Validation of a headspace gas chromatography-flame ionization detector (HS GC-FID) method for determination of terpinen-4-ol in volatile oil from *Zingiber montanum* rhizome

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Abstract

This study aimed to develop a headspace GC-FID method for the analysis of terpinen-4-ol in *Zingiber montanum* (Plai) essential oil. The volatile oil was extracted from fresh *Z. montanum* rhizomes by steam distillation. The amount of terpinen-4-ol was determined by a headspace gas chromatography-flame ionization detector (HS GC-FID) method using benzyl alcohol as an internal standard. The developed method was validated according to the parameters recommended by the International Conference on Harmonization (ICH). Linearity was accessed across the concentration range of 2.5 – 15 µL/mL. The plot of the peak area ratio versus the concentration provided a good linear of this method with a correlation coefficient (r^2) of 0.9989. The LOD and LOQ were found to be 0.20 and 2.5 µg/mL, respectively, which indicated a high sensitivity of the method. The instrument (n = 6), intra-day (n = 9), and inter-day (n = 3) precision as indicated by %RSD were 1.77, 1.73, and 1.77 %, respectively. The recoveries at 3 different levels of terpinen-4-ol were between 100.23 to 100.85%. The content of terpinen-4-ol in Plai essential oil was 15.53 ± 0.27 % v/v. The developed method can be used in routine analysis of Plai essential oil and its products in both cosmetic and pharmaceutical industries.

Keywords: *Terpinen-4-ol, Zingiber montanum, Plai, Gas chromatography, Validation*

1. Introduction

There has been a rising interest in using herbal medicinal products for the treatment of many diseases and symptoms in the primary healthcare system in Thailand because they are considered harmless and inexpensive. The problem that arises regarding the use of herbal medicines is mainly related to the lack of suitable quality control of the raw plant material and its derived products. Standardization of herbal products is essential for the evaluation of the quality of drugs, based on the content of their active constituents. The development of an analytical method for quantitative analysis of bioactive compounds or markers within the herbal preparations is an important step for the herbal products to display a consistent biological activity. For that, the validation of analytical methods is established to ensure that the methods are suitable for their intended purpose.

Zingiber montanum (Koenig) Link ex Dietr. or previously named as *Zingiber cassumunar* Roxb. (Zingiberaceae), locally known in Thai as "Plai" is a perennial herb with underground rhizomes with camphoraceous aroma. Plai rhizome has been usually found in Thai Traditional remedies for the treatment of various diseases, namely gastrointestinal disorders, dysmenorrhea, wound healing, muscle pain & swelling, rheumatoid arthritis, dermatitis, cough, and asthma (The Institute of Thai Traditional Medicine, 1995). Several studies have reported that biologically active compounds in Plai rhizome possessing anti-inflammatory action includes compound D [(*E*)-4-(3',4'-dimethoxyphenyl)but-3-en-2-ol] (Panthong et al., 1997), (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene) (Ozaki, Kawahara & Harada, 1991), DMPBD [(*E*)-1-(3,4-dimethoxyphenyl) butadiene)] (Jeenapongsa et al., 2003), TMPBD [(*E*)-4-(2,4,5-triimethoxyphenyl)but-1,3-ene)], cassumunaquinones, and cassumunins (Nakamura et al., 2009). Plai oil obtained from frying with vegetable oil (hot oil extract) has been widely used as a rubbing oil in Thai folk medicine to relieve musculoskeletal pain. A randomized placebo-controlled clinical study of Plai oil extracted by frying with hot palm oil revealed a similar analgesic effect as 1% diclofenac gel (Wisuitiprot et al., 2019).

