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Standardization of Myristicin in Nutmeg (*Myristica fragrans* Houtt.) Fruit using TLC-Densitometric Method

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Abstract

Background*: Myristica fragrans Houtt. (Myristicaceae family), with the main content of myristicin, has been immensely used in herbal medicine. Standardization is essential to ensure the safety of natural extracts and the quality of herbal medicines using various chemical analysis techniques. Method validation is necessary to ascertain the reliability and reproducibility of the method. Myristicin is a member of the phenylpropene group, a natural organic compound found in small amounts in nutmeg fruit, which has pharmacological effects.* ***Objective:*** *This study aims to determine the myristicin content in nutmeg fruit using TLC-Densitometry.* ***Methods****: Determination of myristicin in nutmeg fruit extract was performed using TLC-Densitometry with silica GF_{254} as stationary phase, mobile phase n-hexane: ethyl acetate (8:2 v/v), and spot visualized at 285 nm. In this study, the content of myristicin in nutmeg fruit was determined using compendial methods (AOAC), thus requiring method verification with parameters including selectivity, linearity, precision, LOD, and LOQ.* ***Results****: The validation of this method showed good linearity and selectivity with y = 0.0001x + 0.0226 (r = 0.9996) and 1.53 (>1.5), respectively. The LOD and LOQ results were low with values of 0.11 µg/spot and 0.33 µg/spot, respectively. The percentage coefficient of variation for precision was below the requirement value of not more than 4%. The average myristicin content in nutmeg fruit extract was approximately 0.0017 ± 0.0003% (w/w).* ***Conclusion****: The developed method was valid and sensitive for the quantification of myristicin content in nutmeg fruit.*

Keywords*: densitometry, Myristica fragrans Houtt., myristicin, standardization, validation*

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INTRODUCTION

Method validation is an analytical technique based on laboratory experiments to demonstrate that the validation parameters satisfy the needs of the method's users and that the findings are nearly identical to the actual value and repeatable (Sugihartini *et al*., 2012). Quantitative analysis of substances or drug components in a biological sample, for example, in plants, must meet the requirements of the method validation. Therefore, method validation is crucial to determine and ensure the accuracy, specificity, reliability, and reproducibility of a method to be used for analytical purposes (United States Pharmacopeial Convention, 2004). Method validation parameters include accuracy, precision, specificity/selectivity, limit of detection (LOD), limit of quantification (LOQ), and linearity. If a standard method (Compendial method) is used, there is no need for method validation but verification. For data collected, data standardization will apply (Sudjarwo *et al*., 2019).

Standardization is necessary to guarantee herbal medicine's quality (Kadian *et al*., 2016). Standardization of herbal medicine is the process of comparing levels to features, constant factors, and qualitative and quantitative values that offer genuine assurance of safety, efficacy, quality, and repeatability (Kumari & Kotecha, 2016; Shulammithi *et al*., 2016; Butt *et al*., 2018). The standardization emphasized the determination of compounds with pharmacological effects, a process of ensuring quality assurance and good standards for both drugs and herbal products (Kunle, 2012).

The nutmeg plant (*Myristica fragrans* Houtt.), belonging to the *Myristicaceae* family, contains small amounts of naturally occurring chemical substances called myristicin, also known as phenylpropenes. Myristicin is known to be responsible for a disease's pharmacological properties or biological activity. The concentration of myristicin in nutmeg oil varies depending on the origin of the fruit, extraction technique, drying process, and part of the fruit used (Liunokas & Karwur, 2020).

In the standardization of myristicin, spectroscopic, TLC, and HPLC methods can be used (Naikodi *et al*., 2011). The TLC-Densitometry method, which is versatile enough to identify nearly every constituent in plants, is one of the most popular analytical techniques for examining the chemical components of plants. Considering the enormous demand for herbal medicines in the global market, many methods are well-developed for standardizing raw materials. This current study determined myristicin content using the compendial method (United States Pharmacopeial Convention, 2012; AOAC, 2019).

