement of the current that is recorded when an analyte is present resulting from an oxidization/reduction reaction of the analyte at a suitable electrode. The level of the current is directly proportional to the analyte concentration. They are versatile detectors with good detection limits that can be used to detect a wide variety of organic functional groups (*44*).

Columns Used in Reversed-Phase HPLC

Columns for HPLC are almost always made from stainless steel with the most common dimensions in the range from 25 cm long and about 4 to 5 mm internal diameter. Pellicular or porous packing materials are usually used. Pellicular packing is non-porous glass or polymer beads ranging from 30 to 40 μm. Porous packing is mostly silica, alumina, or polymeric resin with diameter of 3 – 10 μm. Most porous particles are usually coated with a bonded- phase. As mentioned before, the most popular bonded-phases are usually octadecyl (C-18), octyl (C-8) functional group and phenyl groups as stationary phases in reversed phase HPLC. The choice of the mobile phase depends on the column stationary phase and the analyte. In revered-phase HPLC, the most polar analyte elutes first because it is the most soluble in the mobile phase, and increasing mobile phase polarity decreases its elution time. Often, solvents are mixed to produce a mobile phase of a suitable strength for a particular separation. For instance, various

mixtures of methanol and water are used to produce variety of different polarities. The strength of the mobile phase can be increased or decreased by controlling the water content to produce a mobile phase of a suitable strength to retain the analyte long enough for good separation to take place and elute late peaks more quickly and prevent excessive band broadening (*44*).

The C-8 is the most popular functional group on reversed phase column in which a low/medium polarity mobile phase is used. Methanol/water mobile phase is a good choice for the C-8 column. The octyl (C-8) bonded phase shows good separation of components in a sample with a wide range of polarity (*44*).

Resolution in Reversed-Phase HPLC

To obtain optimal separation, resolution, R_S, is often used to measure how well species have been separated (*44*). For instance, the resolution of two analytes, A, B can be defined as:

$$R_S = 2\,[t_B - t_A]/\,(W_A + W_B) \qquad [1]$$

where R_S is the resolution, t_A and t_B are the retention times for analyte A and B, respectively, and W_A and W_B are the widths at the base of the two peaks respectively.

To achieve better resolution, it is useful to relate the resolution to the number of theoretical plates of the column, the selectivity factor, and the retention factor as given in the following equation:

$$R = ¼\,[(\,k'/1 + k')\,(\alpha - 1\,/\,\alpha)]\,N^{½} \qquad [2]$$

where k' is the retention factor, α is the selectivity factor, and N is the number of theoretical plates in the column. To obtain high resolution, these three terms must be optimized. The first term, retention factor, k', is also called the capacity factor and is

often used to describe the migration rate of the analyte through a column (*44*). The retention factor for an anlayte is defined as:

$$k' = (t_R - t_M) / t_M \quad [3]$$

where t_R is the time between the sample injection and an analyte peak reaching a detector at the end of the column, and t_M is the time taken for the mobile phase to pass through the column. If an analyte's retention factor is less than one, then it is very difficult to determine an accurate retention time because the elution is so fast. On the other hand, high retention factors greater than 20 mean that elution takes a very long time. Therefore, a retention factor for an analyte between one and five is ideal. The separation can be greatly improved by controlling the retention factor, k', and this can be achieved by changing the composition of the mobile phase in HPLC.

The second term is the selectivity factor, α, that describes the separation of two species on the column (*44*). For instance, selectivity factor, α, for species A and B is defined as:

$$\alpha = k'_B / k'_A \quad [4]$$

where k'_B and k'_A are the retention factors of the two species. The selectivity factor, α, can be manipulated and increased to improve separation by changing mobile phase composition, changing column temperature, or changing the composition of the stationary phase.

The third term is the number of theoretical plates in a column, N. It is important to know that the theoretical plates do not really exist. They are a theoretical concept that helps to understand the process at work in the column (*44*). If the length of the column is L, then N is

$$N = L / H \quad [5]$$

where H is the plate height. The more theoretical plates, N, the higher resolution can be obtained. It is, however, not desirable to increase the length of the column because it will lead to an increase in retention time and maybe band broadening. Instead, the plate height can be reduced by reducing the size of the stationery phase particles to increase the number of the theoretical plates, N, without lengthening the column (*44*).

Proposed Research

Based upon the discussion in Chapters 1 and 2, it is important to develop methods to quantify or identify the composition of essential oils in medical herbs. Most of the research involved chromatography because it exhibits excellent recovery, reproducibility, or precision.

There are different methods that have been developed for the analysis of essential oils as were described in Chapter 2. The normal – phase liquid chromatography method by Rauber and his co–workers (*40*) analyzed the citral in lemon grass volatile oil. However, they used a hexane: ethanol mobile phase and the retention time was about 13.8 minutes for citral. Thus the method was rather time consuming, its major disadvantage. The gas chromatography method by Xiuqin and his co-workers (*28*) used the distillation method to extract the essential oils. However, distillation requires a large, bulky apparatus and it is also affected by interferences. Cost is another important consideration for any analysis. On the other hand, both methods show excellent recovery and good precision that indicates chromatography is an accepted analytical technique used today.

Based on the discussion mentioned above, a research project with the following objectives is proposed:

1. To establish optimum reversed-phase chromatographic conditions for quantification of citral in lemon grass samples.
2. To extract the essential oil from lemon grass without any technical support and to carry out the analysis using a mobile phase that is economical and environmentally friendly.
3. To apply the HPLC method to lemon grass samples that have been grown in East Tennessee and harvested at different times of the year.
4. To establish the figures of merits for the reversed-phase HPLC method: reproducibility, linear dynamic range, recovery, and accuracy.

CHAPTER 4

EXPERIEMENTAL PROCEDURES, RESULTS, AND DISCUSSION

Experimental Procedures

This chapter describes the experimental procedures preformed in order to develop and establish the validity of an HPLC method for the quantitative determination of citral in locally grown lemon grass harvested at different seasons. This method is evaluated based upon its precision, accuracy, and it applicability to the determination of citral in lemon grass leaves. To validate the method, linearity, reproducibility, and recovery studies were conducted.

Reagents

Deionized Water, obtained from US Filter Company (Pittsburgh, PA).

Methanol, Glacial Acetic Acid, and Buffer solutions of pH 4.0 and 7.0; all are ACS certified reagents obtained from Fisher Scientific (Fair Lawn, NJ).

1- Octanesulfonic acid, and Sodium dodecyl sulfate, obtained from Sigma Chemical Company (St. Louis, MO).

Cetyltrimethylammonium bromide, and Citral, 95% mixtures of cis and trans obtained from Aldrich Chemical Company (Milwaukee, WI).

Lemon grass samples were obtained from my research advisor's garden (Johnson City, TN) harvested at different times of the year.

Commercial lemon grass sample was obtained from Health Barn Store (Johnson City, TN)

Preparation of Reagents, Stock, and Working Solutions

The following reagents, stock, and working solutions were prepared.

1. Citral stock solution (8.88 mg/mL): 1 mL of citral, 95% mixtures of cis and trans, was pipetted into a 10-mL volumetric flask and then diluted to the mark with methanol.
2. Citral working solution (88.8 µg/mL): 100 µL of citral stock solution was pipetted into a 10-mL volumetric flask and diluted to the mark with methanol.
3. Mobile phase: 900 mL of methanol and 100 mL of deionized water were mixed well. Then 2.5 g of 1-octanesulfonic acid was added such that it is 0.25 % of mobile phase. The mobile phase was degassed by passing helium through it for 15 minutes before use.