Previous studies have been reported that essential oil from *Z. montanum* possessed several biological activities such as anti-oxidant (Leelapornpisid et al., 2007), antimicrobial (Boonyanugomol et al, 2017; Lertsatitthanakorn et al., 2006; Pithayanukul, Tubprasert, & Wuthi Udomlert, 2007), and insect repellent (Cotchakaew & Soonwera, 2019). Sabinene, terpinen-4-ol, and DMPBD are the major active constituents found in Plai essential oil from Thailand by using gas chromatography coupled with a mass spectrometer (GC-MS) and GC with flame ionization detector (GC-FID) (Bua-in & Paisooksantivatana, 2009; Chaiyana et al., 2017; Leelarungrayub, Manorsoi & Manorsoi, 2017; Manaprasersak & Karpkird, 2020; Mektrirat et al., 2020; Huong et al., 2020). The content of chemical constituents described as relative amounts of individual components in Plai essential oil was calculated based on the GC peak area. The relative content of sabinene was more than that of terpinen-4-ol. The major chemical compositions of Plai essential oil from Thailand, Vietnam, and India are relatively similar. Whereas the main compositions of Plai essential oil from Malaysia and Bangladesh differs from Plai essential oil from Thailand (Bhuiyan, Chowdhury & Begum, 2008; Kamazeri et al., 2012). A variation of chemical compositions depends on many factors such as geographical origin, growth environment, and maturity stage.

Terpinen-4-ol is effective against a wide range of pathogenic bacteria (Carson & Rile, 1995) whereas sabinene showed antibacterial activity against *Salmonella typhi* (Arunkumar et al, 2014). Both terpinen-4-ol and sabinene exhibited significant *in vitro* anti-inflammatory activity (Hart et al., 2000; Nogueira et al., 2014; Valente et al., 2013, Chaiyana et al., 2017). Plai essential oil has been incorporated in skincare and pharmaceutical products due to its pharmacological activities. Several clinical studies supported the effect of 14% Plai essential oil in Plai cream on pain relief (Chongmelaxme et al., 2017). Gel formulation (1% Plai essential oil) is commercially available for the treatment of acne lesions. The inclusion of Plai essential oil in nanoemulsions (Surassmo et al., 2013), microemulsions (Chaiyana et al., 2017), and niosomes (Leelarungrayub et al., 2017) delivery systems to improve the chemical stability and efficacy of Plai products were reported.

Chromatographic methods including high-performance liquid chromatography (HPLC) and GC have been usually reported for the determination of constituents in volatile oils. GC method is more preferable for quality control of all volatile oils than HPLC due to its speed, high sensitivity, ease of use, and low operation costs. Even though GC-MS is the most selective and sensitive instrument, it is rarely available in most laboratories in Thailand due to its exceedingly high price. GC-FID is more affordable and widely used for the detection of the amount of almost all organic compounds. The drawback of GC is that non-volatile compounds are not allowed to directly be injected into the GC system. The sample extraction is a tedious procedure and time-consuming. This issue can be overcome by adopting the headspace instrument. Headspace sampling technique is essentially a gas extraction technique permitting the direct analysis of volatile compounds in a non-volatile matrix.

The development of the well-validated analytical method of herbal medicinal plants to ensure their quality and efficacy are necessary. Terpinen-4-ol is undoubtingly a good anti-inflammatory and antimicrobial agent. The concentration of terpinen-4-ol in Plai volatile oil is essentially related to its efficacy. This work aimed to develop the headspace GC-FID method for the quantitation of terpinen-4-ol in Plai essential oil. The proposed method should be helpful for the quality control of Plai essential oil and its products in both cosmetic and pharmaceutical industries.

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2. Objectives

This work aimed to develop the headspace GC-FID method for quantitative analysis of terpinen-4-ol in volatile oil from *Z. montanum*. The proposed method could be considered as a powerful analytical tool for the quality control of Plai essential oil and its products in both cosmetic and pharmaceutical industries.

3. Material and method

3.1 Materials

Terpinen-4-ol (95%), α-pinene, citronellal, and cineole, was purchased from Sigma-Aldrich, USA. Benzyl alcohol, dichloromethane, diethyl ether, dimethylsuldoxide (DMSO), and petroleum ether (b.p. 50-

70°C), and anhydrous sodium sulfate were purchased from Merck, Germany. All reagents were of analytical grade.

