

Rapid Method for The Gas Chromatographic Quantitative Analysis to Determinate Safrole in Commercial Essential Oils

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Synopsis

Safrole is a well-known carcinogenic agent that is present in camphor trees. In this study, a gas chromatographic method was established to quantitate the levels of safrole in essential oils using n-decyl alcohol as an internal standard. The method used a nonpolar column and was able to detect concentrations of safrole as low as 5 µg/ml in the samples. Following addition of 2–10 mg of safrole into 1 g of essential oil extracted from Stout Camphor wood (*Cinnamomum kanehirai* Hayata) or 1–10 mg of safrole into 1 g of essential oil extracted from Small-flower Camphor wood (*Cinnamomum micranthum* Hayat), the recovery rates of safrole were determined. With direct injection of samples into the gas chromatograph, the results showed that the recovery was more than 96.1%, with a coefficient of variation below 5.6%. We then analyzed 23 commercially available Stout Camphor and other essential oil samples and found that 21 of them contained safrole in the range of 37.65–355.07 mg/g. In addition, in the heavier essential oil distilled from Small-flower Camphor wood, the safrole level was up to 642.98 mg/g. Our results demonstrated that most camphor essential oils on the market have a carcinogenic potential due to their high safrole levels.

INTRODUCTION

Safrole (4-allyl-1,2-methylene dioxybenzene) is a principal component of essential oils obtained from several herbs and spices, such as the piper betle flower, camphor, nutmeg, and saffras (1–7). In Taiwan, the term “camphor tree” indicates several species of trees belonging to the *Cinnamomum* genus, which can be subclassified into four groups based on the recovery rate of camphor (8): camphor tree, linalool tree (*C. camphora* CT linalool), *C. longepaniculatum*, and *C. camphora* (Linn.) Presl var. *camphora*. These four groups of “camphor trees” are very difficult to distinguish according to their morphology. Based on

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differences in the main component of essential oils obtained from their leaves, camphor trees can be subclassified into five subgroups: (a) camphor tree (*C. camphora* subsp. *formosana* var. *orientalis*), in which the main component is camphor; (b) linalool tree (*C. camphora* CT linalool), in which the main component is linalool; (c) cineole tree (*C. camphora* (Linn.) *Presl* var. *camphora*), in which the main component is cineole; (d) sesquiterpene tree, in which the main component is nerolidol; and (e) safrole tree, in which the main component is safrole. Although it is not easy to distinguish them according to their morphology, the main component in their leaf essential oil can help to classify these trees. A study of camphor tree essential oils (Fujita et al., [9]) showed that oils extracted from the leaves and fruits of *C. camphora* Sieb contain 85% and 30–43% camphor, respectively, and oils extracted from the fruits also contain 40–59% safrole.

Safrole is a known carcinogenic substance that causes liver tumors (10–12). The metabolites of safrole, including 1-hydroxysafrole, isosafrole, and dihydrosafrole, are all carcinogens (11). A study has also shown that orally administered safrole in humans resulted in four major metabolites, of which safrole 2',3'-oxide (SFO) is a reactive electrophilic metabolite and a high concentration of safrole (0.5%) increased the incidence of cancer in a mouse model (12). Exposure to SFO caused an increase in micronuclei in mouse red blood cells and significantly induced DNA strand breaks, indicating that the toxicity of safrole is due to its metabolites having carcinogenic effects (13–15).

At present, methods for the analysis of safrole include gas chromatographic methods (16–20) and HPLC methods (6,7,21,22). Among these methods, the Association of Official Analytical Chemists (AOAC) method (19,23,24) uses steam to distill safrole, followed by chloroform extraction and subsequent analysis using gas chromatography. However, the protocol is tedious and time-consuming, and the yield of safrole is low because losses occur during the multiple steps of the process. In addition, chloroform is a toxic and controlled reagent. Therefore, the method is complicated and has many limitations. Currently, aromatic therapy is becoming popular, and many essential oils are used in body sprays, evaporative aromatherapy, and even for oral administration. If essential oils have high safrole levels, serious negative health effects will result. Therefore, the development of a simple and rapid quantitative method is required for screening the safrole contents of essential oils on the market.