MATERIALS AND METHODS

Materials

Myristica fragrans Houtt. was obtained from Haruku Island, Oma Village, Central Maluku Regency, Maluku Province, Indonesia, and identified by Herbal Materia Medica Laboratory, Batu, Malang, East Java (067/259/102.20/2023). Myristicin standard (pharmaceutical grade, Chemfaces), technical ethanol 96%, pro-analytical ethanol (Merck), ethyl acetate (Merck), n-hexane (Merck), and Silica Gel TLC 60 F_{254} (Merck).

Instruments

This current study utilized several instruments which include OHAUS analytical balance, rotary vacuum evaporator (BUCHI), sonicator, 20×10×5 cm3 chromatography chamber (CAMAG), densitometer (CAMAG), UV CAMAG lamp, TLC Scanner 4 (CAMAG), Linomat 5 (CAMAG), and VisionCATS software (CAMAG).

Methods

Plant determination

Determination of the plant samples was carried out at the Herbal Materia Medica Batu Laboratory. Based on the letter number 067/259, 102.20/2023 issued in Batu on February 08, 2023, the plant used was *Myristica fragrans* Houtt from the *Myristicaceae* family.

Dried plant powder preparation and extraction

Nutmeg (*Myristica fragrans* Houtt.) fruit has been cleaned and washed, chopped into small pieces, dried by aerating at room temperature 15-30 ° C, and not exposed to direct sunlight. The dried plant then was ground or mashed to form a powder. The powder of nutmeg (*Myristica fragrans* Houtt.) was weighed as much as 200 grams and extracted with 96% ethanol (1:4) using the UAE (Ultrasonic Assisted Extraction) method for 45 minutes while occasionally shaking. Re-maceration was performed twice, using the same type and volume of solvent. A rotary vacuum evaporator concentrated the extract at 50–60°C until a thick extract was produced (Budiastra *et al*., 2013).

Determination of moisture content

The thick extract of nutmeg fruit and dried plant powder was each weighed 1 gram in a preheated porcelain crucible with a lid at 105 °C for 3 hours until their constant weight was achieved. Subsequently, the materials in the crucible were flattened, dried at 105°C for 1 hour, and then weighed. The step was repeated

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twice until a constant weight of the heating product was obtained (no more than 0.25%) (Courtney, 2017; Sri *et al*., 2021).

Preparation of myristicin standard solution

The myristicin standard (10 mg) was dissolved in ethanol p.a in a 10 mL volumetric flask and added ethanol p.a until the limit mark reached 10 mL.

Sample solution preparation

The thick extract of nutmeg fruit (500 mg) was dissolved in ethanol p.a in a 5 mL volumetric flask.

Wavelength determination

Each blank, myristicin standard, sample, and sample solution with myristicin standard addition were photographed on the silica GF_{254} plates with a Linomat 5 applicator. The plate was developed using the selected mobile phase and observed at 200 - 400 nm wavelength region spectrum with a densitometer.

Method verification

Selectivity

Each 10 µL of blank solution, myristicin standard, sample solution, and myristicin standard-added sample solution were spotted on silica GF_{254} 6 × 10 cm TLC plates. The TLC plate was developed with the selected mobile phase; then, the chromatogram was observed with a densitometer, as well the Rf value and the degree of resolution (Rs) were calculated at the selected wavelength.

Limit of detection (LOD) and limit of quantification (LOQ)

An amount of 10 µL of blank solution and a series of myristicin standard solutions with an increasing concentration were spotted on the silica GF254 TLC plate. The plates were developed with a selected mobile phase, and the areas were observed at a selected wavelength. The standard deviation (σ) of the blank area and the linear regression equation (y = bx + a) between the weight of the myristicin standard solution (µg) and the response area, slope (b) were calculated (Bhardwaj *et al*., 2020):

$$LOD = 3.3\ \sigma/b \quad (1)$$

$$LOQ = 10\ \sigma/b \quad (2)$$

Linearity

Myristicin standard series solution was photographed with equal volume on silica gel GF254 TLC plates with selected mobile phase and then observed at a selected wavelength with the densitometer.

