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A Rapid and Simple Gas Chromatographic Method for Direct Determination of Safrole in Soft Drinks

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ABSTRACT

A simple and rapid method was developed to determine the safrole and isosafrole in soft drink using a megapore semi-polar column (CP-SIL 8CB, 30 m × 0.53 mm, 1.5 μm) with direct injection gas chromatography. Direct quantitative analysis of safrole and isosafrole in soft drinks was carried out without any sample pretreatment procedure. The water soluble compound 1,4-dihydroxybenzene (DHB) was used as the internal standard. The detection limit for safrole and isosafrole was 0.25 μg/mL. A recovery study was performed using one of the soft drinks by spiking 1 mL with safrole and isosafrole at 5.0 and 10.0 μg, respectively. The recovery was found in the range of 98-108% with coefficients of variation being less than 8.7%. Twenty-five commercial soft drinks were analyzed by the current method, and results indicated that 20 out of 25 soft drink samples contained safrole andlor isosafrole, and the amount of safrole was 3-5 fold over the regulated amount, 1 μg/mL.

Key words: soft drink, safrole, isosafrole, direct injection, gas chromatography, quantitative analyses

INTRODUCTION

Safrole (4-allyl-1,2-methylene dioxybenzene) is one of the components of refined oils in more than fifty kinds of vegetables. Many of them can be made into seasonings, and are one of the major components of essential oils, such as sassafras, camphor, nutmeg, black pepper and piper betle flower[1-7].

Safrole and isosafrole were once used exten have a connection with inductive liver tumors[8-10]. A high concentration (0.5%) of safrole and isosafrole has been shown to increase the occurrence rate of malignant tumors in mice[10]. The major toxicity of safrole and isosafrole come from their carcinogenic nature after oxidation. Safrole is oxidized into 1-hydroxysafrole by many mam mals and whose derivatives including isosafrole and dihydrosafrole, which are all both carcinogenic[9].

Safrole and isosafrole were once used extensively as a seasoning in soft drinks. For example, Sarsaparilla and Coke have used sassafras oil as seasoning[11-13] which contained nearly 85% safrole and isosafrole. Since safrole and isosafrole are carcinogens, adding sassafras oil in soft drinks has been prohibited in the US since 1970, while it was defined as a kind of food additive and treated as a special element of seasonings in the Republic of China. However, it can be used only in soft drinks with the use limit below 1 μg/mL, according to food additive regulations.

Currently, the methods for analysis of safrole and isosafrole include GC[14-18], HPLC[6-7,19-20] etc. Among them, AOAC is one of the methods to determine safrole and isosafrole in soft drinks. The principle of AOAC is to distill safrole and isosafrole with steam, extract with an organic solvent (such as $CHC1_3$), and then analyze with gas chromatography (GC). Sample pretreatment in AOAC is complicated and time-consuming, and safrole is likely to disappear causing low recovery. Therefore, it is not a good method for routine analysis. In addition, it is necessary to use $CHCl_3$ in the process of extraction, a toxic solvent that can be used only within certain limits and is not available easily. Therefore, it is urgent and important to develop a new simple and rapid analysis method for quantitative determination of safrole and isosafrole.

Our laboratory has been engaged in the research of GC analysis for years, and has found that megapore GC columns purchased from the market were high water-tolerant in the process of separation[21-27]. Even when water-soluble samples were injected directly into the GC column, the recurrences of separation effect and retention time were the same as the original unused column. In addition, salts and impurities that remain in the injector of the glass liner where glass wool was inserted, need not be washed frequently. Clean up could be done only after finishing analysis of more than 100 samples. In addition, it is very simple to clean the glass liner. Simply remove and soak in hydrochloric acid solution for 10 minutes, then remove the glass wool and rinse with water, replace with new glass wool and place back into the injector for use after drying it out. By adopting this direct injection gas chromatography, our research has developed a rapid method for analyzing liquid foods, such as levulinic acid[24] and preservatives[25] in soy sauce, caffeine[26] in tea and coffee beverages, and nicotinamide[27] in tonic drinks.

