A Simple Method for Isolation of Citral using Column Chromatography

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Abstract: Citral is the main component of lemongrass (*Cymbopogon citratus*) oil. This compound has biological activities such as antibacterial, antifungi, analgesic, and antiinflammation. Citral also has importance for its use as starting material for the synthesis of Vitamin A. Due to the broad utilisation of citral, it is important to develop method of isolation which is relatively simple, low cost, but able to give pure citral in high yield. Materials and method. Citral was isolated from commercially available lemongrass oil by simple column chromatography using silica gel as stationary phase. Elution was carried out in isoctaric mode. Mobile phase was chosen among hexane – diethyl ether, hexane – ethyl acetate, and hexane – ethanol based on separation factor and Rf value on thin layer chromatography. Optimum ratio of sample and stationary phase was also optimized based on isolation yield. Isolated citral was analyzed by gas chromatography – mass spectroscopy. The best separation factor on TLC was obtained from hexane – ethyl acetate (97:3) as eluent. The best yield (49,61 ± 2,59 %) was obtained when stationary phase was used at ratio 20:1 to sample.

1 INTRODUCTION

Lemongrass (*Cymbopogon citratus)* is a fast-growing aromatic grass, growing to about 1 meter (3 feet) high with long, thin leaves (Joga Rao *et al*., 2015). It is native to Sri Lanka and South India and is now widely cultivated in the tropical areas of America and Asia, including Indonesia (Ravinder *et al*., 2010). Lemongrass is also one of the main essential oil producing plants in Indonesia (Marques & Kaplan, 2013). In Indonesia, it is commonly known as *sereh dapur* and its stem is used as a spice because of its distinctive lemon-like aroma. This lemony odor is due to its high content of the aldehyde citral, which ranges from 65% to 85% (Ravinder *et al*., 2010).

Citral is a component of essential oils that can be found in a variety of plants of the genus Citrus. It is volatile, has a lemon-like odor and a form of light yellowish oil (Pushpakumari & Vatakencherry, 1985). Citral is a mixture of two compounds, namely geranial (Citral-a or *trans*-citral) and neral (Citral-b or *cis*-citral) (Carbajal *et al*., 1989). Besides in plants of the genus Citrus, citral is also contained in essential oils of lemon myrtle (90-98%), *Litsea citrate* (90%), *Litsea cubeba* (70-85%), and *Cymbopogon citratus* (65-85%) (Purwanto *et al*.,

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2016). This compound has various benefits, such as antibacterial, antifungal, antiprotozoal, ascaricidal, analgesic, antiinflammatory, and antioxidant effects as well as hypoglycemic, hypolipidemic and hypochesterolamic effects (Ravinder *et al*., 2010). Citral is also an important starting compound for the synthesis of vitamin A (retinol) (Purnamasari *et al*., 2016). Due to the broad utilisation of citral, it is important to develop method of its isolation which is relatively simple, low cost, but able to give pure citral in high yield.

One of the oldest isolation methods of citral is by the reaction of adducting with sodium bisulfite (Carbajal *et al*., 1989). Unfortunately, it is not the purest method because sodium bisulfite can adduct other aldehydes and methyl ketones available in lemongrass oil. Therefore, other compounds which are also present in lemongrass oil such as aldehyde compounds (e.g. citranelal), or methyl ketone compounds (e.g. methyl heptenone), can also be adducted together with citral (Carbajal *et al*., 1989).

Steam distillation as well as partial fraction distillation methods have also been used. In these methods, isolation occurs successfully and produces high-purity citral (Joga Rao *et al*., 2015; Carbajal *et al*., 1989). However, these methods have their shortcomings. Separation of components such as geraniol and nerol from citral is difficult because they have a boiling point which differ only a few degrees Celsius from citral. Furthermore, citral is labile to high temperature, hence overheating can lead to rearrangement, polymerization, and even destruction of the citral (Oxtoby *et al*., 2008).

Other methods already used include preparative thin layer chromatography and column chromatography (Carbajal *et al*., 1989; Oxtoby *et al*., 2008). Preparative thin layer chromatography is only able to produce isolates in very small amounts (Pushpakumari & Vatakencherry, 1985; Oxtoby *et al*., 2008). On the contrary, column chromatography can be used on a large scale. Column chromatography is very important in industrial use because its methods can easily be adopted from the laboratory scale to the production scale (Bidlingmeyer, 1989).

