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Development And Validation Of A RP-HPLC Method To Determine Dehydrodiisoeugenol, Myristicin, And Safrole In Ethanol Extract Of Nutmeg (*Myristica fragrans* Houtt).

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ABSTRACT

A simple and validated analytical method to determine myristicin, safrole, and dehydrodiisoeugenol (DDIE) in nutmeg extract has been developed. High Performance Liquid Chromatography (HPLC) using C-18 LiChroCART 250-4, LiChrospher 100 RP 18e (5 µm) 250 mm column as stationary phase and methanol: water (73:27) as a mobile phase with the flow rate 1 mL/min was selected. Detection was done by using ultraviolet (UV) spectrophotometer at 282 nm. Retention time for myristicin, safrole, and DDIE were 8,260; 10,507; 13,900 minute. Limit of detection and limit of quantitation for myristicin, safrole, and DDIE were 0,991 µg/mL and 3,004 µg/mL; 0,668 µg/mL and 2,023 µg/mL; 0,981 µg/mL and 2,973 µg/mL, respectively. The recovery for myristicin, safrole, and DDIE were 99,754 ± 0,788 %; 101.421 ± 0,855 %; 100,242 ± 1,327 %, while the coefficient of variance for myristicin, safrole, and DDIE were 0,802 %; 0.838 %; 1,324 %. Mean concentration of MYR, SAF, and DDIE were 17,226%; 10,979%; 4,662%.

Keywords: HPLC, dehydrodiisoeugenol, myristicin, safrole, validation

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INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt) is Indonesian plant that has antidiabetic and hallucinogenic activity, but it also has carcinogenic activity. Antidiabetic activity is given by dehydrodiisoeugenol (DDIE) through its capability on PPARγ receptor inhibition, hallucinogenic activity is given by myristicin (MYR), while the most toxic substance due to its carcinogenic activity is given by safrole (SAF) [1,2,3,4,5]. The maximum dose of safrole as stated by UK and French governments is 1 mg/day [5].

In Indonesia, tablet formulation from nutmeg extract is developing due to its DDIE activity on PPARγ receptor. A simple, rapid, and accurate method for determining DDIE, MYR, and SAF is interesting to be developed. Several HPLC and GC methods using variety of columns and mobile phase for determination of myristicin and safrole have been reported [4,6, 7,8,9], but there was no reported method to determination of myristicin, safrole, and DDIE simultaneously. In the present study, HPLC is also used based on its advantages on high resolution, efficiency, and fast separation. The aim of this study was to validate and determine the concentration of DDIE, MYR, and SAF in ethanol extract of nutmeg.

EXPERIMENTAL

Reagents and Equipment's

DDIE, MYR, and SAF standards (Fluka), ethanol extract of nutmeg (Kimia Farma), aquabidest (IPHA Laboratories), methanol HPLC grade (JT Baker). Analysis was carried out using a HPLC Dionex Ultimate 3000, with a UV-Vis detector (Dionex Ultimate), RP 18 column (LiChroCART 250-4, LiChrospher 100 RP 18e 5 µmx 250 mm).

Preparation of Mixed Standard Solution

Mixed standard solution was prepared by diluting DDIE, MYR, and SAF with metanol (16 µg/mL).

Preparation of Extract Solution

Nutmeg extract solution was prepared by diluting 100 mg extract in 10 mL methanol [10] (10000 µg/mL)

Chromatographic conditions

Chromatographic analysis of DDIE, MYR, and SAF was done at 28°C. The maximum wavelength was 282 nm, with the flow rate 1 mL/min. Optimization was done by using different composition of mobile phase. Then, the resolution and capacity factor for each composition of mobile phase were measured to get the best composition on the effectivity of separation.

Validation of Analytical Method

Linearity

Mixed standard solution of DDIE, MYR, and SAF of 16 µg/mL was used for preparation of subsequent aliquots. The solution adjusted to obtain 1, 2, 4, 8, and 16 µg/mL. 20 µl of the solution was injected into sample load. The calibration curve of the area under curve versus concentration were recorded. Linearity is determined based on the correlation coefficient (r) in the linear regression $y = bx + a$ [11].

Accuracy and Precision

Six tubes of 4 µg/mL mixed standard solution was made then injected into HPLC system. Accuracy was calculated by using recovery (%). The precision was measured by determining coefficient of variance (CV)[11].

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

LOD and LOQ were calculated using the following formula [11]:

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

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

SD : standard deviation
B : slope of the linear regression

Determination of DDIE, MYR, SAF in Nutmeg Seed Extract

Nutmeg extract solution was prepared in three flasks. This solution was vortexed and sonicated. Then 20 µL extract solution was injected into sample load after the filtration using 0,22 µm membrane filter. Concentration of DDIE, MYR, SAF was calculated by using linear regression equation.

RESULT AND DISCUSSION

Mobile phase composition used was 73:27 and 80: 20 of methanol and water. The effectivity of separation is presented by its resolution (Rs) and capacity factor (k'). The resolution is a quantitative measure of how well two elution peaks can be differentiated in a chromatographic separation. The method has good separation if the resolution value is above 1,5. Capacity factor is measured to obtain the time effectivity of the analysis. The requirement for k' is between 1 to 15 [13]. The value of Rs and k' shown in Table 1. From Table 1, we can conclude that 73:27 of methanol and water as mobile phase is the best composition.

