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# Separation of N-Methylhydroxamic Fatty Acids Based on Ketapang Seed Oil using High Performance Liquid Chromatography

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**Abstract:** N-methyl fatty hydroxamic acids (N-MFHAs) is a derivative of a hydroxamic acid (FHA), were synthesized from ketapang seed oil (Terminalia catappa L.) with N-methyl hydroxylamine enzymatically using immobilized lipase (Lipozyme TL IM). N-MFHAs that are synthesized are still in a mixed state of triglycerides of the ketapang seed oil. This research aims to determine the optimum conditions for the separation of N-MFHAs into their single components and their percentage composition using High Performance Liquid Chromatography (HPLC). The specification of the HPLC system includes using the reverse phase column SGE C-18 ODS-2 and UV detector 213 nm. The optimum conditions for the separation of N-MFHAs in the HPLC system include using the mobile phase of methanol 100% and flow rate of 0.25 mL/minutes with an injection volume of 20 µL and sample concentration of 10.000 ppm. The percentage of the N-MFHAs composition successfully separated using HPLC were linoleoyl-methyl-fatty hydroxamic acid (13.09%), oleoyl-methyl-fatty hydroxamic acid (62.46%), palmitoyl-methyl-fatty hydroxamic acid (19.51%), and stearoyl-methyl-fatty hydroxamic acid (4.93%).

**Keywords:** Lipase; Ketapang Oil; N-Methylhydroxilamine; N-MFHAs; HPLC

## Introduction

Hydroxamic acid is a derivative of hydroxylamine compounds and carboxylic acids. The general formula is RCONHOH. This compound is one of the compounds that can form stable complexes with various metal ions (Johann et al., 2019). This makes this compound an object of study that is important to be investigated and exploited further because it can be used as a good chelating agent. Recently, its complexes with several metal ions have been used in analytical chemistry as reagents for gravimetry, metal spectrophotometry, chelating for rare earth minerals, and for extracting metal ions from the aqueous phase (Suhendra and Gunawan, 2012).

It is not only hydroxamic acid that has attracted the attention of scientists but also its derivative compounds have also received serious attention because of their broad capabilities. There are several recent studies related to the biological activity of these compounds, including to anticancer agents (Ojha, et al., 2018), antituberculosis agents (Majewski, et al., 2015), anti-HIV agents (Stranix, et al., 2016), antioxidant agents (Otuechere, et al., 2020), inhibitors of bacterial infection (Liu, 2018), inhibitors of malaria (Chance, et al., 2018), and HDAC inhibitors (Schmitt, et al., 2019).

According to Suhendra, et al. (2019), one of the hydroxamic acid derivatives that can be used as chelating agents is N-methylhydroxamic fatty acids (N-MFHAs). This compound can be obtained by reacting with hydroxylamine derivatives and carboxylic acids. One of the carboxylic acid functional groups can be found in fatty acids. As one of the raw materials for the synthesis of N-MFHAs, fatty acids can be obtained in the form of pure fatty acids and fatty acids contained in a triglyceride.

The synthesis of this compound has been successfully carried out by Jahangirian, et al. (2011) and Haron, et al. (2012) using palm oil. However, this oil is an oil that is commonly consumed (edible oil). The use of these oils as raw materials for oleochemical products will result in high demand for these oils, there are demand as edible oil and as end-use industries products. Therefore, other non-edible oil materials are needed in the synthesis of N-MFHAs in order not to interfere with the function of food ingredients.

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One of the raw materials which have non-edible character for the synthesis of N-MFHAs is ketapang oil (Terminalia catappa Linn). Ketapang is non-edible vegetable oil from unused and abundant materials that can be used as an alternative to edible vegetable oils. The fatty acid composition of the triglycerides consists of acids: palmitic (25.05-30.96%), oleic (31.5-38%), stearic (3.1-4.3%), linoleic (12-21%) (Gunawan et al., 2019). Its abundant availability, in addition to the seed that is not utilized, makes the ketapang seed the main alternative in the synthesis of N-MFHAs.