Precision

Ten spotting points were made on a silica GF_{254} TLC plate, each being bottled with 60 µL of a 1,000 µg/mL myristicin standard solution. The TLC plates were developed with the selected mobile phase. Using a densitometer, the average area of the spots was calculated at the selected wavelength, standard deviation (SD), and coefficient of variation (C.V).

Determination of myristicin content in nutmeg (*Myristica fragrans* Houtt.) extracts

The thick extract of nutmeg fruit (0.5 g) was weighed, and myristicin standard solution of varying weight was added. Then, 50 µL of each solution was bottled onto silica GF254 TLC plates using Linomat 5. A constant application rate of 100 nL/s was maintained, with a distance of 15 mm between each band. The slit dimension on the densitometer used was 10 × 0.4 mm, and the scanning speed used was 100 mm/s. The TLC plates were developed with the selected mobile phase. The area was observed at the selected wavelength on the densitometer. This level was determined with as many as four replicates in the same way (AOAC, 2019; Kai & Province, 2008; United States Pharmacopeial Convention, 2012).

RESULTS AND DISCUSSION

Preparation of nutmeg (*Myristica fragrans* Houtt.) fruit extract

Out of 15 Kg (wet weight) nutmeg (*Myristica fragrans* Houtt.), 1,437 grams were dried plants, and they were then mashed and obtained 1,337 grams. After that, 200-gram powder was macerated with 800 mL of ethanol. After it was concentrated with a rotary vacuum evaporator, 35.469 grams (17.7%) of thick nutmeg meat extract (*Myristica fragrans* Houtt.) was obtained.

Determination of moisture content

Based on the values of water content in dried plants and extracts of nutmeg (*Myristica fragrans* Houtt.) fruit was 9.8% (w/w) and 6.22% (w/w), respectively (Table 1). These results proved that the dried plant and extracts met the water content requirements (not more than 16.0%) (Courtney, 2017; Sri & Anggelina, 2021). If the water content exceeds the requirement, microorganisms might contaminate the dried plants and extracts. The contamination happens because microorganisms can use the amount of free water as a breeding source (Sri & Anggelina, 2021).

Determination of maximum wavelength

The spectra of the blank, myristicin standard solution, sample solution, and sample solution added to the myristicin standard had a maximum wavelength of 285 nm with a pH of 5. Thus, the selected wavelength was 285 nm (Figure 1). Other researchers reported a wavelength of 254 nm, but no pH value was reported

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(Naikodi *et al*., 2011). The difference in wavelength and spectrum will change at different pH conditions. The higher the pH, the larger the wavelength (red shift) (Balashov *et al*., 1991; Suharyani *et al*., 2021). The selection of different wavelengths could be adjusted according to the analytical needs.