Therefore, in this study, column chromatography method was selected to isolate citral from lemongrass oil. For the optimization of mobile phase to be used for the isolation method, different mixtures of mobile phase used in other experiments were compared, such as mixtures of hexane-ether based on Pushpakumari and Vatakencherry (1986), mixtures of hexane-ethyl acetate based on Scott *et al.* (1989) , and mixtures of hexane-ethanol based on Purnamasari *et al.* (2016) of different ratios. The goal was to determine which mobile phase mixture at which ratio can produce the best resolution to isolate citral. In addition, silica gel as stationary phase and different sample-to-silica ratios were compared to determine which ratio is the most efficient for sample-loading based on the isolate's yield percentage obtained from each ratio. The isolation results were further tested qualitatively and quantitatively using gas chromatography -mass spectrometry (GC-MS) and compared with commercial citral as a standard.

2 MATERIALS AND METHODS

2.1 Materials

Lemongrass (*C. citratus*) oil was obtained from CV M & H Farm. Commercial citral was obtained from Aldrich. Hexane p.a., ethanol p.a., ethyl acetate p.a.

as well as silica gel 60 (0.063-0,200 mm) were obtained from Merck. Ether p.a. were obtained from Riedel-de Haen AG. The anisaldehyde reagents were obtained from Merck. Iodine used to stain the spot on TLC plate was obtained from Kimia Farma.

Filter paper, thin layer chromatography (TLC) chamber, TLC silica gel 60 F254 were obtained from Merck. To apply the lemongrass oil and the fraction onto the TLC plate for the determination of mobile phase mixture to be used for column chromatography, 2 µl capillary pipes were used. Chromatography was then performed in a 2,5 x 50 cm column and the fractionation results were collected in 10 ml vials. The products were subjected to gas chromatography (GC). GC was performed on an Agilent Model 6890N, equiped with mass spectrometer (MS) Agilent 5973 with inert mass spectrum detector (MSD) and Head Space Sampler Model 7697A HSS

2.2 Methods

2.2.1 Determination of Mobile Phase Mixture for Column Chromatography

The solvent mixtures to be compared were hexane - ether (97:3, v/v), hexane - ethyl acetate (97:3, v/v), and hexane - ethanol (97:3, 98:2, 99:1, v/v).

Both the essential oil of lemongrass and the commercial citral were applied on to the TLC plate. The plate was then inserted into the saturated chamber to be eluated with 5 ml of each solvent mixture. Spots from the eluation process were observed under UV lamps of λ 254 nm and also stained by spraying anisaldehyde reagents or by putting the TLC plate into chamber containing iodine.

The best eluent for column chromatography was chosen by taking Rf value and the separation factor (α) of the standard spot (Rf 1) against the nearest spot (Rf 2) into account. The separation factor (α) is calculated by the formula:

$$\alpha = \frac{Rf\ 1}{Rf\ 2}\ x\ \frac{1-Rf2}{1-Rf\ 1} \quad 10$$

2.2.2 Preparation of the Column for Column Chromatography

The amount of silica gel needed for fractionation depends on the results of the solvent mixture optimization. The sample- to - silica gel ratios were selected according to the guideline from Reichsstein *et al.*, (1960), as follows: 1:20, 1:35 and 1:50 if the obtained separation factor (α) ≥1,5 ; 1:65 and 1:80 if the obtained α ≤1,5.

2.2.3 Fractionation of Citral from Lemongrass Oil Using Column Chromatography and the Determination of the Optimum Sample-to-Adsorbent Ratio

Selected eluent was prepared for column chromatography and 1 gram of lemongrass oil was weighed before the fractionation. The flow rate was set to approximately 1 drop per second. The droplets of the eluent from the column were collected in vials, each would contain 10 mL of droplets.

The TLC test was performed on every fifth vial. The TLC test used the mobile phase of the column chromatography. Spots from the elution were observed under UV light 254 nm and stained by anisaldehyde reagent or iodine vapor.