Based on the above reasons, our research selected soft drinks as a liquid sample without any pretreatment procedure, and direct injected into GC after adding an appropriate internal standard solution in coordination with proper columns and gas chromatographic conditions used. Thus, we

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developed a simple and rapid analysis method for quantitative determination of safrole and isosaf-role in soft drinks.

MATERIALS AND METHODS

I. *Materials*

Twenty-five various commercial soft drinks, including Coca-Cola, Pepsi, Sarsaparilla, apple cider and root beer were purchased from convenience stores and supermarkets in Tainan and Pingtung between January and March 1998.

Safrole, its isomer isosafrole and other standards like 1,5-pentanediol, 3-methyl-1,5-pentanediol, 1,6-hexanediol and 1,4-dihydroxybenzene (DHB), each have a purity of over 98%, and were all obtained from TCI Co. in Tokyo, Japan. Among them, isosafrole consisted of 10% cisisosafrole and 90% trans-isosafrole.

II. *Methods*

(I) *Preparation of Standard and Internal Standard Solution*

0.1 g of safrole, isosafrole and 1,4-dihydroxybenzene (DHB) were accurately weighed in 100 mL volumetric flasks and dissolved to volume in methanol solution respectively to form 0. 1 % (w/v) of standard solutions.

(II) *Determination of the Relative Response Factor (RRF) of Safrole and Isosafrole to 1,4-Dihydroxybenzene (DHB)*

0.1% (w/v) of safrole and isosafrole were mixed with 0.1% (w/v) of internal standard 1,4dihydroxybenzene (DHB) in methanol solution or water in various ratios: safrole or isosafrole / DHB = 2/1, 1/l, 1/2. The relative response factor of safrole and isosafrole to DHB was calculated according to their peak area ratio and concentration ratio in a gas chromatographic device:

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

A_S = GC peak area of safrole or isosafrole; A_{IS} = GC peak area of DHB; W_S = weight (μg) of safrole or isosafrole (cis- or trans-); W_{IS} = weight (μg) of DHB.

(III) *Quantitative Determination Of Safrole and Isosafrole*

1. Direct injection GC method

CO_2 was removed by stirring before sampling, and then 1 mL of soft drink samples were individually added into 7 mL vials followed by adding 50 μL of 0. 1 % (w/v, which was equal to 50 μg) internal standard DHB in methanol solution. After mixing well, 0.1 μL of mixture was injected directly into GC for analysis. The contents of safrole and isosafrole can be calculated by the following equation (2):

Safrole or isosafrole (μg/mL) = $(A_S/W_S) \times (W_{IS}/RRF) \times$ 1/V .....(2)

V = volume of sample (mL).

2. AOAC[22-24] method

Safrole was analyzed with GC after isolating by steam distillation followed by extracting with organic solvent ($CHC1_3$).

(IV) *The Lowest Qantitatively Detenninable Concentration*

The standard solutions (1000 μg/mL) of safrole and isosafrole were diluted with pure water into concentrations of 5.0, 2.5, 1.0, 0.5 and 0.1 μg/mL respectively. 50 μL, 0.01% (w/v) of internal standard solutions (DHB) were added into 1 mL of the above solutions respectively and the mixtures were analyzed with GC after mixing well. The lowest quantitatively determinable concentration (CV% was set below 15%) of safrole, cis- and trans-isosafrole were then evaluated. Each concentration was carried out in triplicate.

(V) *The Recovery Test*

10.0 μg and 5.0 μg of safrole and isosafrole were added into 1 mL of soft drink samples (S-1) in 7 mL vials, respectively, followed by adding 50 μL 0.1% (w/v) of DHB internal standard solutions. After mixing well, 0.1 μL of mixture was added directly into GC for analysis. Each addition was conducted in triplicate while the blank test was operated at the same time, and the recoveries of safrole and isosafrole were then calculated.

