International Journal of Chemistry; Vol. 6, No. 3; 2014
ISSN 1916-9698 E-ISSN 1916-9701
Published by Canadian Center of Science and Education

Determination of Safrole in Ethanol Extract of Nutmeg (*Myristica fragrans* Houtt) Using Reversed-Phase High Performance Liquid Chromatography

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Received: February 17, 2014 Accepted: April 27, 2014 Online Published: June 28, 2014

doi:10.5539/ijc.v6n3p14 URL: http://dx.doi.org/10.5539/ijc.v6n3p14

Abstract

Dehydrodiisoeugenol (DDIE), myristicin, and safrole are chemical compounds contained in fruit and seed of nutmeg (*Myristica fragrans* Houtt). DDIE shows antidiabetic activity on PPARγ receptor, while myristicin is hallucinogenic agent. Of the three compounds, safrole is the most toxic substance due to its carcinogenic activity. In this work, we developed an analytical method to determine safrole in ethanol extract of nutmeg. Reversed-Phase High Performance Liquid Chromatography (Dionex Ultimate 3000) using C-18 LiChroCART 250-4, LiChrospher 100 RP 18e (5 µm) 250 mm column as stationary phase, was selected as the method of analysis. A mixture of methanol:water (73:27) at flow rate 1 mL/min was used as mobile phase. Detection was done at 282 nm. Using such conditions, retention time for safrole was 10.45 minutes. The recovery was 101.421%, while the value of CV was 0.838%. LOD and LOQ were 0.668 µg/mL and 2.023 µg/mL, respectively. Mean concentration of safrole in the nutmeg seed extract was 10.979%.

Keywords: HPLC, safrole, validation

1. Introduction

Dehydrodiisoeugenol (DDIE), myristicin, and safrole are chemical compounds contained in fruit and seed of nutmeg (*Myristica fragrans* Houtt). DDIE shows antidiabetic activity on PPARγ receptor, while myristicin is hallucinogenic agent. Of the three compounds, safrole is the most toxic substance due to its carcinogenic activity (Lestari, 2010; Li & Yang, 2012). Safrole and isosafrole (0.5%) has been shown to increase the occurance rate of malignant tumours in mice (Benedetti, Malnoe, & Broillet, 1977). The major toxicity of safrole is caused by its metabolite character. Safrole is oxidized into 1-hydroxysafrole in human body, which is carcinogenic (Peele Jr. & Oswald, 1978). The maximum dose of safrole as stated by UK and French governments is 1 mg/day (European Commission, 2002).

Currently Indonesian pharmaceutical industry is developing a formula of nutmeg extract as antidiabetic drug, based on its DDIE activity which inhibits PPARγ receptor, therefore a rapid and accurate method for the quantification of safrole in nutmeg extract is interesting to be developed. At present, methods of analysis of safrole are HPLC and GC (Archer, 1988; France, Association Francaise de Normalisation, 1986; Choong & Lin, 2001). AOAC described a procedure to determine safrole and isosafrole in soft drinks, where both compounds have to be distilled with steam, extracted with organic solvent, e.g $CHCl_3$, and then injected into GC column for separating and analyzing steps. HPLC using small particles with a high-pressure pump system and sensitive detector. Advantage of HPLC is to provide high-resolution, efficient and fast separation (Skoog, Holler, & Nieman, 1992; Willard, Merrit Jr., Dean, & Settle Jr., 1988; Nagore, Vinod, Pankaj, & Tushar, 2013; Chan, Herman, Lee, & Zhang, 2004). The aim of this study was to determine the concentration of safrole in ethanol extract of nutmeg.

2. Methods

2.1 Materials and Equipments

Materials used are safrole standard (Fluka), ethanol extract of nutmeg (Kimia Farma), double distilled water (IPHA Lab.), and methanol for HPLC (JT Baker). The equipments used are liquid chromatography (Dionex Ultimate 3000), C18 chromatography column (LiChroCART 250-4, LiChrospher 100 RP 18e 5 μm × 250 mm) and ultraviolet spectrophotometer (Dionex Ultimate).

2.2 Preparation of Standard Solution

Standard solution was prepared by diluting safrole in methanol (16 μg/mL).