3.2 Plant materials

The fresh rhizomes of *Z. montanum* cultivated in Chantaburi Province were purchased from Talaad Thai market, Pathumthani, Thailand, in September 2018. The plant samples were identified by a botanist, Mr. Nirun Vipunngeun, at the College of Pharmacy, Rangsit University. A voucher specimen (ORM-017/2018) was deposited at the College of Oriental Medicine, Rangsit University, Thailand.

3.3 Volatile oil extraction

The fresh rhizomes (10 kg) were cleaned, peeled, cut into thin slices, and immediately transferred to the extracting tank filled with distilled water (20 L). The extraction was performed by steam distillation for 4 hours. The distillate was collected into a 500-mL conical flask and anhydrous sodium sulfate (20 g) was added to remove any residual water. The flask was shaken for 2 minutes and then allowed to precipitate. The final essential oil was filtered through cotton wool and stored in a refrigerator until further use.

3.4 Headspace GC-FID procedure

The Clarus 680 GC-FID coupled with the static headspace autosampler (Turbomatrix 40) was obtained from PerkinElmer, Connecticut, USA. A capillary column, Velocity-Wax (Polyethylene glycol), 30 m × 0.32 mm, 0.50 µm film (PerkinElmer) was used as a stationary phase. The samples (2.0 mL) were transferred into a 20-mL headspace vial separately and immediately covered with polytetrafluoroethylene (PTFE) coated silicone septum and tightly sealed with an aluminum cap. Headspace vials were then heated at 100°C for 20 minutes in the HS40 oven prior to headspace injection. The vials were pressurized at 20 psi for 1.0 minutes and then injected for 0.04 minutes. The withdrawal time was 0.2 minutes. The temperature at the transfer line and Needle were set at 115°C and 110°C, respectively. The oven temperature programming was as follows: 60 °C (0 min), increment at 4.0 °C/min until 105°C (0 min) and then to 190°C at a rate of 6.0°C/min, total run time 25.4 minutes. Injector temperature was set at 120°C (with a split ratio of 1:14). Helium was used as carrier gas at a flow of 1.40 mL/min and a constant linear velocity. The detector temperature was set at 200°C. The flow of air and hydrogen were 450 and 45.0 mL/min, respectively. Analytical data were processed using the TotalChrom™ Version 6.3.2 software.

3.5 Method development

GC-FID has been extensively used in the quality control of essential oils because of its advantages such as high efficiency and sensitivity. FID is a universal detector and is commonly used for detecting volatile organic compounds. Solvent, internal standard, equilibration time, and oven temperature suited for the analysis of Plai essential oil were determined as follows.

3.5.1 Selection of solvent

The solubility of Plai essential oil was tested using methanol, ethanol, propanol, diethyl ether, dichloromethane, DMSO, and petroleum ether. Plai volatile oil was gradually added into 10 mL of the tested solvent. The appearance of the resulted solution was observed. A solvent that gives a clear solution at the highest concentration was considered suitable for further use.

3.5.2 Selection of internal standard

The internal standard is used to improve the precision of quantitative analysis. A known concentration of internal standard is equally added in both standard and sample preparation to quantify the components of the sample. The calibration curves were achieved by plotting the ratio of the analyte signal to the internal standard signal as a function of the analyte concentration of the standards. The internal standard peak must not be overlapping with the sample peaks. Investigation of the appropriate substance was carried out by injecting cineol, α-pinene, citronellal, and benzyl alcohol into the GC system. The peak of the internal standard must not overlap with any sample peaks in the chromatogram.

3.5.3 Test for suitable equilibration time for heating headspace sample

Headspace is the gas phase in the vial containing sample preparation. When heated for a while, the volatile components are vaporized to the headspace area over the bulk sample. The relative concentration of the volatile components that are distributed between the two phases in the vial will reach a steady value or "equilibrium". At equilibrium, the compound concentration in the headspace vapor is directly proportional to its concentration in the sample. Equilibration times for the headspace sample were tested at 15, 20, and 25 minutes. The volume of the liquid sample must not more than half of the size of the vial. In this study, two mL of standard and sample preparations were used for the entire experiment.