This study aimed to develop a simple and reliable method to quantify safrole that only requires the addition of an internal standard (IS) to essential oil samples without pretreatment, and the sample can be directly analyzed using gas chromatography.

METHODS AND MATERIALS

MATERIALS

Essential oil samples were purchased from the market, including 15 Stout Camphor essential oil (prepared from *C. kanehirai* Hayata) samples from four different manufacturers in Taiwan (Joben Bio-Medical co., Pingtung; I Chuan Bio-Tech Corp., Tainan; Golden Dapu Biotech Crop., Chaoyi; and Yu-jang Biotech Co., Taoyuan), one sample each of the upper layer and the lower layer of Small-flower camphor essential oil, and a cypress oil sample (from Joben Bio-Medical co.), all of which were included in the analyses in this study.

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 *Antrodia cinnamomea* (Taoyuan, Taiwan). α -terpineol and vanillin at a purity >99% were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Other essential oils that are widely used in several Asian countries, including one sample each of natural peppermint oil (Li Ping Co., New Taipei City), China oil (derived from the blossoming, above-ground parts of *Menthae x piperitae* L. by distillation; Bio-Diaet-Berlin GmbH), Pak Fah Yeow (also called White Flower Analgesic Oil, which is made from a blend of aromatic herbs, contains 6% camphor; How Hin Pak Fah Yeow Manufactory Limited, Taipei, Taiwan), Green oil (a popular oil in China that helps to relieve minor body aches and pains in the muscles and joints and contains 3% camphor; Hsin Wan Jen Pharmaceutical Co., Taichung, Taiwan), and cardamom flower oil (Cheong Kim Chuan (CKC) co., Penang, Malaysia), were purchased from Tainan Pharmacy, and the essential oils were prepared by steam distillation. Safrole, n-decanol (or n-decyl alcohol, DA), and methanol of analytical grade at a purity >99% were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan).

PREPARATION OF ESSENTIAL OIL FROM STOUT CAMPHOR WOOD

Stout Camphor wood (50 g) was ground into small pieces using a Chinese medicine grinder (Model 6022; Shin-Jen Co., Taichung, Taiwan) to a size of 1–2 mm and then placed in a glass bottle. After adding 500 ml of ether, the sample was sonicated for 40 min with a sonicator (DC-600H; DELTA, New Taipei City, Taiwan). The solution was filtrated with qualitative filter papers (Grade No. 5A; Advantec Toyo, Tokyo, Japan), and the solvent was removed by heating at 45°C in a water bath. The resulting liquid was pure essential oil, which was used as the positive control in this study.

PREPARATION OF SAFROLE STANDARD AND DA IS SOLUTIONS

Safrole (100 mg) or DA (100 mg) was placed into a 100-ml volumetric flask and dissolved in methanol to 100 ml. The solutions subsequently obtained were used as stock solutions (w/v) of safrole standard solution (1,000 $\mu\text{g/ml}$) and DA standard solution (1,000 $\mu\text{g/ml}$).

RELATIVE RESPONSE FACTOR OF SAFROLE TO DA

The stock safrole standard (S) and DA IS solutions were mixed together at serial ratios of 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10 in methanol, and the mixtures were subjected to chromatographic analysis. The relative response factor (RRF) of a sample was defined as the peak area of the sample in gas chromatographic analysis divided by the concentration of the sample, and, therefore, the RRF of safrole to DA can be calculated based on the following equation (1):

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

where A_S is the peak area of safrole and A_{IS} is the peak area of DA. W_S is the weight of safrole and W_{IS} is the weight of DA.

