Rapid Method for Analysis of Vanillin as the Internal Standard for Gas Chromatographic Quantitation of α-Terpineol—a Key Component of Stout Camphor Essential Oil

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Abstract Stout camphor essential oil has a special scent due to its content of specific aromatic terpenoid compounds. Essential oils prepared from different species，varieties or subspecies have been shown to possess varied compounds. Of the major essential oil components extracted from the Stout Camphor tree，α-terpineol has been shown to be a key component，while essential oils prepared from other camphor species may not contain this terpene alcohol. Therefore，α-terpineol can be used as an index element to represent the purity of Stout Camphor essential oil. However，a method that can quantitatively examine the quality and purity of commercial Stout Camphor essential oils is necessary. By adding an internal standard to essential oil samples，this study aimed to develop a simple and reliable method for determining the level of α-terpineol in Stout Camphor essential oils on the market. Capillary column gas chromatography has the advantages of high resolution and high sensitivity，and is still one of the most important spectral analysis techniques in modern times. Therefore，this study developed a rapid method that used vanillin as an internal standard to determine the level of α-terpineol，a key component of the essential oil extracted from the stout camphor tree，using gas chromatography. The analysis of each sample only required 30 min. The lowest limit of quantitation was as low as 1 $\mu g \cdot mL^{-1}$. Add α-terpineol 1.0 and 10.0 mg to commercially available *Cinnamomum micranthum* Hayata essential oil and stout camphor wood essential oil，and the recovery rates were more than 98% (98%～103%) with a coefficient of variation below 10.8%. We then analyzed 15 commercially-available essential oil samples and one essential oil sample directly extracted from stout camphor wood. We found that the levels of α-terpineol in these samples were within the range of 21.3%～51.6%. In conclusion，this method has a high accuracy，and the α-terpineol levels can be used as an index to rapidly determine the quality of the stout camphor essential oil on the market.

Keywords Stout camphor tree essential oil；Key component；α-Terpineol；Gas chromatography spectrum；Quantitation

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Introduction

Camphor laurel (*Cinnamomum camphora* (L.) J. Presl) is a large evergreen tree. The whole plant，including the bark，twigs，and leaves，has a strong scent that is derived from its camphor oil-containing aromatic terpenoid compounds. Camphor laurel is native to Taiwan as well as southeast and southwest Asian. The trees grow in areas of bright light and warm temperature，and are mainly distributed in tropical and subtropical regions. It is often a major species of evergreen broadleaf forests in subtropical zones. In Taiwan，during the Japanese Occupation period (1895—1945)，Japan designated that camphor was to be subjected to a government monopoly in Taiwan due to its high economic value. During that period，four different species of camphor trees were con-

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trolled by the government, which were: *C. camphora* Sieb, *C. camphora* var. *nominale* Hayata, *C. kanehirai* Hayata, and *C. micranthum* Hayata[1-3].

Stout camphor (*C. kanehirai* Hayata), one of the five major broadleaf trees in Taiwan, is also a unique native tree of Taiwan[4]. Stout camphor wood has a fine and uniform texture, and is ideal for high-grade furniture and suitable for carving. Its market price is high, approximately US$ 3 500 to US$ 10 000 per ton. An endemic fungi species that only grows on the stout camphor tree in the wild, called stout camphor fungus (*Antrodia cinnamomea*), has been used as a traditional natural medicine with anti-cancer and liver protection properties in Taiwan for many decades. Recent laboratory and clinical studies have demonstrated that extracts of *A. cinnamomea* possess bioactivities including anti-cancer and vasorelaxation, and many more other medicinal applications have been identified in the past few years[5-10]. These developments have increased the demand for *A. cinnamomea*, and methods are being developed that will enable large-scale growth of fruiting bodies of *A. cinnamomea*, including stout camphor wood, indoors. This has increased the demand for stout camphor wood, and has led to the number of stout camphor trees being significantly reduced. Therefore, the stout camphor tree has been listed as an endangered plant by the Taiwan government, and felling and trading of wild stout camphor trees are not permitted.