3.5.4 Test of suitable oven temperature

The initial temperature was set at 80°C and increased to 190°C at 4°C/min. The oven temperature was then gradually adjusted to give the appropriate resolution and analysis time.

3.6 Preparation of calibration curves

Six concentrations of terpinen-4-ol were prepared by separately transferring 25, 50, 75, 100, 125, and 150 µL into a 10-mL volumetric flask. Then, 50 µL of benzyl alcohol internal standard (IS) was added to each flask. Finally, the solution was diluted to volume with petroleum ether and mixed well.

3.7 Sample preparation

The amount of terpinen-4-ol in Plai essential oil was determined by transferring 700 µL of volatile oil samples and 50 µL of benzyl alcohol IS into a 10-mL volumetric flask. The solution was adjusted to volume with Petroleum ether and mixed well.

3.8 Method validation

The GC method validation was carried out according to the recommendation from the International Conference on Harmonization guideline (ICH) (ICH, 1996/2005). The method validation parameters were specificity, linearity, accuracy, precision, limit of quantitation (LOQ), and limit of detection (LOD).

3.8.1 Specificity

The specificity of the analytical method is its ability to differentiate between the analyte and the other substances in the sample. An identification of the analyte is determined by comparing the retention time of authentic standards with those obtained from the samples. The system suitability of the developed method was evaluated using the following responses: retention time, peak area, number of the theoretical plate (N), and tailing factor.

3.8.2 Linearity

Linearity was evaluated by studying the regression of the calibration curves obtained from the peak area ratio between the analyte and internal standard according to the analyte concentration. Six levels of concentration range between 2.5 –15 µL/mL with three replicates were employed. The calibration curves were plotted and the corresponding determination coefficients (r^2) were calculated by the computer program Excel (Microsoft®). The r^2 of the standard curve must be more than 0.999.

3.8.3 Precision

The precision of injection was determined by injecting the same standard solution six times consecutively. Sample preparation was prepared by transferring Plai essential oil (700 µL) with benzyl alcohol (50 µL) into a-10 mL volumetric flask. The solution was diluted to volume with petroleum ether and mixed well. The intra-day (repeatability) was investigated by the injection of nine sample solutions (different preparations) within the same day. The inter-day precision was examined by separate determination of the samples in three replicates on three consecutive days. The results expressed as relative standard deviation (%RSD) must less than 2.0%.

3.8.4 Accuracy

The accuracy of the method was studied by recovery experiments based on the results obtained from three levels of standard separately added into a constant volume of sample solutions. Hence, three different concentrations of standard consisting of the concentrations of 80%, 100%, and 120% of terpinen-4-

ol in sample preparation were prepared. Then, a 1.0 mL aliquot of each concentration level was transferred to a GC vial containing 1.0 mL of sample preparation. Each level of spiked samples was prepared in triplicate. The percentage recovery was calculated as follows: recovery (%) = (the amount found in spiked sample - the amount in the sample) x 100/amount of standard added. The recovery must be in the range of 98.0-102%.

3.8.5 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The LOQ was the lowest analyte concentration that gave a response that can be accurately quantified (the RSD value ≤ 5.0% and the signal-to-noise (S/N) ratio ≥ 10). The LOD was the concentration that the measured at S/N = 3.

4. Results and Discussion

4.1 Method development

4.1.1 Selection of solvent

Plai essential oil was very soluble in dichloromethane, dimethyl sulfoxide, and petroleum ether but practically insoluble in methanol, ethanol, propanol, and diethyl ether. Dichloromethane evaporates rather quickly so it is not a suitable solvent for the quantitative analysis. DMSO is almost non-volatile and is usually used as the diluent in the headspace GC. Unfortunately, it was overlapped with benzyl alcohol. In this study, petroleum ether was selected to be a diluting solvent for standard and sample preparations.