(VI) *The GC Conditions*

GC device: Hitachi G-3000; FID detector: H_2 flow rate = 30 mL/min, Air flow rate = 300 mL/min; temperature detector = 290˚C; temperature of injector = 240˚C; carrier gas : He, flow rate = 5 mL/min; analytical column : CP SIL 8CB (30 m × 0.53 mm, 1.5 μm Chrompack, Netherlands); temperature of oven : The initial temperature was held at 120˚C for 3 min, then programmed to 150˚C at 2˚C/min followed by rapidly increasing to 300˚C at 50˚C/min for 3 min; the injection volume : 0.1 μL (Prior to sampling, a lighter was used to create heat to expel solvent from the syringe); The injection mode: Direct injection mode.

RESULTS AND DISCUSSION

I. *About the GC Conditions*

Polarized CP-Wax, non-polarized CP SIL 200 and semi-polarized CP SIL 8CB (30 m × 0.53 mm) columns were used for trial with respect to the selection of the analytical column. Results demonstrated that the CP-SIL 8 CB column was the most suitable one to be used for analysis of polarized safrole and isosafrole.

The direct injection mode was adopted for sample

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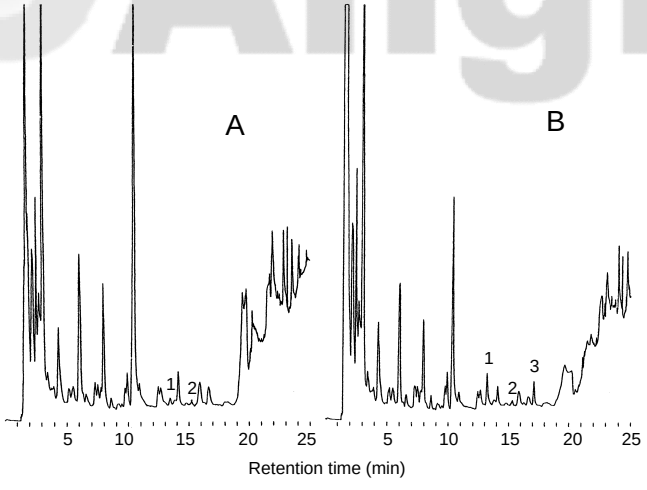

Figure 1. Gas chromatogram of (A) commercial soft drink (C-1) and (B) the spiked safrole, cis-and trans-isosafrole from the commercial soft drink (C-1).
Peak 1 = safrole, Peak 2 = cis-isosafrole, and Peak 3 = trans-isosafrole.

analysis and the appropriate temperatureraising program as described in Method (VI). The retention times of safrole, cis- and trans-isosafrole were 13.22, 15.33 and 17.12 min respectively according to the above analysis conditions. The GC chromatograms of soft drink samples (C-1) and standards were showed in Figure l and Figure 2, respectively.

With respect to the selection of internal standards, a small amount of water-soluble standards of 1,5-pentanediol, 1,6-hexanediol and 1,4-dihydroxybenzene (DHB) were individually added into soft drink samples, including Coca-Cola, Sarsaparilla, Pepsi and Root Sar. According to the GC retention times of various standards with the above GC conditions used, a suitable internal standard was then chosen for determination analysis of safrole and isosafrole. Results showed that the retention times of the above standards were 3.38, 5.83, 8.09 and 11.77 min, respectively. By comparing the retention times of standards to various elements in soft drink samples as shown in Figure 1, the peaks of DHB and all elements in soft drink samples did not display any overlapping. Therefore, DHB was found to be a proper internal standard (IS) used for determination analysis of safrole and isosafrole.

II. *The Relative Response Factor (RRF) of Safrole and Isosafrole to Internal Standard (DHB)*

The water-soluble compound DHB was chosen as the internal standard of safrole and isosafrole for direct quantitative determination of soft drink samples in this research. To quantify accurately, the first step was to determine the RRF of safrole and isosafrole to DHB. The contents of safrole and isosafrole in samples can be calculated by equation (2). The coefficient of linearity (R^2) plotted by peak area ratios (axis Y) of safrole and isosafrole to internal standard versus concentrations (axis X) was over 0.98 (R^2 = 0.98). The relative response factors of safrole, cis-isosafrole and trans-isosafrole to the internal standard DHB were then calculated as the following: 2.33, 2.23 and 2.21 as shown in Table 1.