Therefore, as stout camphor wood is in short supply, it has become very valuable, and illegal logging is increasing. Recently, different methods of culturing *A. cinnamomea* using wood from other camphor species, or using media to grow fruiting bodies of *A. cinnamomea* in solid-state or liquid-state cultures, or mycelia of the fungi in petri dish cultures, have been reported[11-14]. Some studies have shown that adding essential oil extracted from the stout camphor tree to the medium culture significantly improved the growth of *A. cinnamomea*. Therefore, in addition to using camphor wood to directly cultivate *A. cinnamomea*, some researchers have produced camphor essential oil from other camphor laurel species, as other camphor laurel species may contain essential oil components similar to those of the stout camphor tree.

Stout camphor essential oil has a special scent due to its content of specific aromatic terpenoid compounds. Essential oils prepared from different species, varieties or subspecies have been shown to possess varied compounds[15-16]. Of the major essential oil components extracted from the stout camphor tree, α-terpineol has been shown to be a key component, while essential oils prepared from other camphor species may not contain this terpene alcohol. Therefore, α-terpineol can be used as an index element to represent the purity of stout camphor essential oil[17-18]. However, a method that can quantitatively examine the quality and purity of commercial stout camphor essential oils is necessary. By adding an internal standard to essential oil samples, this study aimed to develop a simple and reliable method for determining the level of α-terpineol in stout camphor essential oils on the market[19].

1 Experiment

1.1 Materials

Fourteen stout camphor essential oil samples of different brands were purchased from markets, and stout camphor wood (*C. kanehirai* Hayata) was provided by a company that cultivates *A. cinnamomea*. α-terpineol and vanillin at a purity >99% were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan).

1.2 Extraction of pure essential oil from stout camphor wood

Stout Camphor wood (50 g) was ground into small pieces with a Chinese medicine grinder (Model 6022; Shin-Jen Co., Taichung, Taiwan) to a size of 1～2 mm, and was placed in a glass bottle. After adding 250 mL of ether, the sample was sonicated for 60 mins with a sonicator (DC-600H; DELTA). The solution was filtrated with qualitative filter papers (Grade No. 5A; ADVANTEC TOYO, Tokyo, Japan), and the solvent was removed by heating at 45 ℃ in a water bath. The resulting liquid was the pure essential oil used as the positive control in this study.

1.3 Preparation of stock α-terpineol standard and vanillin internal standard solutions

α-terpineol (100 mg) or vanillin (100 mg) was placed into a 100-mL volumetric flask and dissolved in isopropanol to 100 mL. The solutions used were stock solutions of α-terpineol standard (S) solution (1 000 $\mu g \cdot mL^{-1}$) and vanillin internal standard (IS) solution (1 000 $\mu g \cdot mL^{-1}$).

1.4 Relative response factor (RRF) of α-terpineol to vanillin

The relative response factor (RRF) of the α-terpineol standard (S) to the vanillin internal standard (IS) was measured using the following method. The stock S and IS solutions were mixed together at a serial ratio (S∶IS) of 10∶1, 5∶1, 2∶1, 1∶1, 1∶2, 1∶5 and 1∶10, and the mixtures were subjected to chromatographic analysis. The response factor of a sample was defined as the peak area of the sample in gas chromatographic analysis divided by the concentration of the sample. Thus, RRF can be obtained by Equation (1).

$$RRF = (A_S/W_S)/(A_{IS}/W_{IS}) \quad (1)$$

where A_S is the peak area of α-terpineol and A_{IS} is the peak area of vanillin. W_S is the weight of α-terpineol, and W_{IS} is the weight of vanillin.

1.5 Lowest quantitatively-determinable concentration of α-terpineol

The α-terpineol stock solution (1 000 $\mu g \cdot mL^{-1}$) was

diluted with isopropanol to concentrations of 50, 25, 10, 5, 1 and 0.5 μg · mL^{-1}. One mL of each diluted solution was mixed separately with 1 mL of internal standard solution (vanillin). The mixtures were injected directly into a gas chromatograph in triplicate for analysis. The coefficient of variation (CV%) for α-terpineol recovery was set at 15%; the lowest concentration of α-terpineol obtained was the lowest quantitatively-determinable concentration obtained by gas chromatography.