4.1.2 Selection of internal standard

It was found that peaks obtained from injection of cineol, α-pinene, citronellal were overlapped with the sample peaks. So, benzyl alcohol was chosen as the internal standard in this study.

4.1.3 Test for suitable equilibration time for heating headspace sample

The temperature used for heating the headspace sample was set at 100°C. It was found that the peak ratio obtained from the sample with a heating time of 20 minutes onward was almost unchanged. Hence, the suitable thermostat time for the headspace sample in this study was 20 minutes.

4.1.4 Oven temperature

At first, the oven temperature was set at 60°C up to 190°C with an increment rate of 4°C/min. Terpinen-4-ol and benzyl alcohol were eluted from the column at 22.5 and 27.2 minutes, respectively. To reduce the analysis time, the increment rate was increased to 6 °C/min over the temperature range between 105-190°C. The retention of terpinen-4-ol and benzyl alcohol were reduced to 17.8 and 23.0 minutes, respectively. The third temperature program was tested by increased the increment rate to 6°C/min from 60°C up to 190°C. The two peaks were eluted quickly at 10.4 and 15.2 minutes, respectively. However, peak fronting was found in the third system. Finally, the second system was employed throughout the study.

4.2 Specificity

The specificity of the developed method was evaluated by comparing chromatograms obtained from standard terpinen-4-ol and Plai volatile oil and benzyl alcohol as shown in Figure 1. The retention times of terpinen-4-ol and benzyl alcohol were 17.8 and 23.0 minutes, respectively. It was observed that terpinen-4-ol and benzyl alcohol peaks were completely separated from each other without interference from other peaks in Plai essential oil. Chromatograms of sample and standard preparation are shown in Figure 2. It was seen that the solvent peak did not intervene with the terpinen-4-ol and benzyl alcohol peak. The results confirmed the specificity of the developed method.

4.3 System suitability testing

The column efficiency is expressed by the number of theoretical plates (N). The higher N, the better the efficiency of the column. The N values obtained from the analyte peaks were more than 40,000 with an RSD of 5.46%. Baseline separation between two analyte peaks was evidence indicating the high resolution of the method. Tailing factors that represented peak symmetry were 0.86 for terpinen-4-ol and 0.96 for benzyl alcohol. The tailing factors below 1.0 exhibit some peak fronting but, in practical terms, the values between 0.8 - 1.2 are acceptable. The results of the system suitability test are shown in Table 1.

4.4 Method validation

The GC-FID method was validated for quantitative analysis of terpinen-4-ol in Plai oil. The method validation parameters were evaluated, and the results are concluded in Table 2.

4.4.1 Linearity

The calibration curve across the concentration range of 2.5 – 15 µL/mL (n=3) is depicted in Figure 3. A good linear relationship was achieved with the regression equation; y = – 0.203 + 0.164x (r^2=0.9989), where x and y represented the concentration of standard terpinen-4-ol and the peak area ratio, respectively.

4.4.2 Precision

The instrument precision was shown by six injections of the same standard solution consecutively, and the RSD of peak area ratio was 1.77%. The content of terpinen-4-ol in volatile oil was found at 15.53% v/v for intra-day assay with an RSD of 1.73% (n = 9). The average amount of terpinen-4-ol in Plai essential oil calculated from the inter-day assay was 15.58% v/v with an RSD of 1.77% (n=3).

4.4.3 Accuracy

The concentration of terpinen-4-ol in sample preparation used in the recovery study was 10.9 µL/mL. Three different concentrations of standard consisting of 80%, 100%, and 120% of the concentration of terpinen-4-ol in the sample preparation were made by separately transfer 87, 109, and 130 µL of terpinen-4-ol into a 10-mL volumetric flask containing 50 µL of benzyl alcohol IS. The solution was adjusted to volume with petroleum ether and mixed well. Then, a 1.0 mL aliquot of each concentration level was transferred to a GC vial containing 1.0 mL of the sample preparation (10.9 µL/mL of terpine-4-ol). The results obtained from the recovery study are illustrated in Table 2. The recovery was ranging from 100.23 - 100.85% with an average RSD value of less than 2%. These values indicate that the method is accurate and precise.