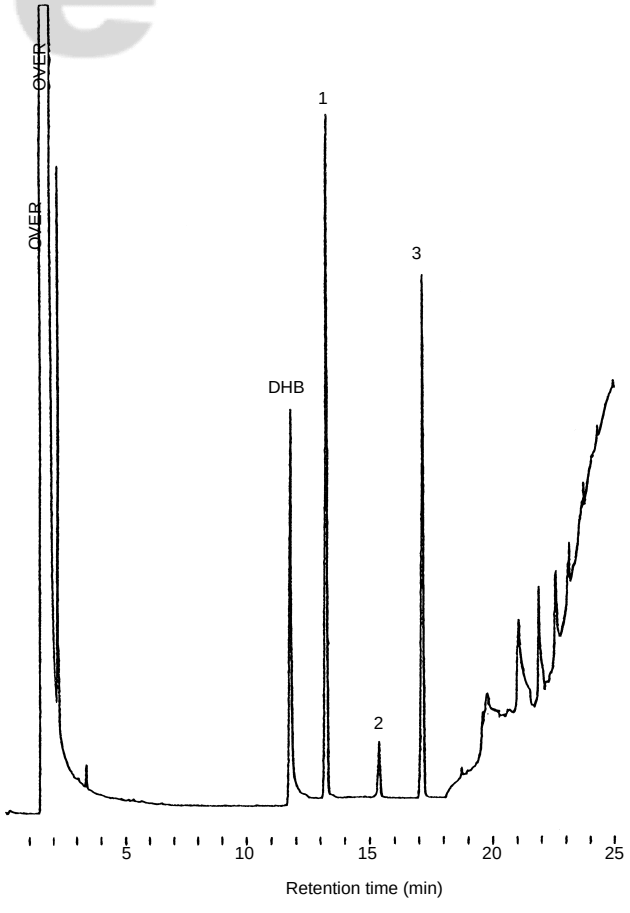

Figure 2. Gas chromatogram of safrole, cis-isosafrole, trans-isosafrole and dihydroxybenzene (DHB, internal standard) authentic standard.
Peak 1 = safrole, Peak 2 = cis-isosafrole, and Peak 3 = trans-isosafrole.

Table 1. Relative response factor (RRF) and GC retention time (RT) of safrole and iso-safrole

Compound	RRF[a]	RT[b]
1,4-Dihydroxybenzene (DHB)[c]	1.00	11.76
Safrole	2.33	13.22
cis-Isosafrole	2.23	15.33
trans-Isosafrole	2.21	17.12

[a] RRF of safrole , cis-isosafrole and trans-isosafrole to 1,4-dihydroxybenzene.
[b] CP-SIL 8CB column (0.53 mm × 30 m, DF = 1.5 μm) was used.
[c] Internal standard.

III. *The Lowest Quantitaively Determinable Concentration of Safrole and Isosafrole with GC-FID*

A standard solution of 0.1% (w/v 1000 μg/mL) safrole and isosafrole was diluted into a series of concentrations of 5.0, 2.5, 1.0, 0.5, 0.25 and 0.1 μg/mL. After adding 50 μL 0.01% (which was equal to 5 μg) of internal standards, mixtures were injected directly into GC equipped with an FID as the detector. The signals were set as follows: FID range = 1 with attenuation = 1. Using the above analysis conditions, the CV% of recovery of safrole and isosafrole was set at 15%. That is to say, the lowest quantitatively determinable concentration was around 0.25 μg/mL, which was within the use limit of 1 μg/mL according to the food additive regulations,

Table 2. Lowest quantitatively determinable concentration of safrole and iso-safrole by gas chromatography with FID detector