Preparation of Citral Standard Solutions

The following procedure was used to generate a series of standards solutions for the calibration curve. Into separate 10-mL volumetric flasks, 10, 50, 100, and 400 µL of the citral working solution were pipetted and then diluted to the mark with methanol to make standards solutions of 0.89, 4.44, 8.88, and 35.52 µg/mL, respectively.

Preparation of Fall, Winter, Summer Cuts and Commercial Samples

Fall, winter, summer (three different cuts), and commercial lemon grass samples were first cut into very small pieces. Then about 5 grams of each sample was weighed out accurately and 50 mL of methanol was added to each sample and the samples were soaked for 48 hours. After this, each of the soaked mixture was filtered through a # 42 Whatman filter paper. The filtrate was further diluted to the mark in a 50-mL volumetric flask with methanol. Finally, 1 mL from each sample solution was pipetted into 10-mL

volumetric flasks and then diluted to the mark with methanol.

Instrumentation

The Waters HPLC system (Milford, MA) used in the project is shown in Figure 3.

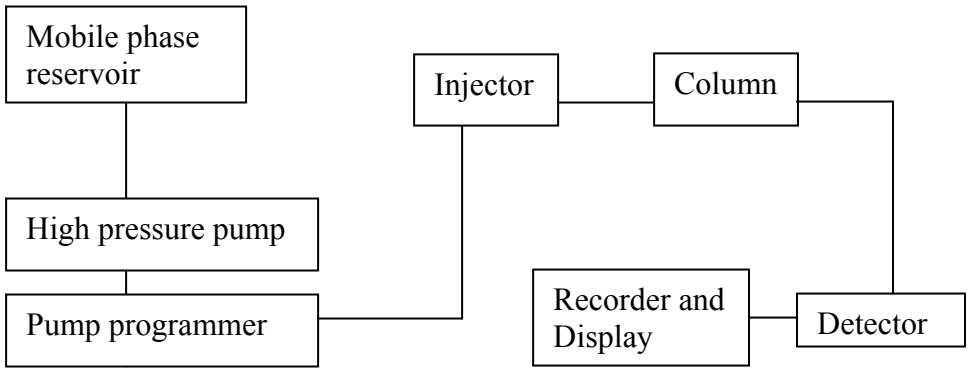


Figure 3. Schematic Diagram of the HPLC System

The system included a Model 501 reciprocating pump, and a Model 484 Tunable UV absorbance detector. The pumps were controlled by a Model 680 Automated Gradient Controller, and the data were recorded and processed on a Model 745B data Module. A Model 7725 Rheodyne loading sample injector with 20 μL loop was used. The analytical column used was a Zorbax 4.6 mm x 25 cm with reversed phase C-8 packing.

HPLC Conditions

All HPLC separations were performed with isocratic elution at a flow rate of 1.0 mL/min with an average pressure of between 1400 to 1600 psi depending on the components of the mobile phase. The mobile phase contained three components; surfactant, deionized water, and methanol. The solvent composition finally chosen was a 90:10 ratio of methanol to deionized water at pH 5.5 with 0.25% of 1-octanesulfonic acid. The UV detector was set at 233 nm wavelength. All the mobile phase solvents

used in this project were degassed by passing helium through them for 15 minutes before use to eliminate air bubbles in the HPLC system.

Data Analysis

The data obtained from the experiments were analyzed using the Excel Program, Microsoft Windows 2000. The averages and standard deviations were obtained using the statistical functions in Excel. Linear regression was also performed using the software, which gave the regression equations and the plots of the results obtained.

The data generated from the procedures described in the experimental section were tabulated and discussed in the following sections. In all instances, experiments were carried out in triplicates of the standard solutions or samples. Then each solution or sample was injected into the HPLC system also in triplicates. The peak areas of the three injections were averaged and the relative standard deviations calculated.

Results and Discussion

In this section, the results of different experiments to develop, optimize, and determine the applicability of the proposed procedure are tabulated and discussed. A series of experiments were performed to optimize the experimental conditions for the determination of citral in locally grown lemon grass samples harvested at different seasons. The proposed method was evaluated by comparisons of studies of reproducibility, linear dynamic range, and recovery with other methods that have been developed for analysis of essential oils as were described in Chapter 2.

Choice of Wavelength of Detection

The detection wave length chosen was 233 nm because it gave large analyte peak absorbance. It also showed a quieter and better baseline that meant improved signal to

– noise ratio. Rauber and his co-workers (*40*) also used 233 nm as the detection wavelength for citral. Therefore, 233 nm was chosen to be the detection wavelength used in all of the experiments.

Optimization of Mobile Phase

It is preferable to use a mobile phase that is commonly used in reversed phase HPLC, and one that is economical and environmentally friendly. To find the optimum mobile phase for analysis, different aliquots of the citral working solution was used, and diluted further if they were too concentrated, to test the applicability of different compositions of mobile phase. The mobile phases with different ratios of water and methanol were tried first. All the chromatograms obtained using mobile phase with ratios of methanol to water 20:80, 30:70, 40:60, 50:50, and 60:40 had a shoulder or overlap with other peaks or have wider peaks and longer retention times.

Higher proportions of methanol in mobile phase such as 80:20 and 90:10 methanol: water were also tried. But the citral was eluted by these mobile phases too quickly. So this mobile phase could not be used for separation. In Figure 4, the chromatograms obtained with methanol:water 80:20, 90:10 mobile phases are shown. Chromatogram (a) was that obtained with mobile phase methanol: water 80:10; while chromatogram (b) was that obtained with methanol: water 90:10. The retention time was 1.45 min and 1.40 min, respectively. We can also see from Figure 4, that there was an unwanted small shoulder (retention time 2.16 min) on the peak of standard citral at retention time of 1.45 minutes.