1.6 Quantitation of α-terpineol in essential oil extracted from the stout camphor tree

Twenty-five or fifty mg of each stout camphor essential oil sample were mixed with 5 mL of internal standard solution (vanillin; 1 000 μg · mL^{-1}). A volume of 0.1 μL of the mixture was injected into a gas chromatograph for analysis. The recovery of each sample was measured in triplicate. The levels of α-terpineol in the essential oil samples were calculated by Equation (2):

$$\text{α-terpineol content (mg} \cdot \text{g}^{-1}\text{ essential oil)} = (A_S/A_{IS}) \times (W_{IS}/\text{RRF}) \times 1/W \quad (2)$$

where W is the weight of the sample.

1.7 Recovery of samples fortified with α-terpineol

In a 20-mL vial, α-terpineol (10 or 1 mg) was mixed with 25 mg of essential oil samples extracted from Small-flower Camphor (C. micranthum Hayat; sample S14) or stout camphor (sample S2). A blank sample was prepared without addition of α-terpineol. After adding 5 mL of internal standard solution (vanillin; 1 000 μg · mL^{-1}), 0.1 μL of each mixture was injected into a gas chromatograph. The recovery level of each was measured in triplicate.

1.8 Gas chromatograph conditions

A gas chromatograph (GL Sciences 390B, Tokyo, Japan) equipped with a flame ionization detector (FID) was used with the H2 flow rate at 30 mL · min^{-1} and the air flow rate at 300 mL · min^{-1} in this study. The temperatures of the injection port and detector wese 250 and 310 ℃, respectively. The flow rate of the carrier gas (N_2) was set at 5 mL · min^{-1}. A CP-Sil 8 CB column (30 m×0.53 mm i. d./1.0 μm; Chrompack, Netherlands) was used.

The oven temperature was qrogrammed to initiate at 80 ℃ and hold for 3 min. The temperature was raised to 150 ℃ at a rate of 6 ℃ · min^{-1}, and hold for 1 min. Finally, the temperature was increased to 300 ℃ at a rate of 30 ℃ · min^{-1}, and hold for 10 min. The injection volume was 0.1 μL in the direct injection mode.

2 Results and Discussion

2.1 Gas chromatography conditions

We performed a test to select a suitable gas chromatography column and appropriate analytical conditions. In terms of gas chromatography column selection, a high-polar column, CP-Wax (30 m×0.53 mm), a non-polar column, CP SIL 200 (30 m×0.53 mm), and a weak polar column, CP-SIL 8CB (30 m×0.53 mm), were tested. The results indicated that the weak polar column (CP-SIL 8CB) was suitable for the analysis of terpineol due to the high polarity of terpineol. Samples were applied using the direct injection mode under column conditions of initiation at 80 ℃ followed by holding for 3 min. The temperature was raised to 150 ℃ at a rate of 6 ℃ · min^{-1}, and held for 1 min. It was finally increased to 300 ℃ at a rate of 30 ℃ · min^{-1}, and held for 10 min. Under these conditions, compounds other than α-terpineol and vanillin were eluted earlier. The retention time for α-terpineol and vanillin was 8.14 and 13.46 min, respectively (Figure 1). The results of the gas chromatographic analyses of the ether-extracted stout camphor sample and the commercial essential oil samples were as shown in Figures 2 and 3.