2.3 Preparation of Extract Solution

Nutmeg extract was accurately weighed 100 mg and dissolved in 10 mL methanol (Chan, Herman, Lee, & Zhang, 2004). The concentration of the extract solution was 10000 μg/mL.

2.4 Preparation of Mobile Phase

Mobile phase was chosen based on the solubility of safrole. In this study, the mobile phase was a mixture of methanol:water (73:27). This composition was selected based on the result of resolution value (> 1.5) in optimization steps, as shown in Table 1.

Table 1. Determination of Resolution of Analytes in different mobile phase composition

Methanol:H_2O	Analyte	Time of retention (minute)	Resolution*
73 : 27	Myristicin	8.260	M-S = 1.605
	Safrole	10.507	S-D = 1.578
	DDIE	13.900	D-M = 2.507
80 : 20	Myristicin	13.308	M-S = 1.597
	Safrol	16.183	S-D = 1.092
	DDIE	18.367	D-M = 2.976

Description: M-S: Myristicin against safrole; S-D: Safrole against DDIE; D-M: DDIE against myristicin.

2.5 HPLC Conditions

Analysis of safrole was performed at room temperature on isocratic conditions at a flow rate 1 mL/min, and was detected at 282 nm using both UV and PDA detectors.

2.6 Validation of Analytical Method [According to ICH Guidelines]

2.6.1 Linearity

Standard solution was pipetted 6.25; 12.5, 25, 50, and 100 mL, respectively, and inserted into the eppendorf tube. The solutions were adjusted using methanol to obtain solutions of 1, 2, 4, 8, and 16 μg/mL, respectively. The solutions were injected into the chromatograph injection gate. Linearity is determined based on the correlation coefficient (r) in the linear regression $y = bx + a$ from the curve of the relationship between area with concentration (Archer, 1988).

2.6.2 Accuracy

Accuracy is measured by determining the recovery (%) (Chan, Herman, Lee, & Zhang, 2004). Standard solution was pipetted @ 50 mL and inserted into six 150 mL flasks diluted with methanol to obtain 4 μg/mL solutions. Recovery is calculated by the formula:

$$\text{Recovery (\%)} = \frac{\text{measured concentration}}{\text{actual concentration}} \times 100\%$$

2.6.3 Precision

Precision is measured by determining three concentrations of analyte with three times replication or minimum six times replication at 100% concentration of analyte (ICH Harmonised Tripartite Guideline, 1996). Standard

solution was pipetted @ 50 mL and inserted into six 150 mL flasks diluted with methanol to obtain 4 µg/mL solutions. The coefficient of variance (CV) was calculated.

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

LOD and LOQ are obtained based on the standard deviation and slope. LOD and LOQ are calculated using the following formula (Ditjen POM, DepKes RI, 1994):

$$\text{LOD} = \frac{3{,}3 \times SD}{b}$$

$$\text{LOQ} = \frac{10 \times SD}{b}$$

SD: Standard deviation; B: Slope of the linear regression.

2.8 Determination of Safrole in Nutmeg Seed Extract

Nutmeg extract solution was prepared in three flasks, each filtered by using 0.45 µm porous filter then degassed. 20 µL extract solution was injected into the chromatograph gate. Concentration of safrole in the nutmeg seed extract was calculated by using linear regression equation.

3. Results and Discussion

Safrole can be analyzed using reverse phase HPLC with methanol:water (73:27) as a mobile phase and flow rate 1 mL/min at 282 nm. This wavelength was selected based on calculation according to Woodward Fieser rule, i.e.:

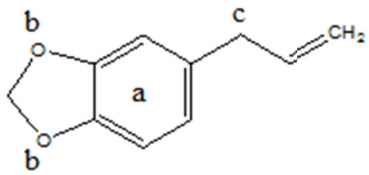


Figure 1. Chemical structure of safrole

a.	Benzene ring	=	256	nm	
b.	O-alkyl	=	12	nm	
c.	Alkyl residue	=	5	nm	+
		=	273	nm	

The obtained value (282 nm) of theoretical maximum wavelength correlates well with experimental value (283 nm).