The synthesis of N-MFHAs from vegetable oil can be used chemically and enzymatically. The chemical synthesis of these compounds from vegetable oils is considered inappropriate because there are fatty acids that makeup the oil that has double bonds. In an alkaline atmosphere and a high enough temperature, the double bond can be oxidized (Suhendra, 2014). The Synthesis N-MFHAs from vegetable oil which is considered the most appropriate by enzymatic method. This is due to the way the enzymatic works in a neutral atmosphere and low temperature, and is environmentally friendly and the enzymes used (lipases) can be reused (Moghaddam et al., 2014). Suhendra et al. (2019) has successfully synthesized N-MFHAs enzymatically from ketapang seed oil with a maximum conversion percentage of 59.7%. However, the compound produced from this reaction is still composed of components that have not been separated, therefore it is necessary to separate them. The separation of N-MFHAs into their components is important considering that the difference in chain length and saturation will affect the selectivity and chelating power of certain metals. For example, linoleate hydroxamic acid (LHA) has a higher reactivity and selectivity to Mn(II) metal than other ALH (Zhou et al., 2015).

The separation of N-MFHAs into their components was carried out using High Performance Liquid Chromatography (HPLC). HPLC was chosen because this tool can be used for qualitative and quantitative analysis (Wahid, 2020). In addition, because of N-MFHAs are compounds that have a low level of volatility and have a molecular weight that is not much difference between components. Therefore, N-MFHAs will be difficult to separate when using column chromatography or gas chromatography.. Majnooni, et al. (2016) have succeeded in separating fourteen fatty acid components in sesame seed oil (C10-C22) using HPLC. In this study, the optimum conditions for the HPLC system to be determined include flow rate, eluent composition, and sample concentration which were determined before separating with HPLC.

## Methods

*Extraction of Ketapang Seed Oil*

Ketapang oil extraction was carried out by the method developed by Suhendra, et al. (2018) with some modifications and adjustments. The mashed ketapang seed core was weighed as much as 50 g and put into a Soxhlet device and then extracted with 250 mL n-hexane at 70$^{o}$C for 6 hours. The extraction results were then evaporated using a rotary evaporator at a speed of 120 rpm at 40$^{o}$C to remove the n-hexane solvent. Ketapang oil is then put into a chromatographic column containing silica gel. Then eluted using n-hexane as eluent.

*Synthesis of N-MFHAs*

The enzymatic reaction of ketapang oil uses the method developed by Suhendra, et al. (2019) with some modifications and adjustments. The reaction mixture consisted of 10 g of ketapang seed oil 75 mL of n-hexane, and 50 mmol of N-methyl hydroxylamine hydrochloride which had been dissolved in distilled water, and the pH was adjusted to 7 with the addition of 6 M NaOH and put into a 250 mL erlenmeyer with a lid. Then 0.1 g of Lipozyme was added, and the mixture was then put into a stirrer water bath at a speed of 100 rpm at a temperature of 30-40°C for 25 hours. The N-MFHAs formed were separated from the aqueous phase and Lipozyme. Then the hexane phase is cooled to <5°C for ± 24 hours and filtered.

*Characterization of N-MFHAs*

The identification of the synthetic product was carried out using the method developed by Suhendra et al. (2019) with some modifications and adjustments. Identification carried out includes FTIR test, color test and analysis of nitrogen content. The results of the FTIR (Perkin-Elmer) Spectrum Two test were compared with the results of ketapang oil. The color test was carried out by dissolving solid N-MFHAs with methanol. Then reacted with 2% FeCl3 and 1 M CuSO4. N-MFHAs will bind to $Fe^{3+}$ ions to form a dark red color and bind to $Cu^{2+}$ ions to form a green color. Analysis of nitrogen content by Kjeldahl test. This analysis was carried out using the Kjeldahl semi macro method with a quality management system according to ISO/IEC 17025 (2017).