4.4.4 Limit of Detection and Quantitation

The limit of detection and quantitation were 0.20 and 2.5 µL/mL, respectively. The RSD value for the LOQ was 4.96%.

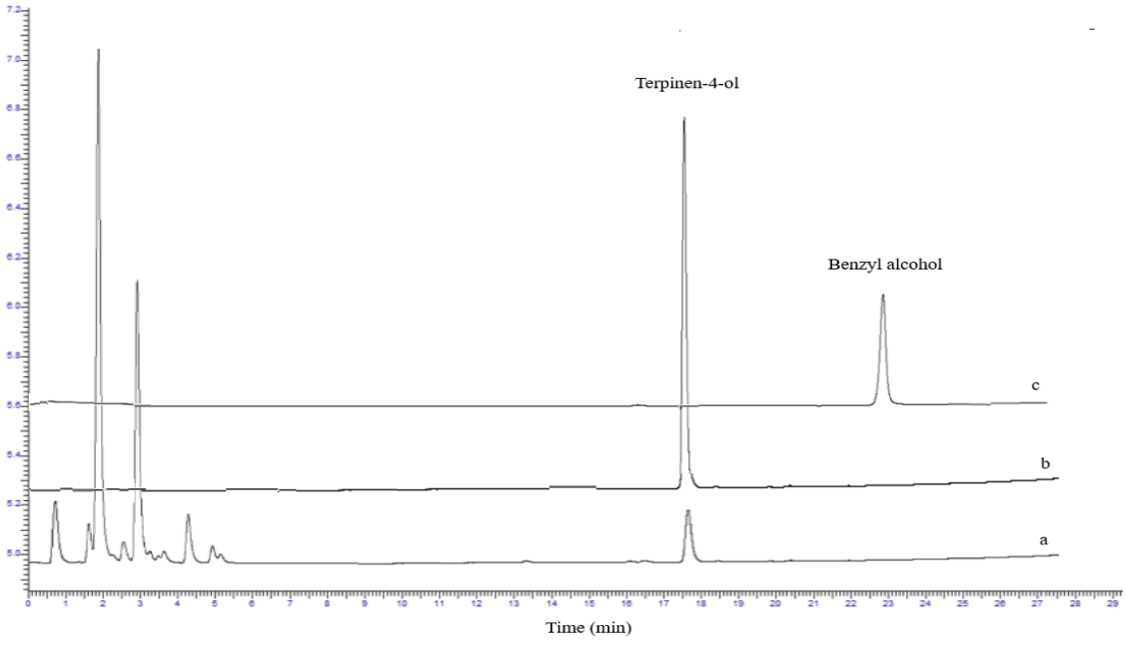

Figure 1 GC chromatograms of (a) Plai essential oil (1 ml), (b) standard terpinen-4-ol (100 µL), and (c) benzyl alcohol (100 µL)