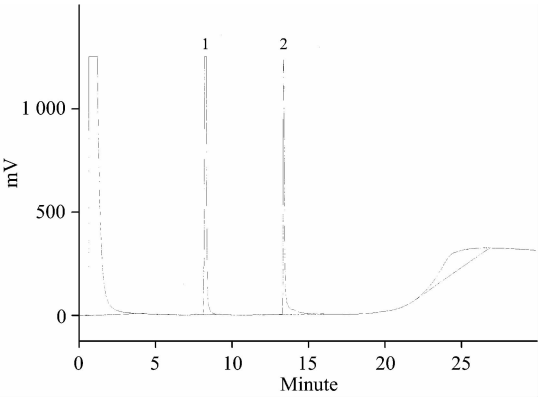

Fig. 1 Gas chromatograph of α-terpineol and vanillin (IS, internal standard) authentic standard. Peak 1=α-terpineol; Peak 2=vanillin (IS)

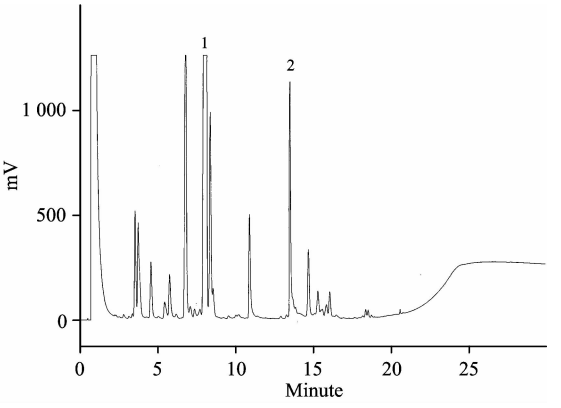

Fig. 2 Gas chromatograph of the ether extract of stout camphor tree camphorate wood essential oil (stout camphor tree essential oil (A) commercial camphor oil). Peak 1=α-terpineol; Peak 2=vanillin (IS)

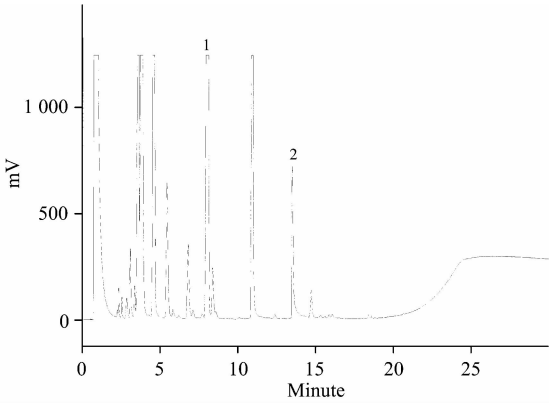

Fig. 3　Gas chromatograph of commercial stout camphor tree camphorate wood essential oil. Peak 1 = α-terpineol; Peak 2 = vanillin (IS)

2.2　Determination of the Relative response factor (RRF) of α-terpineol to vanillin

In order to accurately quantify the content of α-terpineol in the stout camphor essential oil, this study used water-soluble vanillin as an internal standard. The process first determined the RRF of α-terpineol to vanillin, and then, using the RRF value, the content of α-terpineol in each essential oil sample could be calculated according to Equation [2]. Figure 4 shows a plot of the peak area ratios (Y axis: α-terpineol/vanillin) against the concentration ratios (X axis: α-terpineol/vanillin), which demonstrates that the coefficient of determination (R^2) for the linear regression model was >0.999 and the RRF was 2.907 4 (Figure 4 and Table 1).

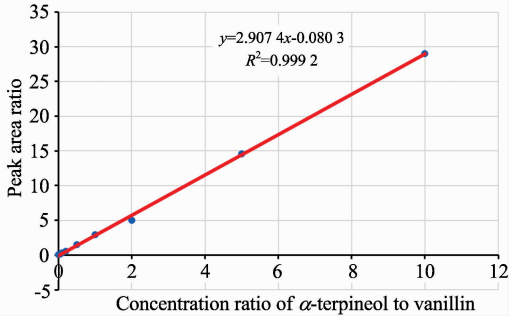

Fig. 4　The calibration curve of α-terpineol to vanillin internal standard

2.3　The lowest quantitatively-determinable concentration of α-terpineol using gas chromatography with a flame ionization detector (FID)

Following the addition of 50 μL of internal standard solution (= 5 μg vanillin) to each serially-diluted standard (50, 25, 10, 5, 1 and 0.5 μg · mL^{-1}), the mixtures were directly injected into the gas chromatograph equipped with a FID under the conditions described in the Materials and Methods section. The result showed that when the CV% for α-terpineol recovery was set at 15%[20-21], the lowest quantitatively-determinable concentration of α-terpineol was 5 μg · mL^{-1} (Table 2).