QUANTIFICATION OF SAFROLE CONCENTRATIONS IN ESSENTIAL OIL SAMPLES

Twenty to fifty mg of different essential oil samples were mixed with 5 ml of IS solution (DA; total 5 mg). A volume of 0.1 μ l of the mixture was directly injected into a gas chromatograph for analysis. The levels of safrole in the essential oil samples were calculated according to the following equation (2):

$$\text{Safrole content (mg / g essential oil)} = (A_S / A_{IS}) \times (W_{IS} / \text{RRF}) \times 1/W. \quad (2)$$

Here, W is the weight of the sample. Analysis of each sample was performed in triplicate.

THE AOAC METHOD (19,23,24)

Safrole in the samples was isolated by steam distillation, followed by chloroform extraction. The chloroform-extracted samples were then analyzed by gas chromatography.

LOWEST QUANTITATIVELY DETERMINABLE CONCENTRATION OF SAFROLE

The safrole stock solution (1,000 μ g/ml) was diluted with methanol to concentrations of 50, 25, 10, 5, 2.5, and 1 μ g/ml. One milliliter of each diluted solution was mixed separately with 1 ml of DA IS solution. The mixtures were injected directly into a gas chromatograph for analysis in triplicate to calculate the lowest quantitatively determinable concentration of safrole. The coefficient of variation (CV%) for α -terpineol recovery was set at 15%.

RECOVERY OF SAMPLES FORTIFIED WITH SAFROLE

Safrole at 2 or 10 mg was mixed with 1 g of essential oil prepared from Stout Camphor wood in the laboratory as described previously, or safrole at 1 or 10 mg was mixed with 1 g of the upper layer of Small-flower Camphor (*C. micranthum* Hayat) essential oil. A control sample was also prepared without the addition of safrole. The samples were then mixed with 0.5–5 ml of DA IS solution (100–1,000 μ g/ml), and 0.1 μ l of each final mixture was injected into a gas chromatograph to calculate the recovery of safrole in the sample. Analysis of each sample was performed in triplicate.

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 and the air flow rate at 300 ml/min in this study. The temperatures of the injection port and detector were 245°C and 315°C, respectively. The flow rate of the carrier gas (N₂) was set at 5 ml/min. A CP-Sil 8 CB column (30 m \times 0.53 mm i.d./1.0 μ m; Chrompack, the Netherlands) was used. The oven temperature was programmed to initiate at 80°C and hold for 6 min. The temperature was raised to 120°C at a rate of 6°C/min. Finally, the temperature was increased to 300°C at a rate of 35°C/min, and held for 10 min. The injection volume was 0.1 μ l in the direct injection mode.

RESULTS AND DISCUSSION

GAS CHROMATOGRAPHY CONDITIONS

Tests using different gas chromatography columns and conditions were performed. The results indicated that a weak polar column, CP-SIL 8CB (30 m × 0.53 mm), resulted in a better outcome than a CP-Wax column (30 m × 0.53 mm) for safrole analysis. In terms of the gas chromatography conditions, with methanol dilution, the samples were directly injected into the gas chromatograph for analysis under the column conditions described in the Materials and Methods section. Other major components could be eluted earlier than safrole, and the retention time (RT) for safrole was 13.90 min.

In terms of selection of the IS, we tested 1,5-pentanediol, 1,6-hexanediol and DA. Adding a small amount of these reagents resulted in RTs of 5.83, 8.09, and 11.38 min, respectively. Comparing the chromatograms of these reagents and those of the essential oil samples, only DA showed no overlap with any peak of the components of the essential oil samples. Therefore, DA was used as the IS for gas chromatographic analysis for quantification of the safrole level in the essential oil samples. The gas chromatographs of safrole and DA are shown in Figure 1. Figures 2 and 3 present the results of the gas chromatographic analyses of the ether-extracted Stout Camphor sample (Figure 2) and the upper and lower layers of Small-flower Camphor essential oil (Figure 3).

DETERMINATION OF THE RRF OF SAFROLE TO DA

DA was used in this study, which has a similar RT to that of safrole. To accurately quantify the contents of safrole in the essential oil samples, the RRF of safrole to DA had to be determined. With the RRF value, the content of safrole in each essential oil sample could be calculated according to equation (2). Figure 4 shows a plot of the peak area ratios of safrole to DA (Y axis) against the concentration ratios of safrole to DA (X axis), which demonstrates that the coefficient of determination (R^2) for the linear regression model was >0.997 and the RRF was 1.2327 (Figure 4 and Table I).