*Separation of N-MFHAs using HPLC*

Separation of N-MFHAs using High Performance Liquid Chromatography (Waters, Breeze 1525, USA) equipped with a UV/VIS detector (Waters, 2489, USA), a binary pump (Waters, Breeze 1525, USA), and a reverse-phase column (SunFire, C18 5 μm 4.6x150 mm, USA). The optimum parameters of the HPLC system determined include the mobile phase and flow rate. Determination of the optimum mobile phase is done by making a sample solution with a concentration of 10,000 ppm which is injected into the HPLC system using methanol: water and methanol: acetonitrile mobile phases in a ratio of (95:5), (90:10), (85:15) and methanol 100 % with a flow rate of 0.5 mL/min. Meanwhile, in determining the optimum flow rate, the sample solution with a concentration of 10.000 ppm was injected into the HPLC system using 100% methanol as a mobile phase with a flow rate variation of 1.5 mL/minute; 1.0 mL/min; 0.5 mL/min; 0.25 mL/min; and 0.1 mL/min.

## Results and Discussion

*Product Identification*

The reaction using chemical catalysts is expected to comprise MFHAs compounds, including linoleoyl-MFHA, oleoyl-MFHA, palmitoyl-MFHA, and stearoyl-MFHA (Suhendra, et al., 2019). Nowadays, immobilized enzymes are preferred to be used as catalysts due to various factors, including time, cost, and materials in reactions proceeding efficiently (Datta, et al., 2013).

Identification of N-MFHAs is shown in Figure 1. Identification of N-MFHAs has been successfully carried out by observing the color changes that occur in Fe(III) and Cu(II) solutions after being added with N-MFHAs. The color complex formed between Fe(III) and Cu(II) with N-MFHAs is a typical reaction (Suhendra, et al., 2019). The result of this color test is a change in the color of the Fe(III) solution from yellow to brown and the Cu(II) solution changes from blue to green. The color change occurs because $Fe^{3+}$ ions and $Cu^{2+}$ ions form complexes with N-MFHAs to produce new color complexes.

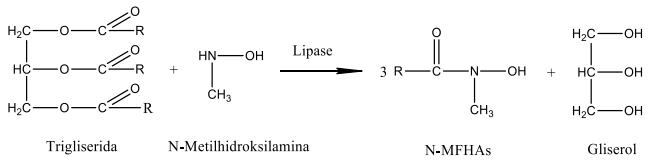


**Figure 1.** Enzymatic reaction for the formation of N-MFHAs

FTIR analysis was performed by comparing the spectrum of ketapang oil with the spectrum of N-MFHAs. The success of the synthesis can be proven by the shift in the wavenumber of the functional groups that are characteristic of ketapang oil and N-MFHAs. The results obtained indicate a shift in the vibrations of C=O ester, =CH, and -CH in the ketapang oil spectrum at wavenumbers 1746 $cm^{-1}$, 3007 $cm^{-1}$, and 2925 $cm^{-1}$ to wave numbers 1719 $cm^{-1}$, 2920 $cm^{-1}$, and 2853 $cm^{-1}$ in the spectrum of N-MFHAs. This indicates that the amide functional group has been formed. There is also an absorption of OH vibration at a wavenumber of 3375 $cm^{-1}$, while the N-tertiary absorption cannot be seen because of the strong influence of the vibration of OH. However, the presence of tertiary N can be identified because there is CN absorption at a wavenumber of 1230 $cm^{-1}$.