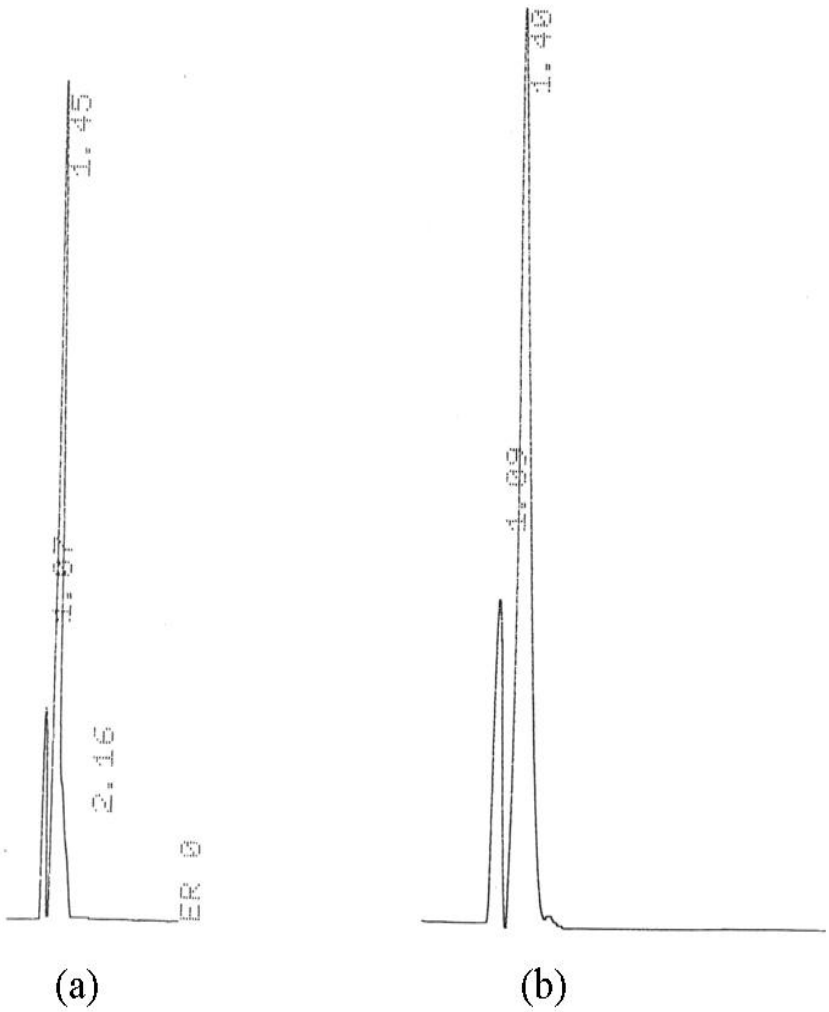


Figure 4. HPLC Chromatograms for 4.44 µg/mL Citral Standard Solution with Different Mobile Phases.
Note: The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The chromatogram (a) 80:20 methanol: water and the retention time of citral peak was 1.45 minutes. The chromatogram (b) 90:10 methanol: water and the retention time of citral peak was 1.40 minutes.

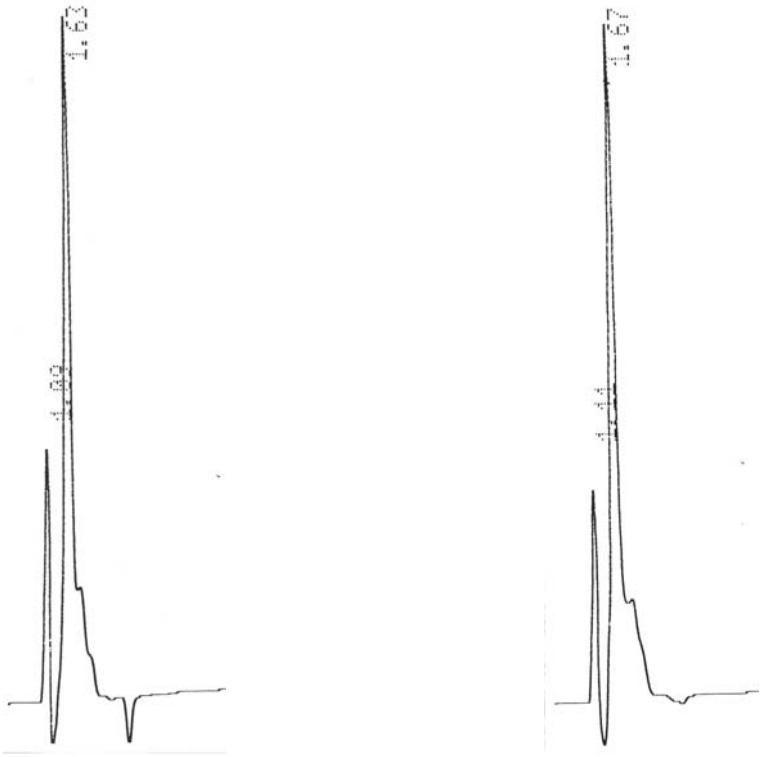
Adjusting the pH of the mobile phase by adding glacial acetic acid was also tried to explore the effects of pH on the retention time and resolution. The mobile phase pH was adjusted to 3.5 and 5.0 - 6.5 by the addition of glacial acetic acid to the mobile phase with different ratios of methanol: water 80:20, 85:15, and 90:10. These pHs values did not appear to have a significant effect upon the resolution or retention time. The peak of

citral has almost the same retention time and it did not significantly affect the chromatographic outcome. The pH value, therefore, was determined to be not as important as just simply to control the mobile phase at a constant pH. The pH, if not controlled, could fluctuate depending upon the ratio of methanol and water used, resulting in a slight lack of reproducibility of the retention times. Therefore, it was decided that the pH be controlled. The pH of the mobile phase was adjusted and maintained at about 5.0 by the addition of glacial acetic acid.

After several trials, better chromatograms were found to be obtained by adding different surfactants to the mobile phase. Surfactants are compounds that have long non-polar hydrocarbon chains with polar groups at the head of the chain, and they are often introduced into the mobile phase (*45*). It is believed that by adding surfactants one can improve resolution and retention time by increasing the interaction of the mobile phase and stationary phase by facilitating the rate of their interaction. The amount of added surfactant in the mobile phase was about 0.25%. The first surfactant used was sodium dodecyl sulfate (SDS) for the mobile phase with ratio of 80:20 methanol: water at pH 5.0. The use of the SDS in the mobile phase gave a little bit longer retention time of 1.63 minutes for citral than when the surfactant was not part of the mobile phase.

Cetyltrimethylammonium bromide (CTAB) was the second surfactant that was attempted in the mobile phase at a ratio of 80:20 methanol: water at pH 5.0. With the use of this surfactant, the retention time was 1.67 minutes for citral that was almost the same retention time of citral as that mobile phase containing SDS. Figure 5 shows representative chromatograms for 8.88 μg/mL of citral standard solution using a mobile phase that included the added surfactants, SDS and CTAB. In both chromatograms (a)

and (b) in Figure 5, a small shoulder on the peak of citral can be seen. The baselines of both chromatograms were not as good as one would like. The SDS and CTAB surfactants appeared to give somewhat similar results.



(a) **(b)**

Figure 5. HPLC Chromatograms for 8.88 µg/mL Citral Standard Solution with Different Surfactants.
Note: The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. Mobile phase was 80:20 methanol: water with 0.25% of surfactant added and pH adjusted to 5.0 with glacial acetic acid. Surfactants used were: in chromatogram (a) Sodium dodecyl sulfate (SDS) and the citral peak corresponds to retention time of 1.63 minutes, (b) Cetyltrimethylammonium bromide (CTAB) and the citral peak corresponds to retention time of 1.67 minutes.

Another surfactant, 1-octanesulfonic acid (OSA), was also tested. This surfactant was added to the mobile phase with 90:10 methanol: water at pH 5.0, but the amount added was the same as that for SDS, and CTAB, about 0.25%. The mobile phase with

90:10 methanol: water at pH 5.0 with OSA surfactant added gave a chromatogram with a sharp peak and retention time of about 3.92 minutes. The increase in retention time by added OSA was due to increasing of the surface tension between the stationary phase and the mobile phase, which affected the transfer of the solute (citral) from the mobile phase to the stationary phase and vice versa. The baseline is quiet with a few barely detectable peaks beside the citral peak. The chromatogram obtained using 90:10 (methanol: water and OSA surfactant) is shown in Figure 6. The other small peaks are traces of other components that are present in the standard solution or mobile phase components.