Table 1. Resolution and k' of DDIE, MYR, SAF

Methanol:Water	Analyte	Retention time	Rs	k'
73:27	MYR	8,260	M-S = 1,605	8,556
	SAF	10,507	S-D = 1,578	10,674
	DDIE	13,900	D-M = 2,507	14,444
80:20	MYR	13,308	M-S = 2,875	13,787
	SAF	16,183	S-D = 1,680	16,981
	DDIE	18,367	D-M = 3,891	19,408

Note: M-S : MYR to SAF
S-D : SAF to DDIE
D-M : DDIE to MYR

DDIE, myristicin, and safrole were completely separated on RP18 column by RP-HPLC using isocratic elution of methanol: water (73:27) as mobile phase with flow rate 1 mL/min at 282 nm. The chromatogram of the mixed standard solution is shown in Figure 1.

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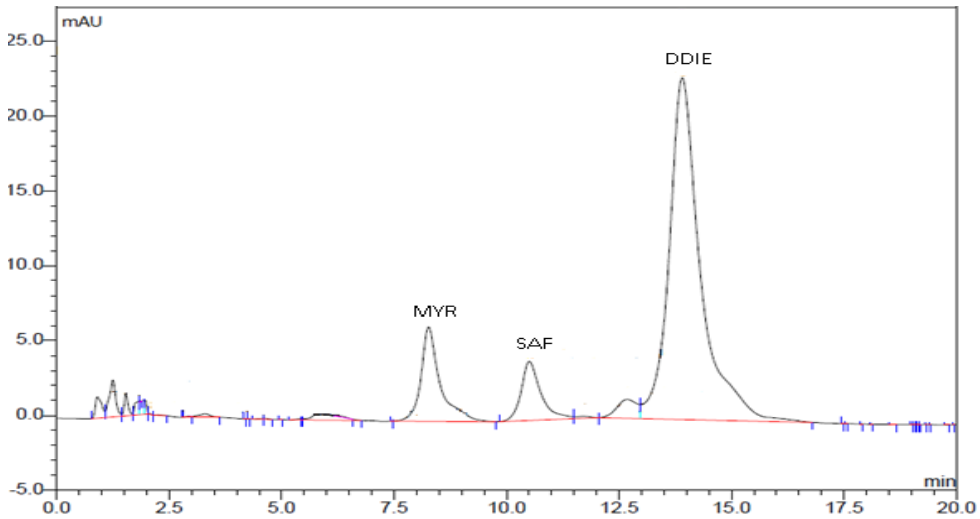

Figure 1. Chromatogram of 16 µg/mL DDIE, MYR, SAF

Linearity

The method gave linear response to DDIE, MYR, and SAF within the concentration 1,2,4, 8, and 16 µg/mL. The value of r and linear regression equation are shown in Table 2. All three r values indicated that the instrument response is proportional with the concentration.

Table 2. Value of r and linear regression equation of DDIE, MYR, SAF

Analyte	r	Linear regression equation ($y = ax + b$)
DDIE	0.9991	y = 1.172010 x – 0.438925
MYR	0.9978	y = 0.198306 x – 0.12042
SAF	0.9996	y = 0.126965 x – 0.04726

Accuracy and Precision

Accuracy and precision are obtained from the calculation of six times measurements of mixed standard solution at concentration 4 µg/mL. The method has good accuracy if the recovery is between 98-102%, while coefficient of varriance is below 2%.The measurement results are shown in Table 3. The values show that the method has good accuracy and pecision.

Table 3. Recovery and coefficient of varriance of DDIE, MYR, SAF

Analyte	Recovery (%)	CV (%)
DDIE	100,242	1,324
MYR	99,754	0,802
SAF	101,421	0,838

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

LOD and LOQ were calculated with the formula previously described. LOD and LOQ for each compound shown in Table 4. This analytical method gave better LOD and LOQ than the study before, that was done by Nagore *et al* (2013).

Table 4. LOD and LOQ of DDIE, MYR, SAF

Analyte	LOD (µg/mL)	LOQ (µg/mL)
DDIE	0,981	2,973
MYR	0,991	3,004
SAF	0,668	2,024

Determination of DDIE, MYR, and SAF in Nutmeg Extract

Determination of DDIE, MYR, and SAF is obtained from the calculation of three times measurements of nutmeg extract at concentration 10000 µg/mL. Concentration of DDIE, MYR, and SAF in nutmeg extract was calculated by entering the instrument's response to the linear regression equation. Average concentration of DDIE, MYR, and SAF in nutmeg extract are 4,662%; 17,226%; and 10,979%, respectively.

CONCLUSION

HPLC method using methanol: water (73:27) as mobile phase, flow rate 1 mL/min, and detection wavelength at 282 nm can be used to determine DDIE, MYR, and SAF simultaneously. All validation criteria's has been fulfilled. Mean concentration of DDIE, MYR, and SAF in the nutmeg extract are 4,662%; 17,226%; and 10,979%, respectively.

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