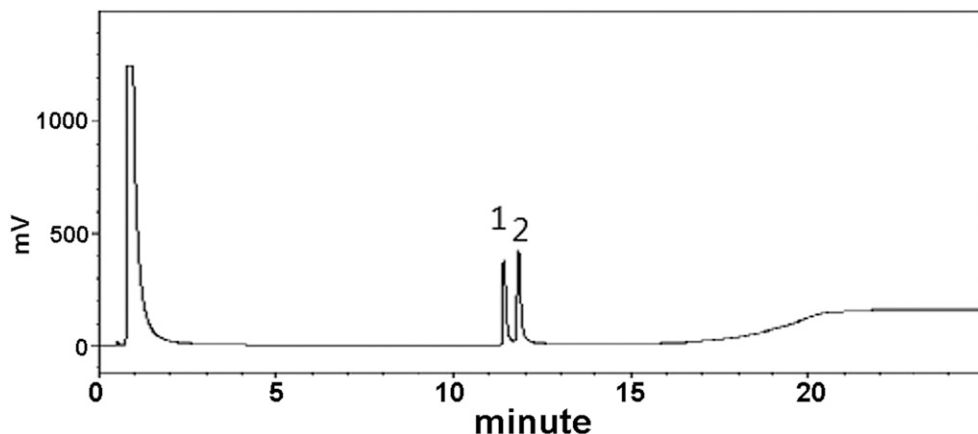


Figure 1. Gas chromatogram of DA (IS) authentic standard. Peak 1 = DA; Peak 2 = safrole.

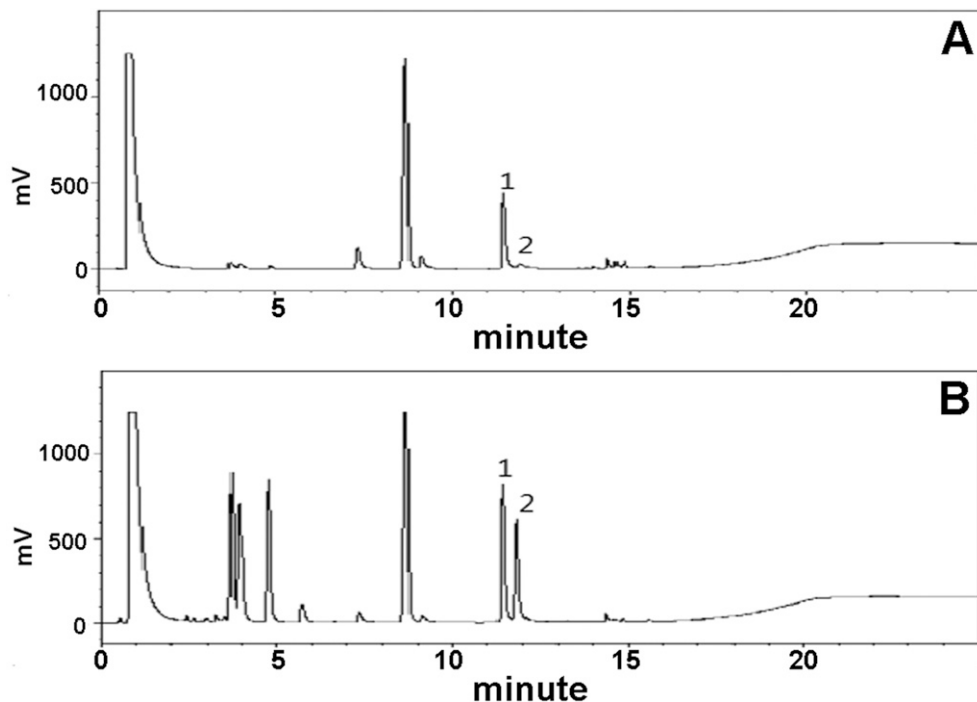


Figure 2. Gas chromatogram of (A) Stout Camphor essential oil (extracted with ether from *C. kanehirai* Hayata) and (B) commercial Stout Camphor essential oil. Peak 1 = DA; Peak 2 = safrole.