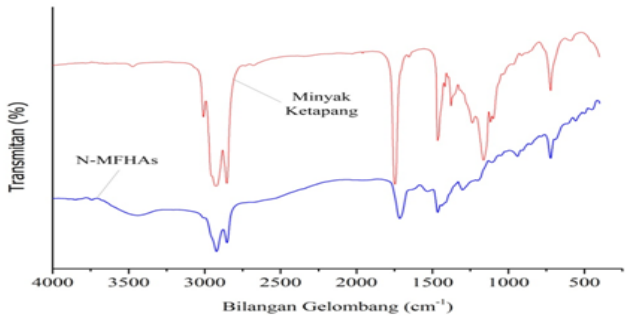


**Figure 2.** FTIR Spectrum of Ketapang Oil and N-MFHAs

Total nitrogen (N) content was determined by the Kjeldahl method and the result was 0.42%. This means that in a 1 g sample of N-MFHAs synthesized from ketapang oil there is 0.3 mmol of the amide group.

*HPLC System Optimization*

*a. Determination of Optimum Mobile Phase*

Mobile phase optimization aims to find the best mobile phase composition that can separate the components that makeup N-MFHAs. The mobile phase plays an important role in analyte separation because the migration of the analyte is regulated by the interaction of the mobile phase and the stationary phase. The migration of the analyte occurs because of the competition between the mobile phase and the analyte to bind to the active sites of the stationary phase. Methanol was prioritized in this separation because the sample N-MFHAs were completely soluble in methanol.

Based on Figure 3, it can be concluded that 100% methanol shows a relatively better separation of the four peaks compared to the methanol:water and methanol:acetonitrile mobile phases. But the resolution value is still less than the recommended value of 1.5. According to Harmita (2006), the recommended resolution value is 1.5. In the mobile phase of methanol:acetonitrile has the greatest resolution value but cannot be used as the optimum mobile phase because it gives rise to many other peaks on the chromatogram. This is because acetonitrile present in the mobile phase solution causes compounds that are polar and nonpolar in the sample solution giving a response around the wavelength of 213 nm. These compounds will be retained in the column longer so that compounds that have almost the same polarity will have overlapping separations. Hence it cannot be used to separate the components of N-MFHAs.

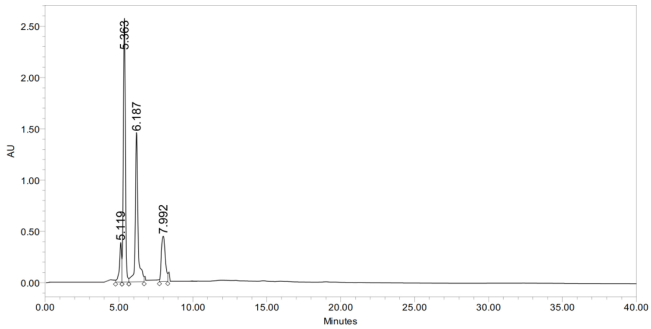


**Figure 3.** Chromatogram of Separation of N-MFHAs Components (Mobile Phase 100% Methanol and Flow Rate 0.5 mL/min)

*b. Determination of Optimum Flow Rate*

The flow rate can affect the success of the measurement because changes in the flow rate can affect the resolution value and elution time. The decrease in flow rate can increase the resolution value but can slow down the elution time (Rosydiati and Saleh, 2019).

Based on Figure 4, the separation of N-MFHAs with a flow rate of 0.25 mL/min is better than that of a flow rate of 1.5; 1.0; 0.5, and 0.1 mL/min. This is evidenced by

the better separation of the four compounds even though the resolution value has not reached the recommended resolution value. In this study, flow rates >1.5 were not carried out because the pump pressure was >200 kgf/cm2. This condition cannot be done because it can cause damage to the column. The value of retention time at a flow rate of 1.5 mL/min is smaller than a flow rate of 0.1; 0.25; 0.5; and 1.0 mL/min. This shows that the faster the flow rate, the smaller the retention time value of the compound. Retention time is affected by flow rate or column length (Calvet, et al., 2014). If the flow rate is slow or the column is long, the retention time value is getting bigger and vice versa (Murniati, et al., 2019). The resolution value of each compound peak can be calculated from the obtained retention time value.