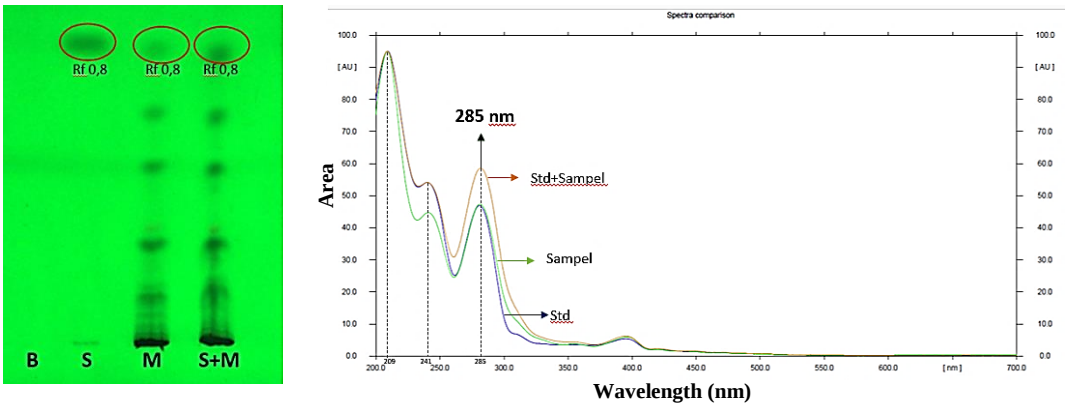
Table 1. Water content results

Material	Sample Weight (gram)	Sample Weight After Heating (gram)	Moisture Content (% *w/w*)
Nutmeg fruit dried plant	1.0044	0.9060	9.80
Nutmeg fruit extract	1.0071	0.9445	6.22

Method verification

Selectivity

The selectivity with the degree of resolution parameter (Rs) was done using the selected mobile phase. The ratio of n-hexane: ethyl acetate (8:2; v/v) showed a degree of resolution (Rs) ≥ 1.5 in each solution (Table 2 and Figure 2). This value proves that the analyte was well-separated from other components. Other researchers using petroleum ether: dichloromethane (30:70; v/v) mobile phase obtained resolution values of 8.5 and 9 (Parsley *et al*., 2014). The distinction was influenced by the different of mobile phase systems. If the mobile phase system is unalike, the polarity will be different (Sudjarwo *et al*., 2019).



(a) **(b)**

Figure 1. UV stain spectra 254; B: blank spectrum; S: standard myristicin; M: nutmeg fruit extract; S + M: extract + standard myristicin (a), standard myristicin spectrum profile at 200-400 nm wavelength (b)

Table 2. Results of Rf-value and Rs-value of myristicin stain

Mobile Phase	Solution Type	R_f-Value	R_s-Value
n-hexane:ethyl acetate (3:2; v/v)	a. Myristicin standard solution	0.91	-
	b. Nutmeg (*Myristica fragrans* Houtt.) fruit extract solution	0.98	1.60
	c. Nutmeg (*Myristica fragrans* Houtt.) extract solution that has been diluted with myristicin standard solution	0.98	1.30
n-hexane:ethyl acetate (8:2; v/v)	a. Myristicin standard solution	0.81	-
	b. Nutmeg (*Myristica fragrans* Houtt.) fruit extract solution	0.82	1.70
	c. Nutmeg (*Myristica fragrans* Houtt.) extract solution that has been diluted with myristicin standard solution	0.86	1.53
Methanol:ethyl acetate (2:8; v/v)	a. Myristicin standard solution	0.97	-
	b. Nutmeg (*Myristica fragrans* Houtt.) fruit extract solution	0.96	1.20
	c. Nutmeg (*Myristica fragrans* Houtt.) extract solution that has been diluted with myristicin standard solution	0.96	1.20