Fractions having the same Rf as the standard were compared. This citral fraction was evaporated in the rotary evaporator, until a constant weight was obtained. Then the yield percentage of citral

obtained from fractionation was calculated with the formula:

$$\%\ yield\ = \frac{constant\ citral\ weight}{weight\ of\ lemongrass\ oil}\ x\ 100$$

The optimal sample-to-adsorbent ratio for column chromatography was determined based on the yield percentage of the obtained citral.

2.2.4 Identification of Obtained Citral Using Gas Chromatography-Mass Spectrometry

Citral obtained from fractionation with column chromatography was injected into GC-MS. The gas chromatography was equipped with a 30 m 0.25 mm, 0.25 mm film thickness column. Helium was used as mobile phase with an average flow of 1.0 ml/min. The condition of the GC-MS was according to the study from Bayala *et al.* (2018): Oven temperature program was from 50° C (3.2 min) to 300° C at 8° C/min, 5 min post run at 300° C. Sample was injected in split mode, injector and detector temperature being at 250° C and 280° C respectively. The peaks generated in the total ion chromatogram are identified by comparing the mass spectra obtained with the mass spectra found in the GC-MS libraries.

3 RESULT AND DISCUSSION

3.1 The Optimum Mobile Phase Mixture for Column Chromatography

The data obtained from the TLC test with various mixture of mobile phase (as described beforehand) are listed in Table 1 below.

The best eluent for column chromatography was chosen by taking Rf value and the separation factor (α) of the spots on the TLC plate into account.

The optimal Rf value is 0.15-0.30.[10] The selected solvent mixture is therefore hexane-ethyl acetate (97: 3, v/v), due to its separation factor of ≥ 2.00 meaning that the separation between two compound occured easily and its Rf value of 0.24 which lied within the accepted range.[10]

Table 1: Rf Values of Lemongrass Oil and Comercial Citral on TLC Using Various Mobile Phases

Mobile phase mixture and its ratio (v/v)	Rf value of essential oil			Rf value of citral standard		α
	1	2	3	1	2	
Hexane - ether (97:3)	0,06	0,18	-	0,06	0,18	3,44
Hexane - ethyl acetate (97:3)	0,03	0,24	0,39	0,03	0,24	2,02
Hexane - ethanol (97:3)	0,09	0,79	0,88	0,09	0,79	1,94
Hexane - ethanol (98:2)	0,05	0,49	0,68	0,05	0,49	2,21
Hexane - ethanol (99:1)	-	0,17	-	-	0,17	-

3.2 Fractionation of Citral from Lemongrass Oil Using Column Chromatography and the Determination of the Optimal Sample-to-Adsorbent Ratio

After fractionation of citral from the lemongrass oil as described before, the results obtained are listed in Table 3.

Table 2: Fractionation Results of Citral From Lemongrass Oil

Sample-to-Adsorbent Ratio	Volume of the eluent (ml)	Volume of the collected fraction (ml)
1:20	400	200,00 ± 10,00
1:35	600	286,67 ± 5,77
1:50	700	326,67 ± 20,82

3.3 Organoleptical Analysis of the Obtained Citral

The results from the organoleptical analysis were compared with the data obtained from literature. The organoleptical characteristics of the citral obtained from the isolation are as follows.

Table 3: The Organoleptics of the Citral obtained from Fractionation Compared with Data from Literature

	Data from literature (Marques *et al.*, 2013)	Obtained citral
Color	Pale yellow	Bright yellow
Form	Thick oil	Thick, oily
Odor	Lemony odor	Lemony/orange-like odor