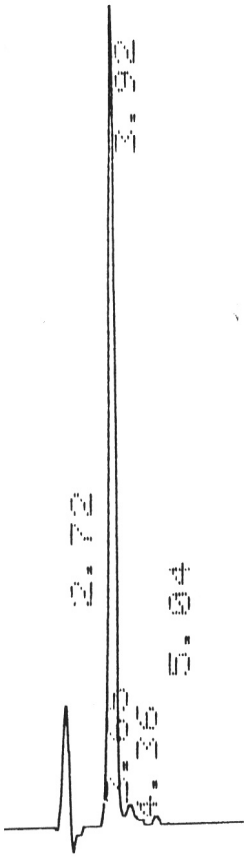
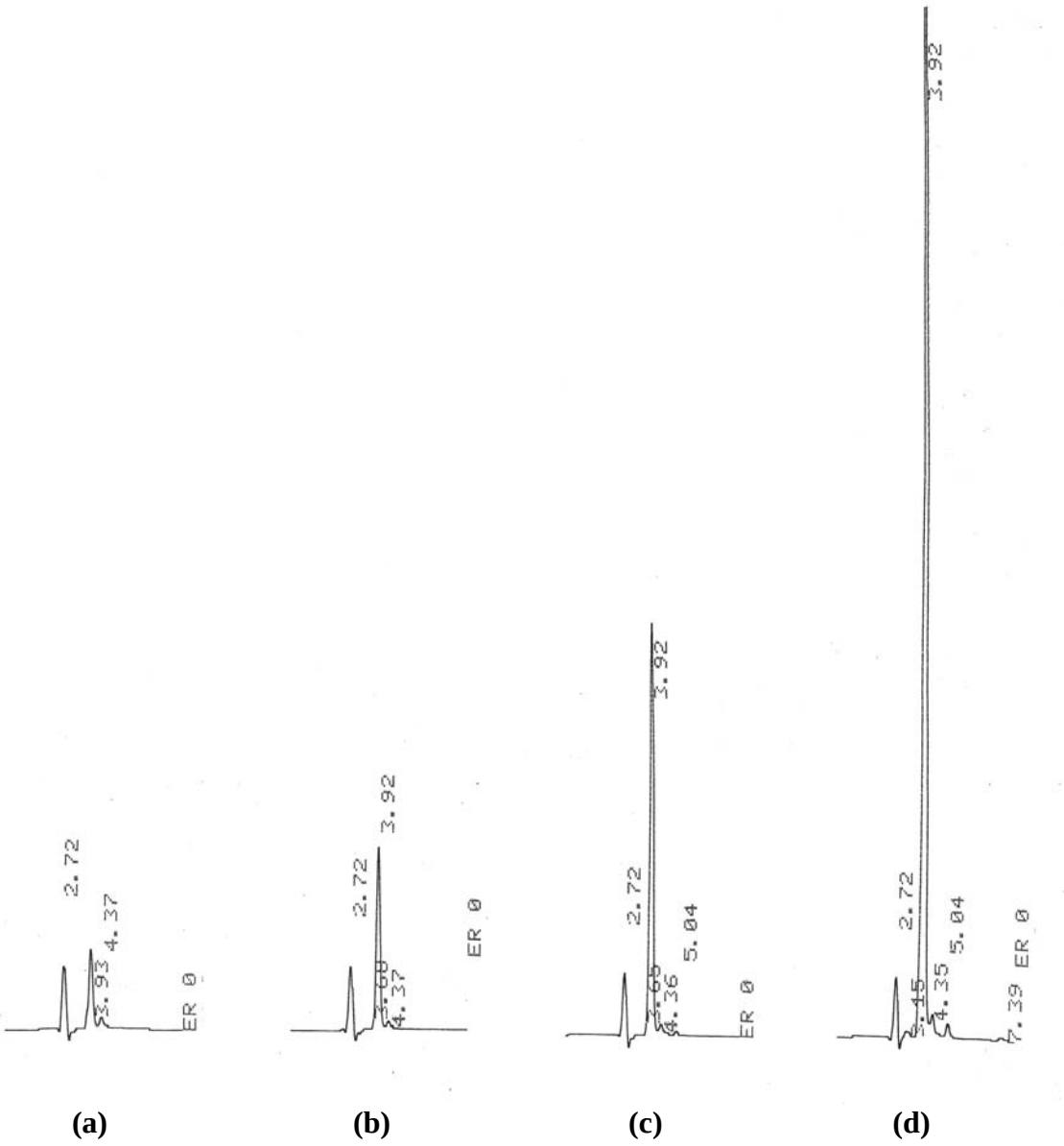


Figure 6. HPLC Chromatogram for 8.88 µg/mL Citral Standard Solution.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The citral peak is the peak at 3.92 minutes.

Figure 7 are chromatograms for 10, 50, 100, 400 µL, respectively, corresponding to 0.89, 4.44, 8.88, and 35.52 µg/mL of citral standard solutions using the 90:10 methanol: water mobile phase that included 0.25 % of OSA.



(a) **(b)** **(c)** **(d)**

Figure 7. HPLC Chromatogram for (a) 0.89, (b) 4.44, (c) 8.88, and (d) 35.52 µg/mL of Citral Standard Solution.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The citral standard solution is the peak at retention time of 3.92 minutes.

We can see from Figure 7 that the values of the retention time remain relatively constant for all citral standard solutions regardless of their amounts with slight variation could be due to fluctuations in instrumental conditions. Height of peaks increased as the concentration of citral increased with a sharp single peak of citral. The other minor peaks all remain mostly constant in area except for disappearance or appearance of some other minor peaks with concentrations. The most significant of the minor peaks at retention time of 2.72 min is most likely a compound found in the components of mobile phase as its size remains constant throughout, with changes in concentration of citral standard. Thus, the mobile phase of 90:10 methanol: water with 0.25 % of OSA was chosen for further studies. Linearity of peak areas with concentration was also confirmed for this mobile phase. Because of the excellent linearity with this mobile phase, it was used for the rest of the experiments. In addition, chromatograms obtained with the mobile phase containing 0.25 % of OSA gave a large sharp peak with a reasonable retention time.

Linear Dynamic Range

In this study, linearity of peak area with citral concentrations was investigated. Two separate experiments were done. For the first experiment, three sets of citral standard solutions ranging in concentration from 0.89 up to 22.20 µg/mL were prepared and determined in succession. Each concentration of each standard solution set was injected into HPLC in triplicates and the data are presented in Table 3.

The correlation coefficients of the three curves was better than 0.99. The calibration curve is plotted for the average of the three trials in Figure 8. The correlation coefficient was 0.9984. The equation of the regression line was $Y = 151320X + 189029$.

Table 3. Areas of Analyte Peak at 3.94 min.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. Three calibration sets of citral standard were prepared in succession. Each area reported is an average of triplicate injections and relative standard deviation (RSD) given in parenthesis.

Citral Conc. (µg/mL)	Trial 1 Area (Average, RSD)	Trial 2 Area (Average , RSD)	Trial 3 Area (Average , RSD)
0.89	381513 (3.75)	362471 (0.84)	359022 (3.94)
4.44	762048 (1.10)	748463 (0.92)	829868 (0.17)
8.88	1882361 (0.97)	1418647 (0.94)	1409282 (0.08)
22.20	3831770 (0.91)	3783869 (0.02)	3027717 (2.20)
Correlation Coefficient	0.9892	0.9943	0.9996

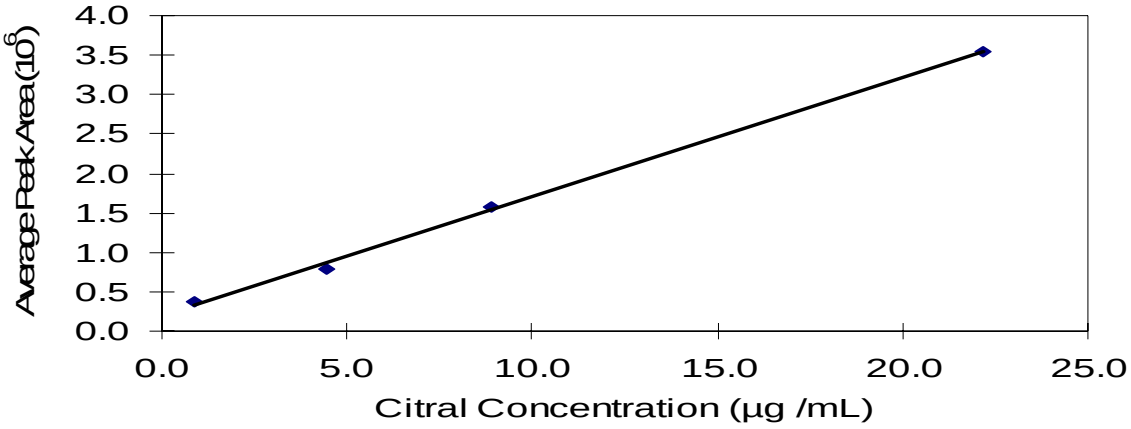


Figure 8. Plot of Calibration Curve Using 0.89, 4.44, 8.88, and 22.20 µg/mL of Citral Standard Solution.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The equation for the regression line is Y = 151320X + 189029 with a correlation coefficient of 0.9984 for the average of the three trials.