P-ISSN: 2406-9388
E-ISSN: 2580-8303

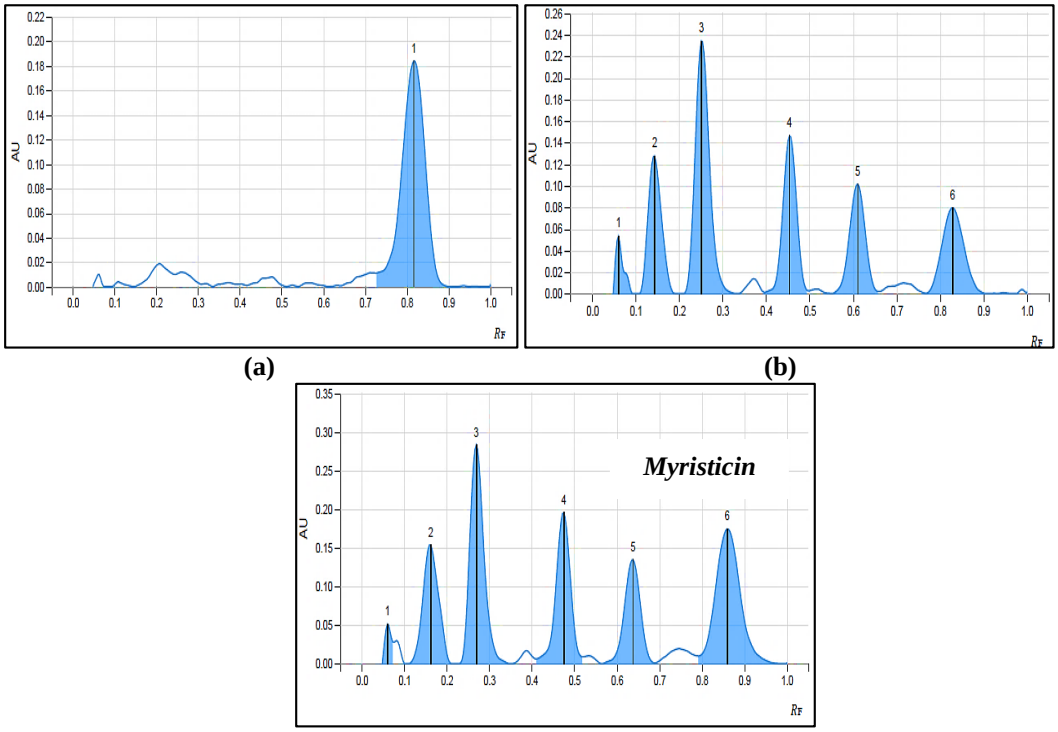


Figure 2. Chromatogram of standard myristicin (a), chromatogram of nutmeg (*Myristica fragrans* Houtt.) fruit extract (b), chromatogram of nutmeg (*Myristica fragrans* Houtt.) fruit extract added with standard myristicin with mobile phase n-hexane: ethyl acetate (8:2)

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

In determining LOD and LOQ, myristicin standard series solutions with concentrations 2 µg to 6 µg were used and then photographed on TLC plates with the same volume of 10 µL. The SD value of the blank was 0.00003346, and the regression equation of the myristicin standard was y = 0.001x + 0.00005 (r = 0.9998), with LOD and LOQ results obtained of 0.11 µg/spot and 0.33 µg/spot, respectively. The TLC-densitometry method developed was sensitive because the LOD and LOQ values were low. The lowest amount of analyte in the sample that can still be detected is indicated by LOD. On the other hand, LOQ indicates the lowest analyte concentration in the sample that can be accurately and precisely identified using quantitative means (Fatmawati & Herlina, 2017). In another study, the LOD and LOQ were obtained at 0.1 µg/spot and 0.4 µg/spot, respectively, for compounds of the *Myrtaceae* family (Rastogi *et al*., 2008). If modest LOD and LOQ values are acquired, it is more sensitive since the difference in LOD and LOQ depends on the accuracy of the study (Table 3).

Linearity

The linearity of myristicin is found from equation y = 0.0001x + 0.0226 (r = 0.9996) (Figure 3). This shows a linear relationship between the weight of the myristicin standard in the bottled (µg) and the response area. The myristicin standard calibration curve provides a good linearity value, and the determination of levels with the calibration curve is guaranteed to be correct (Fatmawati & Herlina, 2017). The linearity from the *Myrtaceae* family found by other researchers showed y = -592.632 + 6.341x (r = 0.9939) (Rastogi *et al*., 2008). The difference depends on the accuracy of the study.

Precision

The precision obtained for the average myristicin area is the coefficient of variation (C.V) of 0.326% (Table 4). This value still met the requirements of C.V (not more than 4%) (Birmingham *et al*., 2021). Another study obtained a precision of 0.76% (Rastogi *et al*., 2008). This difference occurs due to the researchers' different expertise, resulting in different accuracy.