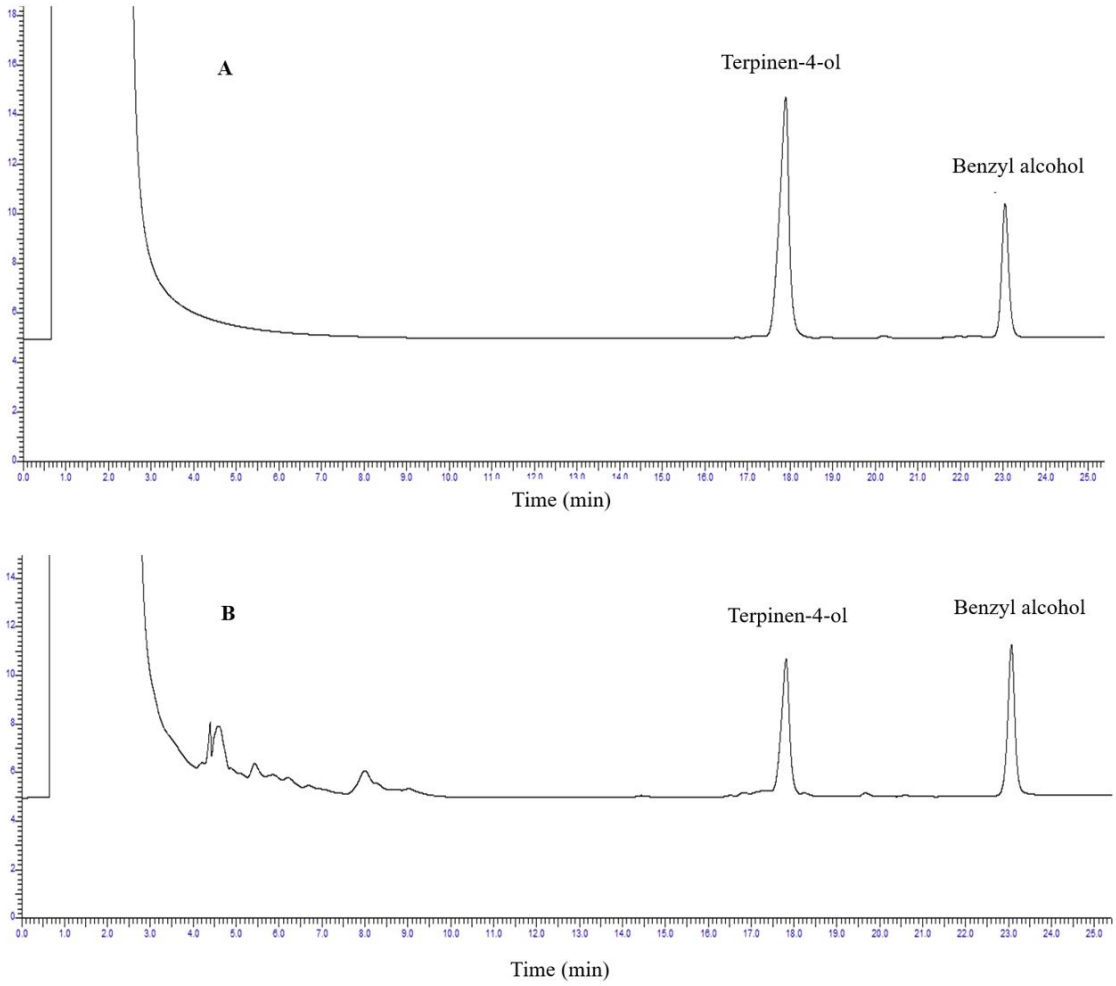

Figure 2 GC chromatograms of (A) standard terpinen-4-ol preparation (15 µL/mL), and (B) sample preparation (70µL/mL)