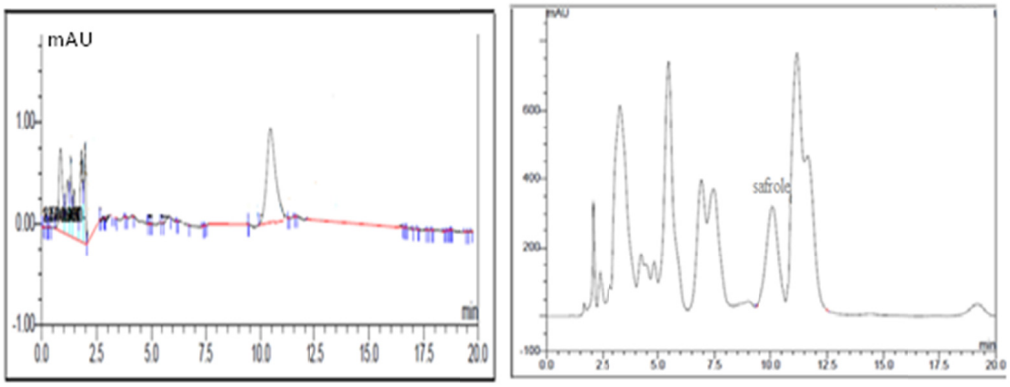
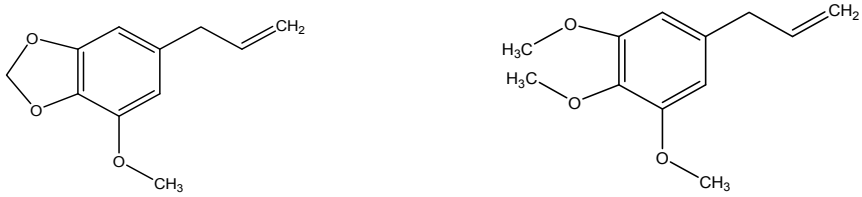


Figure 2. Chromatogram of 16 µg/mL safrole standard (left) and safrole in 10000 µg/mL nutmeg extract (right) using UV detector

Safrole was detected at 10.45 minute (Figure 2), indicating that this compound retained in the nonpolar stationary phase due to its lipophilic character, while myristicin showed a double peak at 7.5 minute. This double peak probably was caused by poor separation of myristicin and its analogue, elemicin (Figure 3) due to their similar polarity.

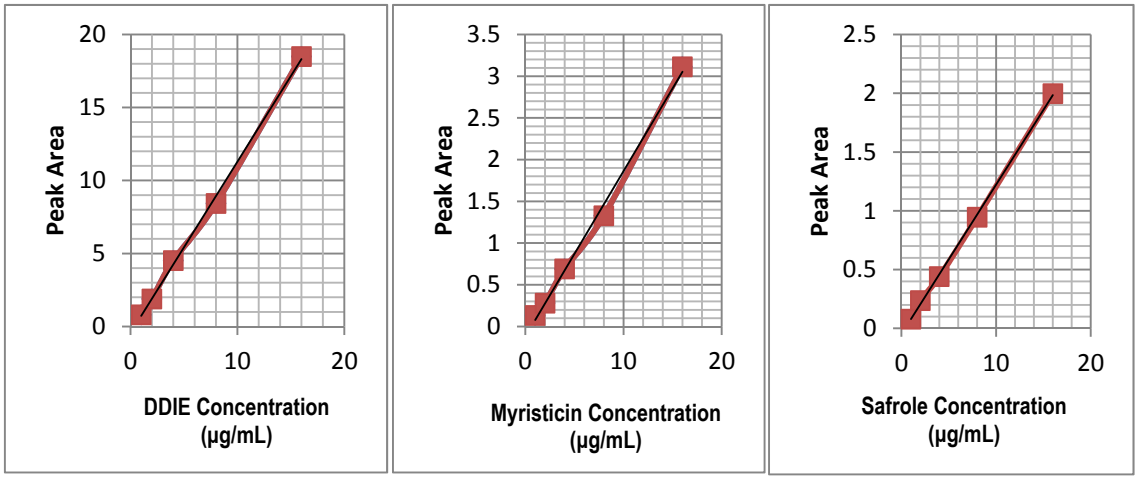


(a) (b)