MEASUREMENT OF THE LOWEST QUANTITATIVELY DETERMINABLE CONCENTRATION OF SAFROLE

The stock safrole standard solution (1,000 $\mu\text{g}/\text{ml}$) was serially diluted to 50, 25, 10, 5, 2.5, and 1 $\mu\text{g}/\text{ml}$. Following addition of DA IS solution (1 mg each), the diluted standard solution containing DA was directly injected into the gas chromatograph equipped with an FID under the conditions described in the Materials and Methods section, with settings of FID range = 2 and attenuation = 2. Under these conditions, the results showed that when the CV% for safrole recovery was set at 15%, the lowest quantitatively determinable concentration of α -terpineol was approximately 5 $\mu\text{g}/\text{ml}$ (Table II).

RECOVERY RATES IN SAMPLES FORTIFIED WITH SAFROLE

When 2 or 10 mg of safrole were added to 1 g of Stout Camphor essential oil prepared in our laboratory, the recoveries of safrole from the samples were 97.6 to 101.5%, with a CV% of 5.6% or lower (Table III). When 1 or 10 mg of safrole were added to 1 g of the upper layer of Small-flower Camphor essential oil, the recoveries of safrole from the samples were 96.1 to 103.5%, with a CV% of 4.2 or lower (Table III). The results indicated that with high or low safrole contents in the essential oil samples, the recovery rates were not only high, but also accurate using our quantitative method.

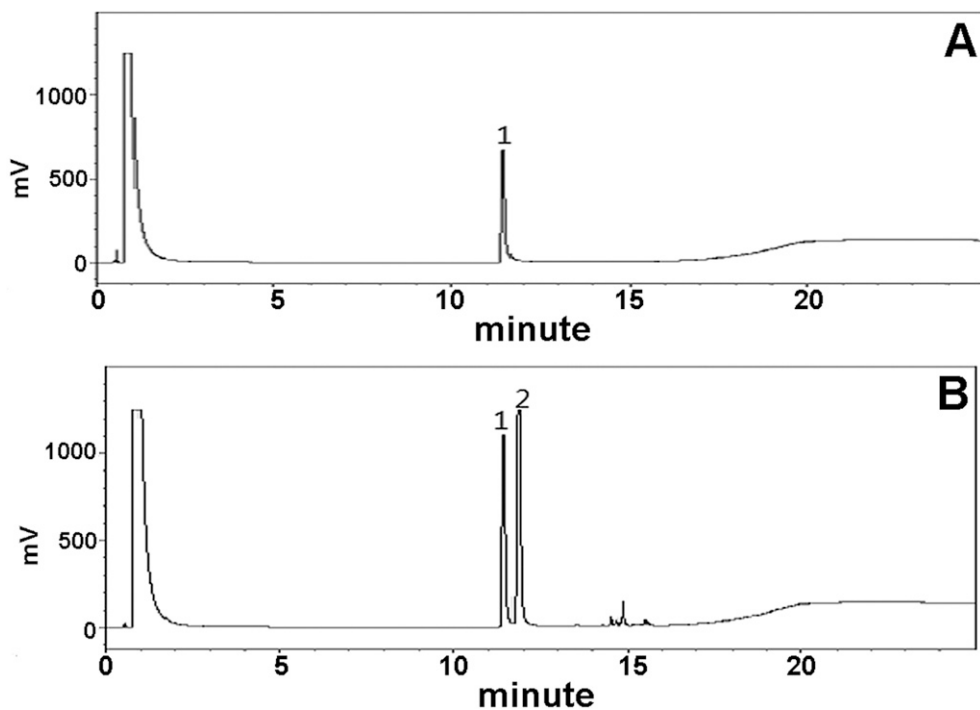


Figure 3. Gas chromatogram of (A) Small-flower Camphor (*C. kanehirai* Hayata) essential oil (upper layer) and (B) Small-flower Camphor essential oil (lower layer). Peak 1 = DA; Peak 2 = safrole.