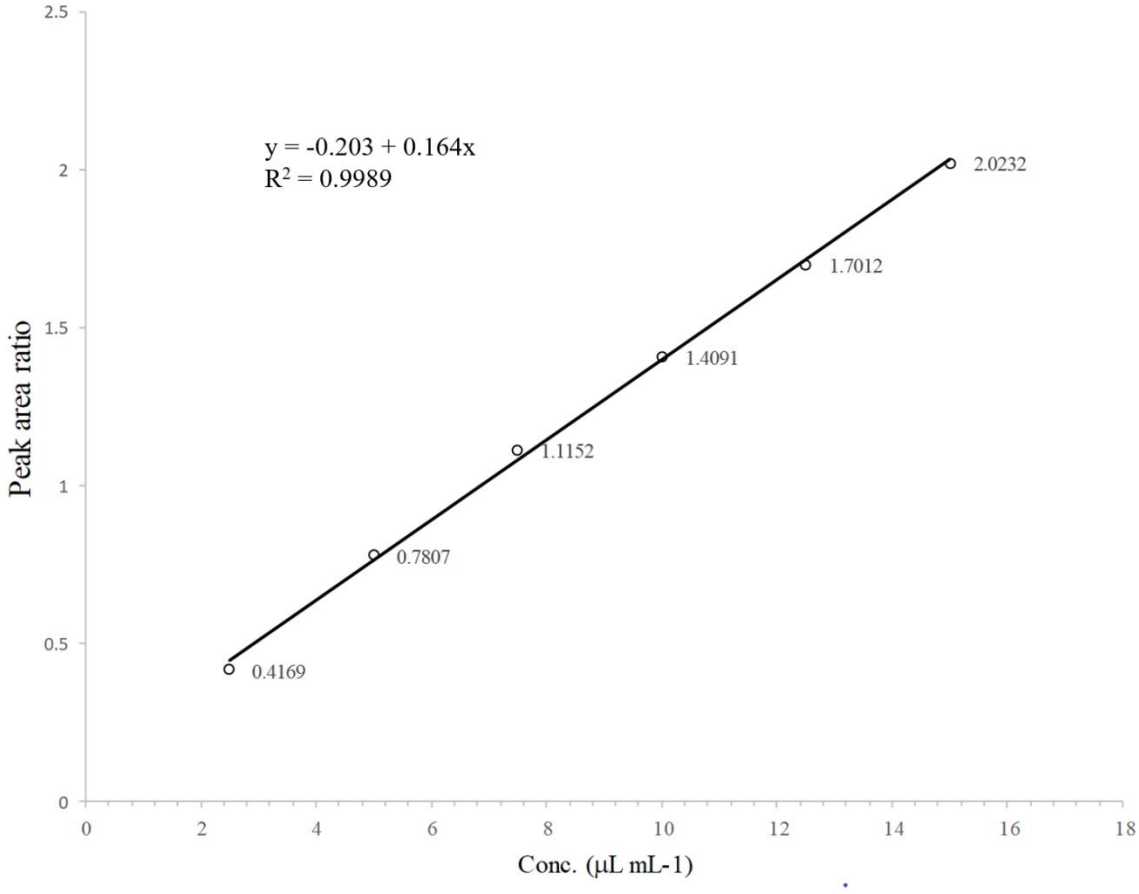

Figure 3 Calibration curve of standard terpinen-4-ol solution

Table 1 System suitability test results.

Compound	Retention time (min)	Theoretical plate (N)	USP tailing factor (T)
terpinen-4-ol	17.80	43628	0.86
%RSD	0.05	5.46	0.90
Benzyl alcohol	23.03	110221	0.96
%RSD	0.03	5.65	1.26

Table 2 Recovery results: sample solution containing terpinen-4-ol at a concentration of 10. 9 µL/mL.

Serial No.	Added (µL/mL)	Amount found (µL/mL)[1]	Recovery (%)	RSD (%)
1	8.7	8.72±0.03	100.23	0.35
2	10.9	10.86±0.02	100.37	0.20
3	13.0	13.11±0.07	100.85	0.52

[1]Expressed as mean ± standard deviation (SD; n = 3)

5. Conclusion

The proposed HS GC-FID method is a reliable and sensitive analytical method for the assay of terpinen-4-ol in Plai essential oil. A validation has been successfully investigated, and the values obtained for all parameters were acceptable. The developed method should be considered suitable for routine quality

control of Plai essential oil and its corresponding products. The use of a headspace sampler provided a very clean method of introducing volatile compounds without prior removal of any interfering non-volatile matrices into the GC system. Then, this HS-GC-FID is also applicable for the analysis of terpinen-4-ol in the non-volatile matrix. The present study provided a useful analytical tool for the screening of Plai essential oil used in the preparation of cosmetics and pharmaceutical products.

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