P-ISSN: 2406-9388
E-ISSN: 2580-8303

Table 3. LOD and LOQ results

Bottling Amount of Blank (µL)	Area
10	0.00001
10	0.00000
10	0.00000
10	0.00006
10	0.00008
Standard Vial Amount (µg)	
2	0.00212
3	0.00312
4	0.00415
5	0.00515
6	0.00624

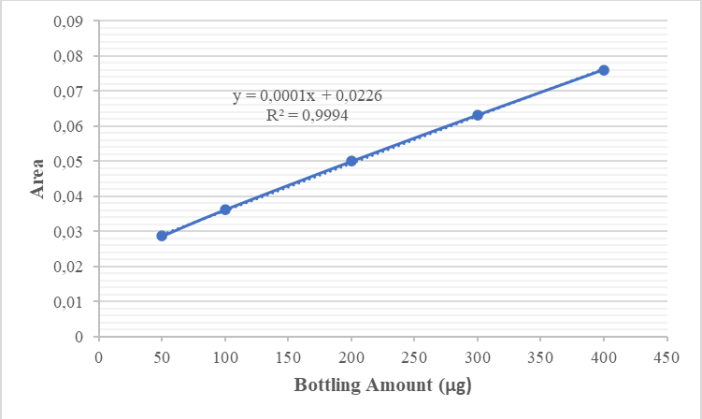


Figure 3. Linearity of *myristicin* standard solution

Table 4. Precision results

Bottling Amount (µg)	Area
60	0.03750
60	0.03848
60	0.03930
60	0.03971
60	0.03988
60	0.03856
60	0.03772
60	0.03734
60	0.03683
60	0.03583
Average (X)	0.03811
Standard Deviation (SD)	0.00124
C.V	0.326

Determination of *myristicin* content in nutmeg (*Myristica fragrans* Houtt.) fruit extracts

The application of the TLC-densitometry method for the determination of myristicin content in nutmeg fruit extract was carried out by the compendial method (United States Pharmacopeial Convention, 2012; AOAC, 2019). Based on the results in Table 5, the average myristicin content in the fruit extract sample of nutmeg is 0.0017 ± 0.1632% (w/w). In another study, the myristicin content in nutmeg was reported to be 109.28% (µg) (Naikodi *et al*., 2011). The differences in myristicin content are attributed to variations in plant parts, sampling locations, genetic factors, growing environments, cultivation practices, and harvest times (Zarshenas *et al*., 2013; Mustafa *et al*., 2017). In this study, nutmeg fruit flesh was used, while in other studies, the aril (mace) part was utilized. As reported by Gayathri and Anuradha (2015), the total phenol content in the fruit was lower than that in the seeds and aril of nutmeg. Another possibility is caused by the use of different methods and solvents.

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Table 5. Myristicin content in nutmeg (*Myristica fragrans* Houtt.) fruit extract

Replication	Weigh (gram)	Obtained (gram)	% (*w/w*)
1	0.5000	0.00000700	0.0014
2	0.5099	0.00000767	0.0015
3	0.5199	0.00000833	0.0016
4	0.5324	0.00001133	0.0021
		Average	0.0017
		Standard Deviation (SD)	0.0003
		C.V.	0.1632

CONCLUSION

The developed method was valid and sensitive for the quantification of myristicin content in nutmeg fruit, producing myristicin in the standardized nutmeg (*Myristica fragrans* Houtt.) fruit extract of 0.0017 ± 0.0003% (*w/w*).

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AUTHOR CONTRIBUTIONS

Conceptualization, SJ.; Methodology, SJ., SK.; Software, D.E.E.; Validation, SJ., SK.; Formal Analysis, D.E.E.; Investigation, D.E.E.; Resources, D.E.E., SJ., SK.; Writing - Original Draft, D.E.E.; Writing - Review & Editing, SJ., SK.; Visualization, D.E.E.; Supervision, SJ., SK.; Project Administration, D.E.E., SJ., SK.; Funding Acquisition, SJ., SK.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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