Table 1　Relative response factor (RRF) and gas chromatographic retention time (RT) of α-terpineol to vanillin

Compound	RRF[a]	RT[b]
Vanillin (IS)[c]	1.000 0	13.457
α-Terpineol	2.907 4	8.137

[a] RRF of α-terpineol to vanillin;
[b] A CP-SIL 8CB column (0.53 mm×30 m, DF=1.0 μm) was used;
[c] Internal standard

Table 2　Lowest quantitatively-determinable concentration of α-terpineol by gas chromatography with a flame ionization detector

Compound	Concentration /(μg · mL^{-1})	Detectability	Recovery[a] /%	RSD[b] /%
α-Terpineol	50.0	Yes	99.7	4.8
	25.0	Yes	104.5	3.9
	10.0	Yes	102.1	5.7
	5.0	Yes	108.7	10.4
	2.5	No	117.8	17.5
	1.0	No	151.9	29.7

[a] Average of triplicate analyses;
[b] Coefficient of variation (CV%)

2.4　Recovery rates in samples fortified with α-terpineol

Next, we studied the recoveries of α-terpineol in two different types of essential oil samples fortified with additional α-terpineol. When 10 or 1 mg of α-terpineol was added to Smallflower Camphor essential oil (a camphor species known to contain no α-terpineol; sample S14), the recovery rates were 102.7% and 98.4%, respectively, with a CV% of 5.24% or lower (Table 3). On the other hand, when 10 or 1 mg of α-terpineol was added to stout camphor essential oil (sample S2), the recovery rates were 103.4% and 98.3%, respectively, with a CV% of 10.8% or lower (Table 4). Based on the aforementioned results, we demonstrated that our method only requires the addition of isopropanol solution that contains a known concentration of internal standard, and the mixed samples can be analyzed by gas chromatography using the direct injection mode. The analysis took only 30 min for one sample, which is fast and simple.

2.5　Determination of α-terpineol levels in commercially-available stout camphor essential oils

We used stout camphor essential oil prepared by ether extraction from stout camphor wood as the positive control, and determined the α-terpineol contents in the sample prepared from stout camphor wood, in commercially-available

stout camphor essential oil samples, and in Small-flower Camphor essential oil (upper layer). Using the method developed in this study, the α-terpineol contents in the 14 essential oil samples were found to range from 213 to 533 mg·g^{-1} (Table 5). This finding indicated that the α-terpineol levels in different samples varied, and a difference of 2.5-fold was observed between the highest and the lowest contents. This is in agreement with several previous studies[20-23], which reported that the components of stout camphor essential oil might vary depending on the which parts of the tree are used as the material to prepare the essential oil. In addition, the age of the tree may also affect the quality of the essential oil. This study also confirmed that essential oil extracted from the small-flower camphor tree did not contain α-terpineol.

Table 3 Recovery of spiked α-terpineol from small-flower camphor essential oil by the direct injection GC method

Compound	Blank[a] /mg (A)	Amount added/mg (B)	Amount found[b]/mg (C)	Recovery[c] /%	CV[d] /%
α-Terpineol	0.00	10.00	10.27	102.70	3.87
	0.00	1.00	0.98	98.42	5.24

[a] α-terpineol in stout camphor Tree essential oil;

[b] Average of triplicate analyses;

[c] Recovery(%)=(C−A)/B×100%;

[d] Coefficient of variation (CV%)

Table 4 Recovery of spiked α-terpineol from stout camphor tree essential oil (S2) by the direct injection GC method

Compound	Blank[a] /mg (A)	Amount added/mg (B)	Amount found[b]/mg (C)	Recovery[c] /%	CV[d] /%
α-Terpineol	213.56	10.00	223.90	103.40	6.28
	213.56	1.00	214.54	98.33	10.79