Figure 1: Pure citral obtained from the fractionation

3.4 Analysis of Weight and the Yield of the Obtained Citral

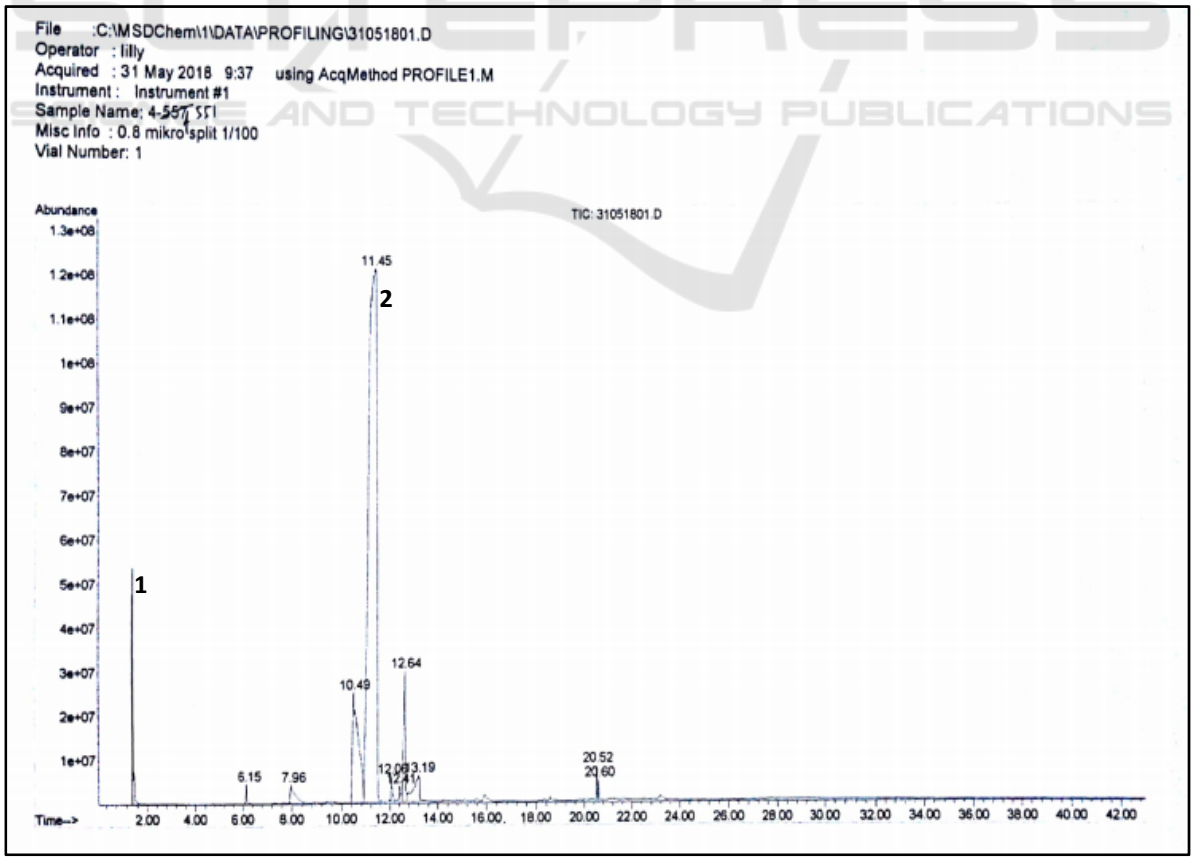
After the isolation of citral with different sample-to-adsorbent ratios, the weight of pure citral and the percentage of citral yield have been obtained as indicated in Table 5. It was obtained from three replications that the sample-to-adsorbent ratio of 1:20 can produce the highest yield percentage when compared to the other ratios.

Table 4: Weight and the Yield Percentage of the Obtained Citral

	Weight and the Yield Percentage of the Obtained Citral		
Sample – to Adsorbent ratio	1:20	1:35	1:50
Mean yield percentage	49,61 ± 2,59 %	45,66 ± 2,84 %	38,30 ± 1,72 %

3.5 Identification of Obtained Citral Using Gas Chromatography-Mass Spectrometry GC-MS

Further qualitative and quantitative identification of the obtained citral from was done using gas-spectrometry mass chromatography (GC-MS) to determine the purity of the isolate. The results of the normalization percentage of neral (**1**) was 11,50% and geranial (**2**) compound was 77,95%. Thus, the obtained citral from the fractionation using 1:20 (w/w) sample – to - adsorbent ratio had the purity of 89.45%.