In the second experiment, a calibration curve chosen for use in the determination of citral in lemon grass was obtained by preparation 0.89, 4.44, 8.88, and 35.52 µg/mL of citral standard solution. Every standard solution was injected into HPLC in triplicate and

the results are presented in Table 4. The results are also graphed and regression analysis done. The linear regression line was plotted in Figure 9. The regression line has an equation of Y = 180356X + 327424. The correlation coefficient is 0.997 showing the calibration curve was linear for citral standard solutions of concentrations of 0.89, 4.44, 8.88, and 35.52 µg/mL indicating that the procedure was satisfactorily linear.

Table 4. Areas of Analyte Peak at 3.94 min.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. Each area reported is an average of triplicate injections and relative standard deviation (RSD) given in parenthesis.

Citral Concentration (µg/mL)	Area (Average , RSD)
0.89	494115 (5.2)
4.44	949796 (1.5)
8.88	2128637 (1.0)
35.52	6705906 (0.43)
Correlation Coefficient	0.997

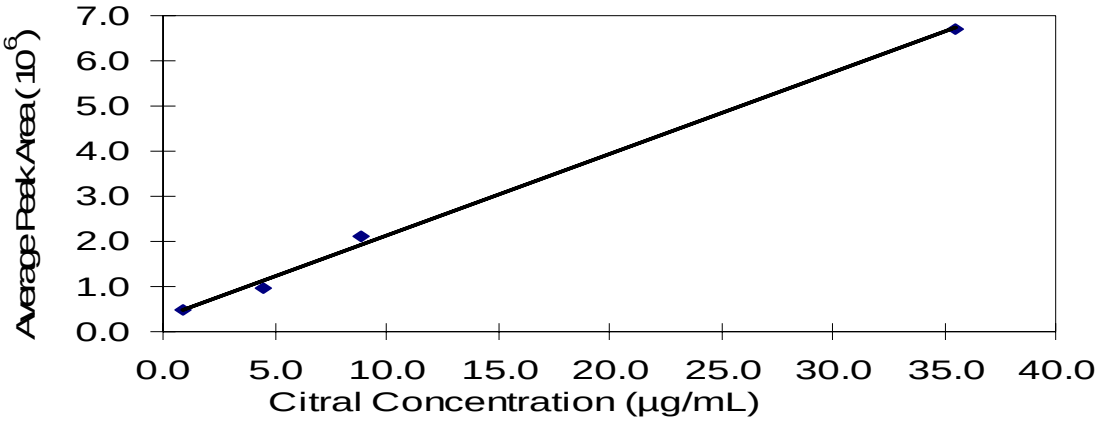


Figure 9. Plot of Calibration Curve Using 0.89, 4.44, 8.88, and 35.53 µg/mL of Citral Standard Solution.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The equation for the regression line is Y = 180356X + 327424 with a correlation coefficient of 0.997.

Reproducibility Studies

The new HPLC method must be reproducible for it to be applicable. Six commercial lemon grass samples were prepared as described previously in this chapter. The reproducibility was studied by injecting the six commercial lemon grass samples and a set of citral standard solutions into the HPLC system on the same day. Each sample and standard solution was injected in triplicates, and the average and relative standard deviation of the triplicate injections were calculated for each sample. A calibration curve of the kind in Figure 14 was used to calculate the concentration of citral in the six commercial samples. The average percentage content of citral for all six commercial samples is 0.14% with a relative standard deviation of 2.8%. The results of reproducibility studies obtained for commercial lemon grass samples are presented in Table 5.

Table 5. Results of Reproducibility Study for the Average Percentage Content of the Citral in Commercial Lemon Grass Sample.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The retention time of analyte peak was 3.94 min. Each percentage content reported is an average of triplicate injections.

Sample	% of Citral found
1	0.067
2	0.068
3	0.064
4	0.069
5	0.067
6	0.069
Average	0.067
R.S.D	2.8 %

In the second reproducibility study, the same format and procedure as in the first reproducibility study was implemented on a summer lemon grass sample. The average

percentage content of citral for the six samples is 0.14 with a relative standard deviation of 10.8%. The inhomogeneity that gave rise to different citral contents among the summer samples in Table 6 maybe because of the extraction step described previously. The results of reproducibility studies obtained for summer samples are presented in Table 6. In Table 6, the citral found in sample # 5 was higher than the other samples that caused the increase of the relative standard deviation for the summer sample. The reason for this deviation was not clear. It could be experimental. .

Table 6. Results of Reproducibility Study for the Average Percentage Content of the Citral in Summer Lemon Grass Sample.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The retention time of analyte peak was 3.94 min. Each percentage content reported is an average of triplicate injections.

Sample	% of Citral found
1	0.13
2	0.13
3	0.14
4	0.15
5	0.17
6	0.15
Average	0.14
R.S.D	10.8 %

Recovery Studies

To determine the accuracy of the method developed, recovery studies were carried out. A set of fresh citral standard solutions with 4.44, 8.88, and 35.52 µg/mL citral was prepared for use in making the calibration curve and summer lemon grass samples were prepared as described previously. Each citral standard solution and the analyte (summer sample) were injected into HPLC system in triplicates. For the recovery study, two aliquots of 1 mL of stock summer lemon grass sample solution were delivered

into two10–mL volumetric flasks (A, B). To the same flasks (A, B), 75 and 150 µL of citral working solution were delivered, respectively, and diluted with methanol. Each sample was prepared in triplicate. Each sample was also injected into the HPLC system in triplicate. In order to calculate the percent recovery, the difference between the average peak area of the analyte plus the amount of the citral working solution added and the average peak area of the analyte was obtained, and then this difference divided by the average area of the analyte as given by the following equation:

% Recovery = [Average peak area of (analyte + citral working solution added)

- Average peak area of analyte] /

[Average peak area of citral working solution added to the analyte]

The calibration curve with 4.44, 8.88, and 35.52 µg/mL of standard citral to calculate the average peak area of citral working solution added to the analyte was shown in Figure 10. The linear regression equation Y = 191255X + 379643 used for all the calculations. The results of the recovery are presented in Table 7.