COMPARISON OF OUR METHOD TO THE AOAC METHOD

To examine the accuracy of the proposed method for safrole measurement, safrole contents in the oil samples were measured by the AOAC method and by our method. With the AOAC method, the safrole levels in a Stout Camphor essential oil sample (S1) and a natural

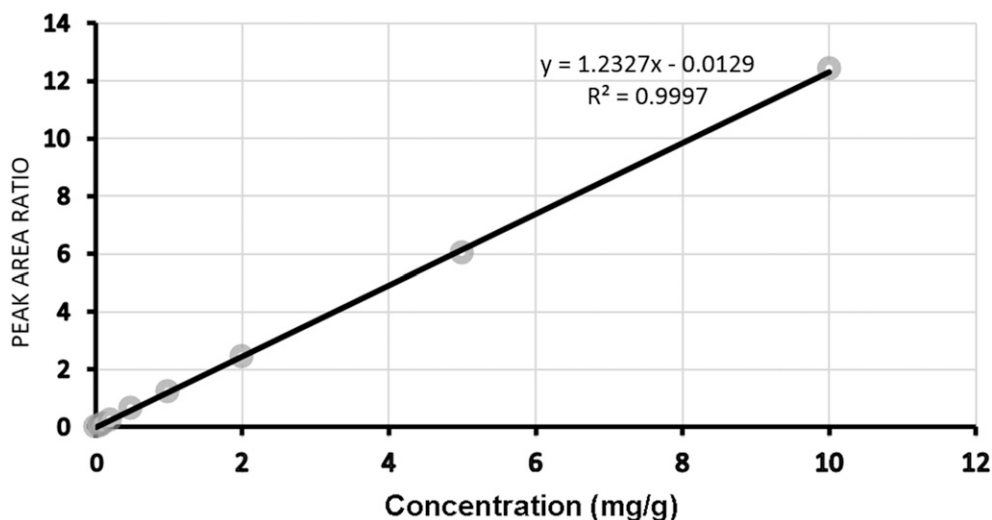


Figure 4. Calibration curve of safrole to DA.

Table I
RRF and GC RT of Safrole

| Compound | RRF ^a | RT ^b |
|-----------------|------------------|-----------------|
| DA ^c | 1.000 | 11.356 |
| Safrole | 1.207 | 11.809 |

^aRRF of safrole to DA.

^bA CP-SIL 8 CB column (0.53 mm × 30 m, DF=1.0 μm) was used.

^cIS.

peppermint oil sample obtained from a market were 36.23 and 24.01 mg/g, respectively, and the CV% values were 5.89% and 6.11%, respectively (Table IV). Using our direct injection method, however, the safrole levels in the samples were 37.65 and 35.28 mg/g, respectively, and the CV% values were only 3.62% and 2.73%, respectively. As the AOAC method requires sample pretreatment with steam distillation and chloroform extraction before gas chromatographic analysis, 4 h are needed to obtain a result. However, with our proposed method, no pretreatment is needed, and, therefore, only 25 min are needed to analyze a sample.

The small CV% values obtained using our direct injection method indicated that it has a higher accuracy than the AOAC method. In addition, the greater number of sample preparation steps in the AOAC method, including steam distillation and chloroform extraction, might lead to a lower yield of safrole, resulting in lower detection values and higher CV% values. Furthermore, chloroform is a toxic and controlled reagent, which renders the application of the AOAC method more difficult than our method (Table V).