Figure 3. Chemical structure of myristicin (a) and elemicin (b)

3.1 Linearity

The method gave linear response to DDIE, myristicin, and safrole (Figure 4) within the concentration 1, 2, 4, 8, and 16 ppm with r^2 = 0.999, 0.998, 0.999, respectively. Linear regression curves for DDIE, myristicin, and safrole were $y = 1.1720x - 0.438925$; $y = 0.1983\ x - 0.12042$; and $y = 0.1270x - 0.04726$, respectively.



(a) (b) (c)

Figure 4. Linear responses of peak area against the concentrations of DDIE (a), myristicin (b) and safrole (c)

All three curves indicated that the instrument response is proportional with the concentration. These data showed that Lambert-Beer law was obeyed by the condition of analytical method for all three compounds within the interval of concentrations selected.

3.2 Accuracy and Precision

Determination of accuracy and precision obtained from the calculation of six times measurements at concentration 4 µg/mL. The measurement results are shown in Table 2. Instrument response (area) entered into the linear regression equation which has been previously calculated, so that the measured concentration was obtained. Then the recovery and coefficient of variance were calculated, i.e. 101.421% and 0.838%. The values show that the method used for safrole quantification presents high accuracy and precision.

Table 2. Accuracy and precision of safrole

Actual concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (%)	SD	CV (%)
4	4.052	101.300	0.034	0.838
	4.033	100.825		
	4.097	102.425		
	4.102	102.550		
	4.029	100.725		
	4.028	100.700		
Mean	4.057	101.421		

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

LOD and LOQ were calculated with the formula previously described, the SD values was obtained by the formula: SD = $\sqrt{\frac{\Sigma(yi-y')^2}{n-2}}$. In Table 3, calculations have been described to obtain the value of $(y-y_1)^2$. By inserting these values into the formula, with n = 5, obtained SD is 0.0257.

Table 3. Determination of LOD and LOQ

x Concentration (µg/mL)	y Area	y_1	$(y-y_1)$	$(y-y_1)^2$
1	0.0796	0.08	-0.0004	0.00000016
2	0.2368	0.207	0.0298	0.000888
4	0.4408	0.461	-0.0202	0.000408
8	0.9456	0.969	-0.0234	0.0005476
16	1.9968	1.985	0.0118	0.0001392
				0.00198296

LOD and LOQ were calculated by using following formula:

$$\text{LOD} = \frac{3{,}3\ x\ SD}{b} = \frac{3{,}3\ x\ 0.0257}{0.127} = 0.668\ \mu g/mL$$

$$\text{LOQ} = \frac{10\ x\ SD}{b} = \frac{10\ x\ 0.0257}{0.127} = 2.023\ \mu g/mL$$

LOD and LOQ of safrole with this method are 0.668 µg/mL and 2.023 mg/mL, respectively. LOD and LOQ indicate safrole concentration limit that can still be detected and quantized using this method.

3.4 Determination of Safrole in Nutmeg Seed Extract

Determination of safrole concentration in the extract was calculated by entering the instrument's response (area) to the linear regression equation. Safrole concentration from three measurements are shown in Table 4. Average concentration of safrole contained in nutmeg seed extract is 10.979%. Standard deviation and coefficient of variance, respectively are 0.005 and 0.045, respectively.

Table 4. Determination of safrole in the extract

Extract concentration (µg/mL)	Area (mAU*min)	Safrole concentration (µg/mL)	Safrole concentration (%)	SD	CV (%)
10000	139.427	1098.525	10.985	0.521	0.047
	139.306	1097.572	10.976		
	139.320	1097.683	10.977		
Mean	139.351	1097.927	10.979		

4. Conclusion

Our HPLC method can be used to determine the concentration of safrole in nutmeg seed extract with methanol: water (73:27) as mobile phase, flow rate 1 mL/min. Detection wavelength was at 282 nm. This method fulfilled validation criterias, excluding specifity and robustness. Mean concentration of safrole in the nutmeg seed extract is 10.979%.

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