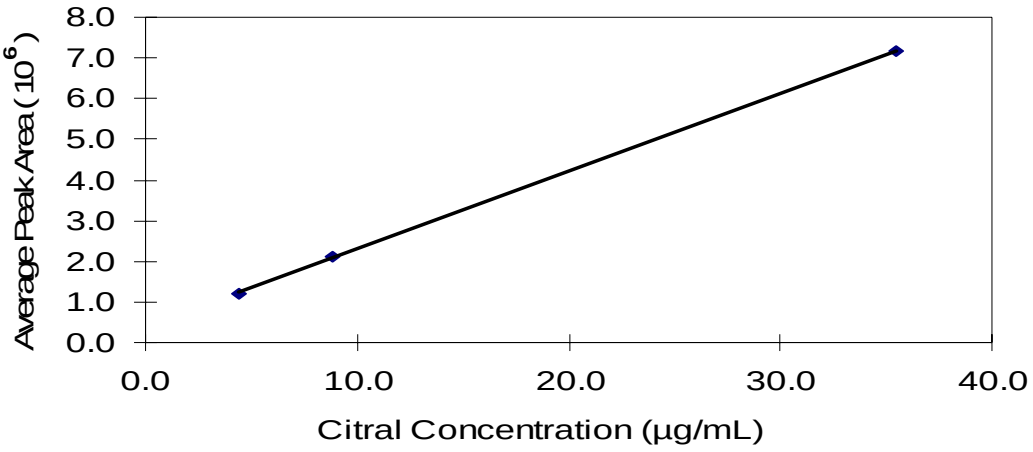


Figure 10. Plot of Calibration Curve Using 4.44, 8.88, and 35.53 µg/mL of Citral Standard Solution.
Note: The linear regression equation is Y = 191255X + 379643 with a correlation coefficient of 1.00.

Table 7. Recovery Results for Analysis of Analyte Peak of Summer Lemon Grass Sample.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The retention time of analyte peak was 3.98 min. Each percentage content reported is an average of triplicate injections.

Flask	µL of working citral added	% Recovery (R.S.D %)
A	75	106.3 (3.58 %)
B	150	113.0 (1.99 %)

A similar recovery study was also performed on a different lemon grass sample. The same format and procedure used in the first recovery study was carried out. However, the amount of the citral working solution added to the flasks that contain1 mL of stock summer lemon grass sample solution was 100, and 200 µL. The calibration curve with 4.44, 8.88, and 35.52 µg/mL of citral standard solution was made (Figure 11) to calculate the average peak area of citral working solution added to the analyte by using the linear regression equation Y = 186308X + 250680 . The results of the recovery study are presented in Table 8.

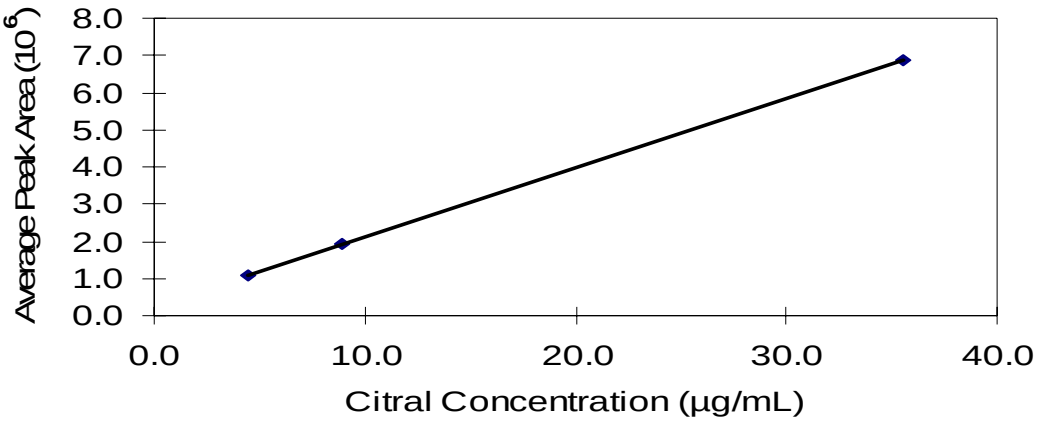


Figure 11. Plot of Calibration Curve Using 4.44, 8.88, and 35.52 µg/mL of Citral Standard Solution.
Note: The linear regression equation is Y = 186308X + 250680 with a correlation coefficient of 1.00.

Table 8. Recovery Results for Analysis of Analyte Peak of Summer Lemon Grass Sample.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. The retention time of analyte peak was 3.98 min. Each percentage content reported is an average of triplicate injections.

Flask	µL of working citral added	% Recovery (R.S.D %)
A	100	99.9 (6.04 %)
B	200	100.0 (0.25 %)

The overall average recovery for all samples tested was 104.8 %. This indicates that the method is accurate for the determination of citral in lemon grass samples. The recovery range is 99.9 – 113.0 with a relative standard deviation of 5.2 %. This is an acceptable level of precision and these results indicted that the proposed method had satisfactory accuracy. The variation of the recovery in the sample was within experimental error.

Application of the Proposed HPLC Method to Fall, Winter, Summer Cuts and Commercial Samples

The proposed method was applied to determine the amount of citral in locally grown lemon grass samples harvested at different seasons and in a commercial lemon grass sample (dried leaves) obtained from a local store. Each sample was prepared as described in the experimental procedure section (preparation of fall, winter, summer cuts and commercial samples). The analysis of each sample was done in triplicate and each sample was injected into HPLC system in triplicate. A calibration curve of the kind shown in Figure 9 with citral standard solutions of concentration 0.89 to 35.52 µg/mL was used to calculate the concentration of citral in the samples, and the relative standard deviation (RSD) was obtained for each trial. Representative chromatograms that show the

citral peak at a retention time of 3.94, 3.94, 3.98 3.84 minutes for fall, winter, summer and commercial sample, respectively are shown in Figure 12. We can see from Figure 12 that citral peak of commercial sample is slightly less in retention time (retention time 3.84), still satisfactory though it was not as well resolved.