[a] α-terpineol in stout camphor tree essential oil;

[b] Average of triplicate analyses;

[c] Recovery(%)=(C−A)/B×100%;

[d] Coefficient of variation (CV%)

Table 5 α-terpineol content in some commercial Stout Camphor Tree essential oil

ample	α-Terpineol content/ (mg·g^{-1})
Stout Camphor Tree essential oil (Extracted by ether)	516.27
Commercial Stout Camphor Tree essential oil (S1)	512.13
Commercial Stout Camphor Tree essential oil (S2)	213.56
Commercial Stout Camphor Tree essential oil (S3)	228.65
Commercial Stout Camphor Tree essential oil (S4)	304.29
Commercial Stout Camphor Tree essential oil (S5)	352.17
Commercial Stout Camphor Tree essential oil (S6)	265.94
Commercial Stout Camphor Tree essential oil (S7)	365.47
Commercial Stout Camphor Tree essential oil (S8)	412.09
Commercial Stout Camphor Tree essential oil (S9)	333.28
Commercial Stout Camphor Tree essential oil (S10)	279.34
Commercial Stout Camphor Tree essential oil (S11)	394.12
Commercial Stout Camphor Tree essential oil (S12)	410.58
Commercial Stout Camphor Tree essential oil (S13)	275.96
Small-flower Camphor essential oil (S14)	ND

[a] Average of duplicate analyses;

[b] ND=not detected

3 Conclusions

In this study, a fast and simple gas chromatographic method was developed to quantitate the quality of commercially-available stout camphor essential oils. The method only required the addition of vanillin as the internal standard, and the samples can then be directly injected into a gas chromatograph to determine the level of α-terpineol, a key component of stout camphor tree essential oil. In the 14 samples we tested, the highest and lowest contents of α-terpineol were 51.6% and 21.3%, respectively. Our findings demonstrated that α-terpineol can be used as an index to rapid determine the quality of stout camphor essential oils on the market.

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以香草素为内标之气相层析光谱法定量牛樟木精油指标成分松油醇的快速分析方法

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摘　要　牛樟精油具有独特浓郁的香气，其中以 α-松油醇(α-terpineol)为牛樟精油主要成分。由于樟树精油制品可依种类、变种、亚种等不同而有不同成分，以松油醇最具独特性，可作为牛樟木精油品质重要指标成分，应用以区别牛樟树与有樟以及香樟等木材精油的方法，因此建立市售牛樟木精油指标成分 α-松油醇含量的简便快速又准确的检验方法便有其必要性。该研究拟以市售精油液体样品，不经任何前处理，加入适当之内标准溶液溶解后，直接注入气相层析光谱仪中，配合适当的分离管柱及气相层析条件，以期建立简便快速又准确的牛樟木精油指标成分 α-松油醇之定量方法。毛细管柱气相层析具有高解析度及高灵敏度等优点，仍为现代最重要分析技术之一，因此研究建立了以香草素为内标准定量牛樟木精油指标成分 α-松油醇(α-terpineol)之气相色谱层析法的快速分析方法。牛樟木精油液体样品，加入适当量之香草素内标准溶液混合溶解后，即可直接注入配有广口径之毛细管柱(megabore column)气相色谱仪中分析，相当简便，每分析一个样品仅需约 30 min。结果显示松油醇之最低定量浓度(limit of quantitation, LOQ)为 1 $\mu g \cdot mL^{-1}$左右。在添加回收试验中添加松油醇 1.0 及 10.0 mg 于市售有樟精油及牛樟精油中，其回收率在 98%～103%，变异系数均在 10.8%以下，显示该方法的精密度相当高。以该研究建立的方法分析 15 件市售牛樟精油中松油醇含量，结果显示，市售牛樟木精油的松油醇含量最高约 51.6%及最低为 21.3%左右，此结果显示以定量松油醇作为市售牛樟精油品质指标是一快速、准确且可行的方法。

关键词　牛樟木精油；指标成分；松油醇；气相层析光谱；定量分析

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