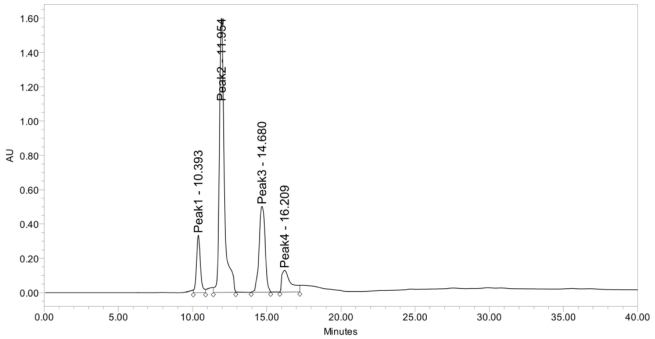


**Figure 4.** Chromatogram for Separation of Components of N-MFHAs (Mobile Phase 100% Methanol and Flow Rate 0.25 mL/min)

*c. Separation N-MFHAs Using HPLC Optimum Conditions*

The synthesized N-MFHAs samples were then separated using the previously obtained HPLC optimum conditions. The optimum conditions for HPLC are shown in Table 1.

**Table 1.** Specifications and Optimum HPLC Conditions

| | |
|---|---|
| Stationary Phase | SunFire C18 (5 μm 4.6 x 150 mm) |
| Mobile phase | Methanol |
| Flow rate | 0.25 mL/min |
| Injection volume | 20 μL |
| Concentration | 10,000 ppm |
| Detector | UV 213 nm |

The HPLC analytical method presents a simple, specific, accurate, and precise quantitative method (Ahmad, et al., 2022). Additionally, it is a beneficial analytical method for analyzing single or combined compounds (Habash, et al., 2020). The results of the chromatogram separation of N-MFHAs at optimum conditions can show that N-MFHAs consists of four main compounds that can be seen according to the four peaks formed on the chromatogram. This main compound is expected to be the four main compounds that makeup triglycerides in ketapang oil. Identification to prove the four compounds is done by comparing the value of the retention time of the sample with the value of the standard retention time.

**Table 2.** Identification of the Components of N-MFHAs

| Peak to- | N-MFHA type | $t_R$ Standard (minutes) | $t_R$ Sample (minutes) |
|---|---|---|---|
| 1 | Linoleic-MFHA | 10.751 | 10.393 |
| 2 | Oleoil-MFHA | 12.323 | 11.954 |
| 3 | Palmitoyl-MFHA | 14.958 | 14.680 |
| 4 | Stearoyl-MFHA | 16.287 | 16.209 |

Based on the chromatogram that has been compared with the standard, the composition of N-MFHAs is obtained, namely linoleoyl-MFHA (13.09%), oleoyl-MFHA (62.46%), palmitoyl-MFHA (19.51%) and stearoyl-MFHA (4.93%). The order of polarity follows: alkyne > alkene > alkane (Suhendra, et al., 2020). First, Linoleoyl-MFHA compounds were eluted and oleoyl-MFHA subsequently as the hydrocarbons polarities having single bonds are lower than those having double bonds. Because of its single bonds, the alkanes reactivity is lower than that of alkenes, the reaction of alkenes occurs at double bonds, which affects the level of polarity. Similarly, palmitoyl-MFHA and stearoyl-MFHA have been bound strongly to the stationary phase of non-polar octadecyl silane (ODS).

## Conclusion

This study aims to determine the optimum conditions for separating N-MFHAs into their single components using High Performance Liquid Chromatography (HPLC). Based on the results of the research and literature review that has been carried out, it can be concluded that N-MFHAs synthesized from ketapang oil can be separated using HPLC. The best conditions for the HPLC system to separate N-MFHAs were (1) methanol mobile phase, and (2) a flow rate of 0.25 mL/min.

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