Compound	Concentration (μg/mL)	Detectability[a]	Recovery (%)[b]	RSD (%)[c]
Safrole	5.0	Yes	103.7	4.8
	2.5	Yes	104.5	3.9
	1.0	Yes	103.1	5.7
	0.5	Yes	108.7	10.4
	0.25	Yes	111.8	11.5
	0.1	Yes	121.9	19.7
cis-Isosafrole	5.0	Yes	101.3	3.8
	2.5	Yes	97.8	5.9
	1.0	Yes	98.6	6.3
	0.5	Yes	112.8	10.4
	0.25	Yes	109.5	12.4
	0.1	No	–	–
trans-Isosafrole	5.0	Yes	98.5	2.9
	2.5	Yes	102.7	5.8
	1.0	Yes	99.1	3.7
	0.5	Yes	107.8	10.4
	0.25	Yes	113.7	11.3
	0.1	No	–	–

[a] FID range =1, Attenuation=1. [b] Average of triplicate analyses. [c] Coefficient of variation (cv%).

Table 3. Recoveries of the spiked safrole, cis-isosafrole and trans-isosafrole from soft drink (S-1) by direct injection method

Compound	Blank[a] (μg) (A)	Amount added (μg)(B)	Amount found (μg)[b] (C)	Recovery (%)[c]	CV (%)[d]
Safrole	4.28	10.00	15.02	105.18	5.41
	4.28	5.00	10.01	107.86	8.69
Isosafrolee	0.00	10.00	10.36	103.6	6.87
	0.00	5.00	4.92	98.40	8.24

[a] Safrole, cis-isosafrole and trans-isosafrole in 1 mL soft drink (S-1).
[b] Average of triplicate analyses. [c] Recovery (%) = (C-A)/ Bx100%.
[d] Coefficient of variation (cv%). [e] The mixture of cis- and trans- isosafrole.

Table 4. Safrole and isosafrole content in soft drink (S-1) analyzed by using direct injection GC and AOAC methods

Method[a]	Content (μg/mL)[b]		
	Safrole (CV%)[c]	Cis-isosafrole (CV%)[c]	Trans-isosafrole
AOAC	3.18(9.7%)	ND	ND
Direct injection	4.31(5.8%)	ND	ND

[a] Direct injection method = proposed method in this study.
AOAC method = steam distillation, distillate was extracted by chloroform and then determined by GC[(4)].
[b] Average of triplicate analyses.
[c] Coefficient of variation (cv%).

indicating that our developed method was conducted legally as shown in Table 2.

IV. *The Recovery Test*

The recovery test data of the additions of safrole and isosafrole in soft drink samples (S-1) are listed in Table 3. Results indicated that the r eries of the additions of 10.0 μg safrole and 5.0 μg isosafrole in 1 mL of soft drink samples (S-1) were 105-108% and 98-104 respectively, with the coefficients of variation less than 8.7%. The above results show that the direct injection gas chromatographic method adopted in this research required no sample pretreatment procedure within only 25 min for each sample operation. Therefore, it was a more rapid and simple method than the AOAC method which involves timeconsuming procedures such as distillation and extraction (the operation time is more than 2 hrs).

V. *In Comparison with Our Developed Method and AOAC Method*

The comparisons between the direct injection gas chromatographic method developed by our laboratory and AOAC method involving distillation-extraction ($CHC1_3$) for determination of safrole and isosafrole in soft drinks are shown in Table 4. Results demonstrated that the contents of safrole and isosafrole detected by our developed method were 4.31 μg/mL and 0 μg/mL respectively, with the coefficient of variation (CV%) than 5.8%. However, the contents of safrole and cis-isosafrole detected by the AOAC method were 3.18 μg/mL and 0 μg/mL respectively, with coefficient of variation less than 9.7%. Therefore, our developed method was more accurate than the AOAC method with respect to the coefficient of variation. Furthermore, the detection value of safrole was lower by the AOAC method involving the distillation-extraction ($CHC1_3$) procedure. Results indicated that ele-