3.6 Discussion

In this research, the column chromatography method of citral isolation from lemongrass oil (*Cymbopogon citratus*) was optimized. The goal was to determine which mobile phase mixture at which ratio can produce the best resolution to isolate citral and which sample-to-adsorbent ratio was the most efficient for sample-loading. Mobile phase affects the separation factor, while sample-to-adsorbent ratio affects the effective theoretical plate number of the chromatographic system.[10] Both separation factor and the number of effective theoretical plates are important parameters affecting the resolution of a chromatographic system.[10,11] At the beginning of the study, eluent mixture was selected using thin layer chromatography (TLC) based on Rf value and the separation factor (α) of the spots. The eluent mixtures tested were hexane ether (95: 5, v/v), hexane - ethyl acetate (97: 3, v/v), hexane - ethanol (97: 3; 98: 2; and 99: 1, v/v). The optimal Rf value is 0.15-0.30, while the price of the optimal separation factor is ≥ 1.5, meaning the separation between compounds in essential oils was relatively easy. Hence, a hexane-ethyl acetate solvent was selected (97: 3, v/v) since the separation factor ≥ 2.00 classified as "easy separation" and the Rf value within the range was 0.24.[10] After determining the solvent mixture used as the mobile phase for fractionation, fractionation process was performed by column chromatography. In this study, an isocratic chromatography method was used. The chosen stationary phase was silica gel. To separate polar compounds such as aldehydes (e.g., citral), a polar stationary phase such as silica gel was required since the surface comprises a highly polar hydroxyl group and interacts with the dipole of a polar solute,[12] hence silica gel was selected. At this stage, the three sample-to-adsorbent ratios were compared based on the yield percentage of the isolated citral. The three sample-adsorbent ratios were 1:20, 1:35, and 1:50 (w/w), respectively, chosen based on guidelines from Reichstein *et al.* (1960). [11] With each ratio, fractionation was performed with three replication. The fractionation results were evaporated until the weight stayed constant and the yield percentage was calculated.

The results of the isolates obtained were yellow liquid compounds smelling like lemon / orange. The results of this organoleptic observation were consistent with the data from the literature, which states that the citral is a pale yellow liquid oil-like compound smelling like lemon [4,8] .

After three replication, the largest yield percentage of 49,61 ± 2,59 %. was obtained by the 1:20 (w/w) sample – to - adsorbent ratio. This may occur because the larger sample-to-adsorbent ratio, more citral was retained on the surface of the silica gel. The more the amount of silica, the greater the surface area of the stationary phase and the greater number of analyte interacting with the stationary phase.[10] Therefore, it can be assumed that more citral interacts with the polar hydroxyl group of silica gel so that in larger amounts of silica the more citral is left in the stationary phase. The variation in the yield percentage of citral in one replication compared to another could be caused by the drying process, where the citral coalesced along with the solvent.

Further qualitative and quantitative identification of the obtained citral was done using gas-spectrometry mass chromatography (GC-MS) to determine the purity of the isolates. In chromatogram the peak of

neral can be seen at retention time of 10.49 min and peak of geranial at retention time of 11.45 min. Citral is a mixture of *cis*-citral compounds (also called neral) and *trans*-citral compounds (also called geranial).[5] The difference in retention time of both compounds could be caused by the interaction of the compound with the stationary phase in the gas chromatographic system. The column used was nonpolar, therefore the polar compound came out first while the more nonpolar compounds would be retained longer in the column. It can be concluded that *trans*-citral compounds (neral) are more polar than *cis*-citral (geranial) compounds.[8] The normalization percentage of neral was 11,50% and from geranial 77,95%. Thus, if added, the isolate obtained by a 1:20 (w/w) sample – to - adsorbent ratio had the purity of 89.45%.

All in all, the method of fractionation using column chromatography optimized in this study has provided pure isolate with high yield, despite using an isocratic elution. Hence, the optimized method has the advantages of an isocratic elution such as the relatively fewer solvents needed if compared to gradient elution. Another advantage of the optimized method would be its suitability for preparative use in large amounts. Yet further studies need to be done in order to adapt this optimized method to larger scale.

4 CONCLUSIONS

The optimal mobile phase mixture to isolate the citral from lemongrass oil (*Cymbopogon citratus*) using column chromatography method is hexane-ethyl acetate with a ratio of 97: 3 (v/v), and the optimal sample-to-adsorbent ratio to isolate citral from the lemongrass oil (*Cymbopogon citratus*) using column chromatography method is 1:20 (w/w).

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