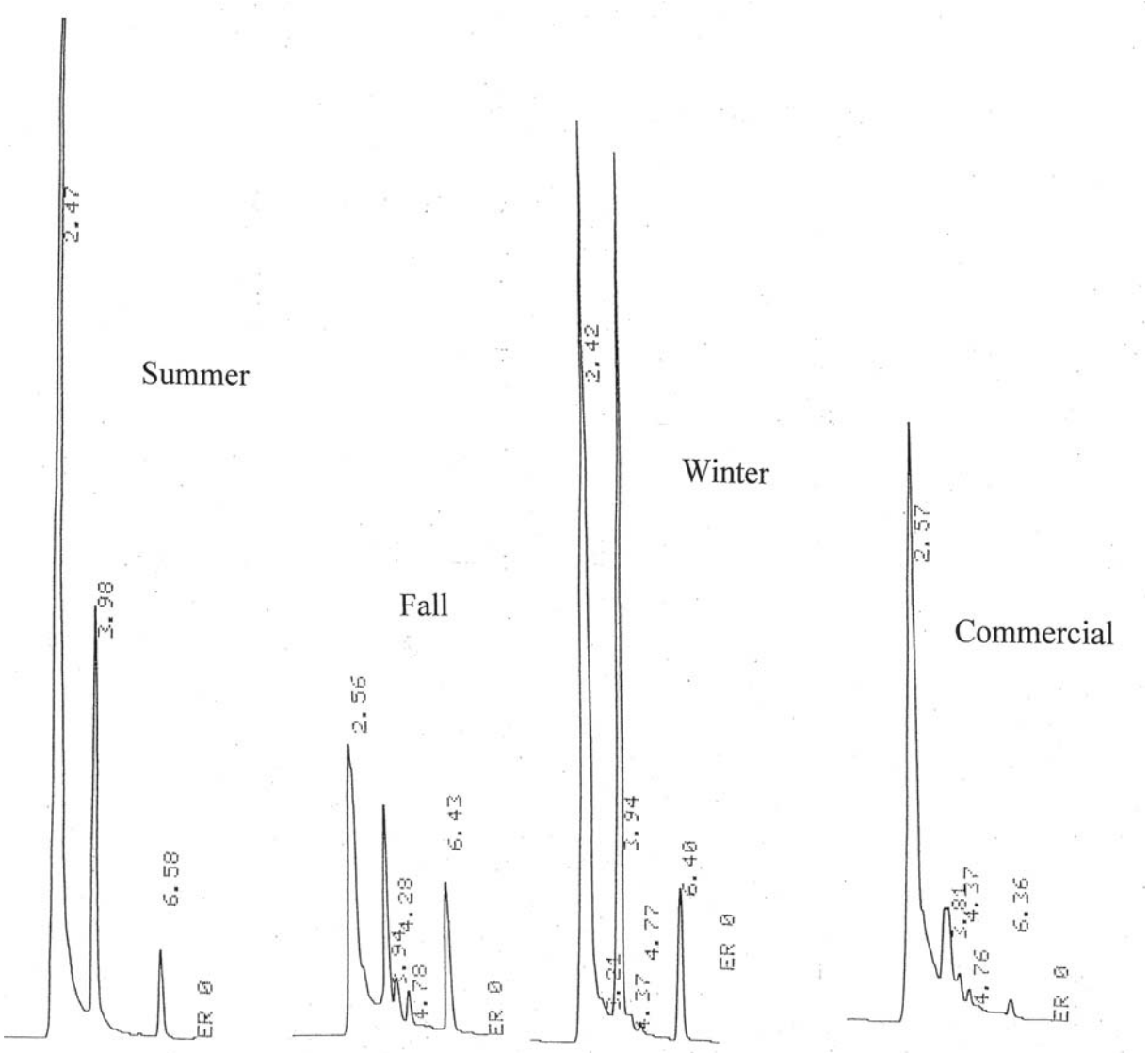


Figure 12. HPLC Chromatogram for Fall, Winter, Summer and Commercial Lemon Grass Samples.
Note: The mobile phase was 90:10 methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and absorption wavelength was at 233 nm. The citral peak corresponds to retention times of 3.94, 3.94, 3.98 and 3.81 minutes for fall, winter, summer and commercial samples, respectively.

The commercial sample was most likely quite old and had been sitting on the shelf for a while. There might be some degradation products present. Thus compounds in the commercial sample may have caused interference in the reversed phase column and overlapping absorption at 233 nm. The results of the HPLC determination of citral in the samples are presented in Table 9.

Table 9. Results From Analysis of Fall, Winter, Summer Cuts and Commercial Lemon Grass Samples.
Note: The mobile phase was 90:10methanol: water with 0.25 % of 1-octanesulfonic acid (OSA) and pH 5.0. The flow rate was 1.0 mL/min and the absorption wavelength was set at 233 nm. Each sample was analyzed in triplicate corresponding to Trials 1, 2, and 3. Each trial reported is an average of triplicate samples.

SAMPLES	TRIAL 1 Citral Cocn. (µg/mL) RSD (%)	TRIAL 2 Citral Cocn. (µg/mL) RSD (%)	TRAIL 3 Citral Cocn. (µg/mL) RSD (%)
Fall	0.072 (5.6)	0.096 (2.7)	0.11 (5.2)
Winter	0.26 (2.3)	0.26 (2.2)	0.30 (2.0)
Summer (Cut 1)	0.093 (0.6)	0.12 (0.0)	0.10 (0.0)
Summer (Cut 2)	0.13 (4.3)	0.13 (4.4)	0.14 (4.1)
Summer (Cut 3)	0.15 (3.9)	0.17 (3.5)	0.15 (4.0)
Commerical	0.067 (0.9)	0.064 (1.9)	0.068 (2.2)

The calculated average percentage of citral for the three trials with relative standard deviation was 0.093(16.1%) , 0.27 (6.6%), 0.10 (11.0%), 0.13 (3.1%), 0.16 (5.6%), and 0.066 (1.5%) for fall, winter, summer(cut 1), summer (cut 2), summer (cut 3), and 1 commercial store sample, respectively. The RSD obtained for the average of three trials of fall and summer (cut 1) samples are relatively higher RSD, but still satisfactory. This could possibly be because of the extraction step from the dried leaves of lemon grass given rise to variations among samples.

The proposed HPLC method of Rauber and his co-workers (*40*) or the study by Wilson and his co-workers (*27*) was to determine the concentration of citral in volatile oil extracted from lemon grass. Therefore, there were no specific values reported for the concentration of citral in dry leaves of lemon grass to compare. The highest concentration of citral was found in the "winter" samples. The "winter" sample was actually the lemon grass that had been growing all summer and fall and kept in the house to winter over. So the leaves had been growing for the longest period. This is maybe the reason for the higher content of citral in them. The lowest citral content was found in the commercial sample. This is expected because it has long shelf-life and also the dried leaves of lemon grass are crushed very fine in the commercial sample. However, degradation over time also might cause less of citral content.

The most interesting results are these given by the summer lemon grass that had been "freshly" growing in the garden during the season. As one progressed during the season, the amount of citral found in the leaves gradually increased (from cut 1 to cut 3). So it seems that the lemon grass leaves did produce more citral as the plants grew older.

CHAPTER 5

CONCLUSIONS

The proposed HPLC method for the determination of citral in lemon grass samples has been shown to be a simple, rapid, precise, and accurate method. The lemon grass leaves were harvested fresh at different times of the year and were soaked in methanol for 48 hours without any mechanical assistance. The extraction is simple, clean, and fast. The samples and reagents are easy to prepare. Different ratios of methanol and water were tried as the mobile phase. To improve the chromatogram, sodium dodecyle sulfate (SDS), 1- octanesulfonic acid (OSA), and Cetyltrimethylammonium (CTAB) as surfactants are added to the methanol- water mobile phase. The chosen mobile phase with 90:10 methanol: water with 0.25% 1-octanesulfonic acid and controlled at pH 5 gave chromatogram with a sharp single peak with large analyte peak area at 233 nm wavelength of absorption by UV detector. With 0.25% of OSA in the mobile phase, the retention time was increased to about 3.94 minutes, and the peak area was larger and better resolved than that of the mobile phase with the other two surfactants.

Form the data and discussion presented in Chapter 4, the proposed method was shown to have good reproducibility from the consistent results during the entire project. In this study, the precision was shown by the results of reproducibility studies obtained for six commercial samples and six summer samples. The RSD obtained was 2.8% for commercial samples and 10.8 for summer samples. These results are deemed satisfactory.

The linearity of the proposed method from many experiments was very

satisfactory and consistent. The linearity studies showed that the proposed method is linear from 0.89 to 35.25 μg/mL of citral standard solutions with a high correlation coefficients greater than 0.99. The linear range is most likely much wider than the data we have obtained.

The HPLC method for analysis of citral was also evaluated in term of its accuracy. The recovery studies indicated acceptable accuracy for the proposed procedure. Recovery studies gave percent recoveries ranging from 99.9 % to 133.0 % with an average recovery of 104.8 % and RSD of 5.2 %.

From the experimental results, the possibility of this method for the determination of citral in locally grown lemon grass harvested at different seasons was established. The amount of citral found as a percentage of the dried leaves are, 0.093, 0.27, 0.10, 0.13, 0.16, and 0.066 for fall, winter, three summer, and 1 commercial store samples, respectively.

An advantage of the proposed method is the widespread availability of the well established and developed reversed-phase HPLC instrumentation. The proposed method has a relatively short analysis time per sample (less than 6 minutes) once the HPLC is set up properly. Therefore, the proposed HPLC method is fairly quick for analysis of citral in lemon grass. The mobile phase, methanol and water with 0.25% of OSA as surfactant, are reasonably economical and environmentally friendly.