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Table 5. Safrole, cis-isosafrole and trans-isosafrole content in some commercial soft drinks

Sample	Content (μg/mL)		
	Safrole	cis-Isosafrole	trans-Isosafrole
S-1	4.28	ND[b]	ND
S-2	3.85	ND	ND
S-3	5.56	ND	ND
S-4	4.12	0.89	ND
S-5	4.34	ND	ND
C-1	4.16	1.38	ND
C-2	3.98	0.78	ND
C-3	4.45	0.73	ND
C-4	3.42	0.62	ND
C-5	4.47	1.05	ND
P-1	2.99	ND	ND
P-2	3.87	ND	ND
P-3	3.64	ND	ND
P-4	3.19	ND	ND
P-5	4.34	ND	ND
A-1	ND	ND	ND
A-2	ND	ND	ND
A-3	ND	ND	ND
A-4	ND	ND	ND
A-5	ND	ND	ND
R-1	4.50	ND	1.36
R-2	3.80	ND	ND
R-3	4.11	ND	0.67
R-4	3.38	ND	ND
R-5	4.06	ND	ND

[a] Average of duplicate analyses. [b] ND = not detected.

ments to be quantified tended to go away causing lower recovery. In addition, $CHCl_3$ was difficult to purchase in the market because it is a kind of restricted solvent. Therefore, the direct injection gas chromatographic method developed by our laboratory is worthy of application.

VI. *The Contents of Safrole and Isosafrole in Commercial Soft Drinks*

25 various soft drinks were purchased from the market including Sarsaparilla, Coca-Cola, Pepsi, Root Sar and Apple Cider. Results showed that the contents of safrole, cis-isosarole and trans-isosafrole were 0-5.56, 0-1.38 and 0-1.36 μg/mL respectively when analyzed with the direct injection gas chromatographic method adopted in this research. 20 out of 25 soft drink samples contained safrole and/or cis-isosafrole and the contents of safrole all exceeded the use limit of 1 μg/mL according to the food additive regulations. A main reason for the above results was that all soft drinks that contained safrole consisted of natural seasoning extracts (which were labeled on the products) and which comprised a high volume of safrole.

CONCLUSIONS

Our research developed a simple, rapid and accurate method for quantitative determination of safrole and isosafrole in various commercial soft drinks purchased in the market. By using this method, various soft drink samples were added with a proper volume of water-soluble internal standard DHB and then 0.1 μL of mixture was injected directly into GC for analysis without any sample pretreatment procedure. Only 25 min was needed for each sample operation. Our developed method was used to determine the contents of safrole and isosafrole in 25 various soft drinks. Results showed that all soft drinks labeled with natural seasoning extracts all contained safrole andlor iso-safrole, and most exceeded 3-5 times the regulated concentration of 1 μg/mL.

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市售清涼飲料中黃樟素之簡易快速氣相層析定量分析法

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摘 要

本研究建立了市售清涼飲料中黃樟素之簡便快速氣相層析方法。選擇水溶性之1,4-dihydroxybenzene（DHB）為內標準加入清涼飲料中，樣品不需經任何前處理，直接注入氣相層析儀中，採用直接注入（direct injection）之方式，配合中間極性之CP-SIL 8CB管柱（30 m × 0.53 mm，1.5 μm）分析定量清涼飲料之黃樟素。最低檢出量為0.25 μg/mL左右。添加safrole及isosafrole 5.0及10.0 μg/mL於1 mL檢體中，直接注入GC分析，其回收率為98~108%，變異係數cv%在8.7%以下。以本方法分析市售不同廠牌之清涼飲料中黃樟素含量。結果顯示25件清涼飲料中有20件檢測出含safroleh及／或isosafrole。且safrole之含量均超過食品添加物使用範圍及用量標準之規定（1 μg/mL）3-5倍。

關鍵詞：清涼飲料，黃樟素，直接注入法，氣相層析，定量分析