In this study, we performed tests to validate the specificity of our method. The process used 0.1-μl solutions of reference standards, test samples, and a negative control (blank solvent) in a gas chromatographic protocol. The results showed that no safrole was detected in the negative control (Figure 3A) and no chromatographic peaks appeared during the range of the RT. This confirmed that the negative control (blank solvent) did not interfere with the chromatographic analysis of safrole in the test samples and standards, which indicated that our method had a good specificity. In addition, near the RT of safrole (11–14 min), only a single symmetrical chromatographic peak was observed, demonstrating a single compound consistent with the RT.

Table II
Lowest quantitatively Determinable Concentration of Safrole by Gas Chromatography equipped with an FID Detector

| Compound | Concentration (μg/ml) | Detectability ^a | Recovery (%) ^b | RSD (%) ^c |
|----------|-----------------------|----------------------------|---------------------------|----------------------|
| Safrole | 50.0 | Yes | 103.8 | 3.8 |
| | 25.0 | Yes | 102.5 | 3.5 |
| | 10.0 | Yes | 98.1 | 5.8 |
| | 5.0 | Yes | 110.7 | 10.9 |
| | 2.5 | Yes | 122.8 | 18.5 |
| | 1.0 | No | — | — |

^aFID range = 2, attenuation = 2.

^bAverage of triplicate analyses.

^cCoefficient of variation (cv%).

Table III
Recoveries of Spiked Safrole from Stout Camphor Essential Oil (Extracted with Ether from *C. kanehirai* Hayata) and Small-Flower Camphor (*C. micranthum* Hayat) Essential Oil (Upper Layer) by the Direct Injection Method

| Sample | Blank ^a (mg) (A) | Amount added (mg) (B) | Amount found (mg) ^b (C) | Recovery (%) ^c | CV (%) ^d |
|---|--------------------------------|--------------------------|---------------------------------------|---------------------------|---------------------|
| <i>Antrodia</i> essential oil | 32.73 | 10.36 | 43.25 | 101.54 | 2.41 |
| | 32.73 | 2.07 | 34.75 | 97.58 | 5.61 |
| Small-flower Camphor essential oil (upper layer) | 0.00 | 10.36 | 10.72 | 103.50 | 3.49 |
| | 0.00 | 1.03 | 0.99 | 96.12 | 4.24 |

^aSafrole in 1 g Stout Camphor essential oil.

^bAverage of triplicate analyses.

^cRecovery (%) = $(C - A)/B \times 100\%$.

^dCoefficient of variation (cv%).

We also explored the reproducibility of the method. Taking six different aliquots prepared from the same commercial source of stout camphor essential oil, our method determined the safrole concentrations in these six samples to be 136.73, 135.28, 131.19, 139.54, 136.05, and 135.92 mg/g (mean = 135.79 mg/g, SD = 2.70 and CV = 1.99%). The results indicated that this method had a high accuracy. We also tested the reproducibility of our method by analyzing three replicates of each of the samples on each of 3 d and found that our method resulted in reproducibility standard deviations of 1.8% and 2.1% for the measurement of safrole. These results indicated that the method was stable and reproducible.

The recovery rate test showed that the recovery rates were all in the range of 96–104%, with CVs all below 5.6%. Our method used direct injection of safrole samples into a gas chromatograph, and the findings demonstrated a higher accuracy of determination of safrole concentration than that of the AOAC method. In addition, we performed experiments using different small amounts of a safrole control standard mixed with IS DA at different ratios and found that the safrole–IS plot showing the peak area ratio (*Y* axis) to the IS (*X* axis) had a linear regression R^2 value above 0.99 in the linear range of 0.05 to 10.10 mg/g (Figure 4). The results indicated that this method had good linearity in the test range.

Table IV
Safrole Content in Stout Camphor Essential Oil (S1) and Natural Peppermint Oil Analyzed Using Direct Injection GC and AOAC Methods

| Method ^a | Safrole ^b mg/g ^b (cv%) ^c | |
|---------------------|---|------------------------|
| | Stout Camphor essential oil (S1) | Natural peppermint oil |
| AOAC | 36.23 (5.89%) | 34.01 (6.11%) |
| Direct injection | 37.65 (3.62%) | 35.28 (2.73%) |

^aDirect injection method = method developed in this study; AOAC method = steam distillation, chloroform extraction and analysis by GC (4).