One aspect of the procedure that can be done in the future work would be the study of the extraction procedure to include time of extraction by methanol, assistance of extraction by sonication and other means without causing volatilization of citral. The other aspect for study may be to look at more closely location and length of growth of the

lemon grass and how they relate to the citral contents found in them.

From the results and discussions, the proposed method is reliable, precise, accurate, economical, and practical for quantitative analysis of citral in lemon grass and may also be applicable to analysis of citral in other natural products.

REFERENCES

1. Parry, E.J. *The Chemistry of Essential Oils and Artificial Perfumes*. 4th Ed.; Scott, Greenwood and Son: London, UK, 1921; Vol. 1, pp 63, 270, 71-73, 270

2. Gerard, J. *The Herbal or General History of plants*. Dover Pub. Inc.: New York, 1975; pp1-3.

3. Le Strange, R. *A history of herbal plants*. Arco Pub. Co. : New York, 1977; pp 1 -13.

4. Clarkson, R.E. *Herbs their Culture and uses*. The Macmillan Company: New York, 1966; pp 46-51, 78-79.

5. Grieve. M. *Culinary Herbs and Condiments*. Dover Pub. Inc. : New York, 1971; pp 39 - 42, 59 – 63.

6. Harris, B. *Better Health with Culinary Herbs*. Barre Pub. : Barre, MA, 1971; pp 3-10.

7. Craker, L.E.; Simon, J.E. *Herbs, Spices and Medicinal Plants: Recent Advances in Botany, Horticultures and Pharmacology*. The Oryx Press: Phoenix, AZ, 1986; Vol. 1, pp 33-34, 81-82.

8. Parry, E.J. *The Chemistry of Essential Oils and Artificial Perfumes*. 4th Ed.; Scott, Greenwood and Son: London, UK, 1922; Vol. 2, pp 1-12.

9. Garin, D.L. *J. Chem. Ed.* 1976, *53*, 105.

10. Practical Action: The Schumacher Centre for Technology & Development. Small-Scale Production of Essential Oils. http://www.itdg.org/docs/technical_information_Service/essentialoils.pdf. 2005

11. Lemon Grass (cymbopogon citratus). http://www.uni-graz.at/~katzer/engl/Cymb_cit.html. 2005

12. Wilson, C.M. *Grass and People*. University of Florida Press: Gainesville, 1961; pp 75.

13. Gilman, E. F.; Florida Cooperative Extension Service: Institute of Food and Agriculture Sciences. University of Florida, Gainesville, 1999. Lemon Grass. http://edis.ifas.ufl.edu. 2005.

14. Sofowora, A. *Medicinal Plants and Traditional Medicine in Africa*. John Wiley & Sons Inc. : New York, 1982; pp 71-72.

15. Schimelpfenig, C.W. *J. Chem. Ed.* 1978, *55*, 306.

16. Simonsen, J.L. *The Terpenes*. 2nd Ed. Cambridge University Press: Cambridge, 1947; Vol. 1, pp 42 - 45, 70 - 85.

17. Bertram. *Schimmel's Report*. 1888, *17*.

18. Semmler, F.W. *Ber. Dtsch. Chem. Ges.* 1890, *23*, 2965-3556.

19. Dodge. *Amer. Chem J.* 1890, 12, 553.

20. Semmler, F. W. *Ber. Dtsch. Chem. Ges.* 1891, *24*, 201.

21. Barbier; Bouveault. *Comp. Rend.* 1894, 118, 983, 1050 ; 1895, *121*, 1159; 1896, *122*, 293.

22. Kruger; Stiehl; Tiemann. *J. Pr. Chem.* 1898, *58*, 51; 1899, *59*, 497.

23. Tiemann. *Ber. DTSCH. Chem. Ges.* 1899, *32*, 117.

24. Zeitshel. *Ibid.* 1906, 39, 1787.

25. Lowless, J. Lemongrass oil. *Complete Essential Oils*. Mustard Books: Bath, 1999, pp 132-133.

26. Tyler, E.V.; Lynn, R. B.; Robbers, J. E. *Pharmacognosy*. 9th Ed. Lea and Febiger: Philadelphia, 1988; pp 123-124.

27. Wilson, N.; Ivanova, M.; Watt, R.; Moffat, A. "The quantification of Citral in Lemon Grass and Lemon Oils by Near-Infrared Spectroscopy." *The Journal of Pharmacy And Pharmacology*. 2002, *54*, 1257-1263.

28. Xiuqin, L.; Jia, Y.; Song, A.; Chen, X. ; Bi, K. "Analysis of the Essential Oil from Radix Bupleuri Using Capillary Gas Chromatography." *The Pharmaceutical Society of Japan*. 2005, *125*, 815-819.

29. Motoo, Y.; Sawabu, N. *Cancer Lett.* 1994, *86*, 91-95.

30. Bermejo, P.; Abad, M.; Silvon Sen, A.; Sonchez Contreras, S.; Diaz lanza, A. *Life Sci.* 1998, *63*, 1147- 1156.

31. Hattori, T.; Ito, M.; Suzuki, Y. *Nippon Yakurigaku Zasshi*. 1991, *97*, 13-21.

32. Husek, P.; Mack, K. *J. Chromatgr*. 1975, *133*, 139-230.

33. Pala-Paul, J.; Perez-Alonso, M.; Velasco-Negueruela, A.; Sanz, J. "Analysis by Gas Chromatography – Mass Spectroscopy of the Volatile Components of Ageratina Adenophora Spreng., Growing in the Canary Islands." *J. of Chromatography*. 2002, *947*, 327-331.

34. Adams, R. *Identification of Essential Oils Compounds by Gas Chromatography – Mass Spectroscopy*. Allured Publishing Co. : IL, 1997.

35. Jennings, W.; Shibamoto, T. *Qualitative Analysis of Flavor and Fragrance Volatile by Capillary Gas Chromatography*. Academic Press: New York, 1980.

36. Libbey, L.; Essent, J. *Oil Res.* 1991, *3*, 192.

37. Swinger, A.; Silverstein, R. *Aldrich Chemical Co.* Milwaukee, WI, 1981.

38. Epstein, W.; McGee, L.; Poulter,C.; Marsh, *J. Chem. Eng. Data*. 1976, 21, 500.

39. Joulain, D.; Konig, A.; Verlag, E. *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons* Hamburg, Germany, 1998.

40. Rauber, C.; Guterres, S.; Schapoval, E. "LC Determination of Citral in Cymbopogon Citratus Volatile Oil." *J. of Pharmaceutical And Biomedical Analysis*. 2004, *37*, 597-601.

41. Sacks, J.; Greenley, E.; Leo. G.; Willey, P.; Gallis, D.; Mangravite. J. "Separation and analysis of Citral Isomers." *J. Chem. Ed.* 1983, *6*, 434-436.

42. Mckone, H. T. "High Performance Liquid Chromatography of Essential Oils." *J. Chem. Ed.* 1979, *56*, 698.

43. Skoog, A.; West, D.; Holler, F. *Fundamentals of Analytical Chemistry*. 7th Ed.; Harcourt College Publishers: Philadelphia, PA, 1997.

44. Skoog, A.; Holler, F.; Nieman, T. *Principles of Instrumental Analysis*. 5th Ed.; Saunders Golden Sunburst Series; Harcourt Brace College Publishers: Philadelphia, PA, 1998.

45. Solomons, T. *Organic Chemistry*. 6th Ed.; John Wiley & Sons Inc. : New York, 1996.

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