^bAverage of triplicate analyses.

^cCoefficient of variation (cv%).

Table V
Safrole Contents of Various Commercially Available Essential Oils

| Sample | Content (mg/g) ^a |
|--|-----------------------------|
| Stout Camphor essential oil (extracted by ether) | 32.73 |
| Small-flower Camphor essential oil (upper layer) | ND |
| Small-flower Camphor essential oil (Lower layer) | 642.98 |
| Commercial Stout Camphor essential oil (S1) | 37.65 |
| Commercial Stout Camphor essential oil (S2) | 306.52 |
| Commercial Stout Camphor essential oil (S3) | 326.98 |
| Commercial Stout Camphor essential oil (S4) | 256.18 |
| Commercial Stout Camphor essential oil (S5) | 281.60 |
| Commercial Stout Camphor essential oil (S6) | 196.48 |
| Commercial Stout Camphor essential oil (S7) | 136.46 |
| Commercial Stout Camphor essential oil (S8) | 176.85 |
| Commercial Stout Camphor essential oil (S9) | 301.65 |
| Commercial Stout Camphor essential oil (S10) | 355.07 |
| Commercial Stout Camphor essential oil (S11) | 269.70 |
| Commercial Stout Camphor essential oil (S12) | 227.09 |
| Commercial Stout Camphor essential oil (S13) | 304.91 |
| Commercial Stout Camphor essential oil (S14) | 199.76 |
| Commercial Stout Camphor essential oil (S15) | 278.37 |
| Cypress oil | 129.11 |
| Natural peppermint oil | 35.28 |
| China oil | 109.56 |
| Pak Fah Yeow | 33.37 |
| Green oil | ND |
| Cardamom flower oil | 22.06 |

^aAverage of duplicate analyses. (24)

ND: not detected.

DETERMINATION OF SAFROLE LEVELS IN ESSENTIAL OIL SAMPLES FROM MARKETS

We used Stout Camphor essential oil prepared by ether extraction from Stout Camphor wood as the positive control and determined the safrole contents in 15 commercially available Stout Camphor essential oil samples, Small-flower Camphor essential oil (upper layer and lower layer) samples, and one sample each of cypress oil, natural peppermint oil, China oil, Pak Fah Yeow, Green oil and Cardamom flower oil (23 samples in total). Using the method developed in this study, safrole was detected in 21 of the 23 samples. All 15 Stout Camphor essential oil samples purchased from markets were found to contain safrole, in levels ranging from 37.65 to 355.07 mg/g. The upper layer of Small-flower Camphor essential oil did not have a detectable level of safrole, whereas the lower layer of the essential oil had up to 642.98 mg/g of safrole. The safrole levels in the remaining essential oil samples were as follows: cypress oil, 129.11 mg/g; natural peppermint oil, 35.28 mg/g; China oil, 109.56 mg/g; Pak Fah Yeow, 33.37 mg/g; Green oil, 0 mg/g; and Cardamom flower oil, 22.06 mg/g. The results indicated that many of the Stout Camphor essential oils and other essential oils available on the market have high levels of the carcinogenic agent safrole.

CONCLUSION

In this study, we developed a fast and simple gas chromatographic method to quantify safrole in essential oils. The method only requires the addition of an IS DA methanol solution,

and the samples can then be directly injected into a gas chromatograph to determine the level of safrole. The analysis of each sample takes only 25 min because no complicated pretreatments are required, in contrast with the AOAC method, using which sample analysis requires 4 h. Our findings indicated that most of the Stout Camphor essential oils on the market have high safrole levels (ranging from 37.65 to 355.07 mg/g). In addition, several other essential oils that are very popular in many Asian countries and some European countries also have high safrole contents (0–129 mg/g). To protect public health, the safe, cheap, and fast quantitative method developed in this study has the potential for use in the assessment of the safety of essential oils.

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