

## Research Article

# Analysis of Glycerol in Nipa (*Nypa fruticans Wurmb.*) Kernel Extract (NKE) with High-Performance Liquid Chromatography (HPLC) Method

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**Abstract:** The quantification of glycerol reveals application areas in research as well as industrial prospects for supplies of biodiesel. Although petroleum and fossil fuels are not sustainable sources of energy, these fuels are on the verge of being exhausted. The study aims to establish a novel, calibrated, simple, and accurate analysis of the glycerol content using High-Performance Liquid Chromatography (HPLC) method. The quantification of glycerol from Nipa Kernel Extract (NKE) using the HPLC method resulted from a 4.06% concentration using hydrolysis reaction with methanol and KOH catalyst. The pure and hydrolyzed NKE were monitored using isocratic elution with Hydrophilic-Interaction Chromatography (HPLC-HILIC). The chromatograms extracted from the LC were analyzed and validated and showed good performance in terms of linearity as implicated ( $R^2=0.9928$ ), repeatability (%RSD range from 2.6758% and 16.6130%), intermediate precision (p-value (0.00119)  $< \alpha$ ), the limit of detection 0.0001458% (w/w), the limit of quantification 0.0004182% (w/w), and accuracy (76.7105% to 82.8505%) of hydrolyzed Nipa Kernel Extract (NKE). The results produced from the employed approach are sufficient for determining the concentration of glycerol, and the method itself can serve as an alternate way to conduct chemical analysis. Additional research revealed that the contaminants and free fatty acids connected to glycerol absorbed the same absorbance units. It is anticipated that the investigation and identification will include quantifying the concentration of other unknown compounds found in the matrix.

**Keywords:** Concentration; Glycerol; Hydrolysis; Hydrophilic-Interaction Chromatography; Nipa Kernel Extract (NKE)

## 1. Introduction

Nipa fruit (*Nypa fruticans Wurmb.*) is a good source of biofuel due to its sugar-rich sap that can be transformed into alcohol upon fermentation [1]. A tropical mangrove plant, blooming throughout the year, nuts grow in clusters and contain edible meat from its kernel. The edible meat is soft, sweet, and translucent with oily tannin-like [2].

Quantification of glycerol yields application in research and industries possibilities for biodiesel sources. Petroleum and fossil fuels are approaching extinction despite these energy sources being non-renewable.

However, the goal of creating alternatives for fossil fuels urges to development of renewable fuels from biomass and biofuels, which supports green energy and the production of its by-products [3], [4]. This potential breakthrough pushes researchers to assess and evaluate sources of biofuels using different indicators [5].

Glycerol is a result of the transesterification process of plant oils. This polyhydric alcohol can be applied to pharmaceutical, cosmetics, food, and construction industries [6]. Its water-coating effect and usually applied as solvents in food additives [7]. Glycerol can also be used as an anti-inflammatory agent in cosmetic products [8].

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Glycerol can be applied in syrups, cream-based products, lubricants, and ointments. It is also an antimicrobial agent and solvent for hand sanitizers, plasticizers, sweeteners, and tonics [9].

Transesterification is a process of converting glycerol using methanol and using homogenous catalysts such as NaOH and KOH. Unlike acid catalysts, essential solutions minimize excess alcohol and lower the temperature for more extended periods [10], [11]. In transesterification, glycerol sinks at the bottom into a separated mixture. In addition, biodiesel, which dissolves on top of the glycerol, is an important by-product essential for cosmetic processes [12], [13].

In 2015, the rise of biodiesel production created an avenue for glycerol production for more than 300,000 cubic meters globally. Glycerol is a renewable chemical used in the chemical industry as solvents and beverages [14].

In the study of Alang et al. [15], through the fiction process, the yield of about 10% w/w glycerol as a product together with in the tests conducted using IR spectrum reports absorption peaks that fall on the carbonyl compound, alcohols and esters which validates the occurrence of glycerol on the sample [15], [16]. The presence of triglyceride in oil samples can produce glycerol derivatives and biodiesel in transesterification.

According to Muniz et al. [17], using HPLC, two proposed analytical methods were used to determine the glycerol content from biodiesel. The data collected were compared based on linearity, the limit of quantification, the limit of detection, repeatability, and precision. In the run tests by Harabi et al. [18], the glycerol content was about 30.40% w/w from waste frying oil compared to the result of Kongjao et al. [19], the yield of approximately 28.56%.

The study of Belasin-Prieto, et al. [20] employed the HPLC method analysis of oxidative glycerol products. Precision, accuracy, sensitivity, detection, and quantification limits are used to validate the analytical method. The interesterification process was used by Santoro et al. [21] to analyze the Physio-chemical properties of fatty acids using silver ion HPLC combined with mass spectrometry (MS) and another constituent triglyceride. Triacyl glyceride (TAG's) constituents were measured in the % composition of different oil samples.

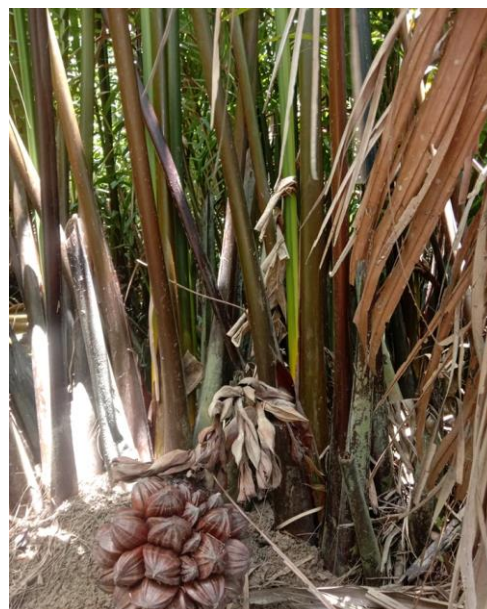
Testing and analysis of potential sources of glycerol is an indicator of significant products together with biodiesel. HPLC is an analytical method for analyzing single and attached molecules. Some studies focused on analyzing oil samples' fatty acid and glycerol profiles [22]–[24]. However, no study has validated or developed to analyze the glycerol profile of nipa kernel oil. The study aims to establish a novel, calibrated, simple, and accurate analysis

of the glycerol content using High-Performance Liquid Chromatography (HPLC) method.

## 2. Material and Methods

### 2.1. Material

The nipa kernel meat is obtained from Rizal, Odiongan, and Romblon Tablas Island mechanical pressed for oil extraction. The soxhlet extractor and rotary evaporator yield oil extraction from the nipa kernel. Glycerol is a standard solution to measure glycerol concentration in nipa kernel oil extract. The AR-grade chemicals that will be used in the study are glycerol (99.9% purity), potassium hydroxide pellets, and methanol (99.9%) and are obtained from Vivanto Chemical Company, Terran Alchemy and Mt. Zion Chemicals [2].

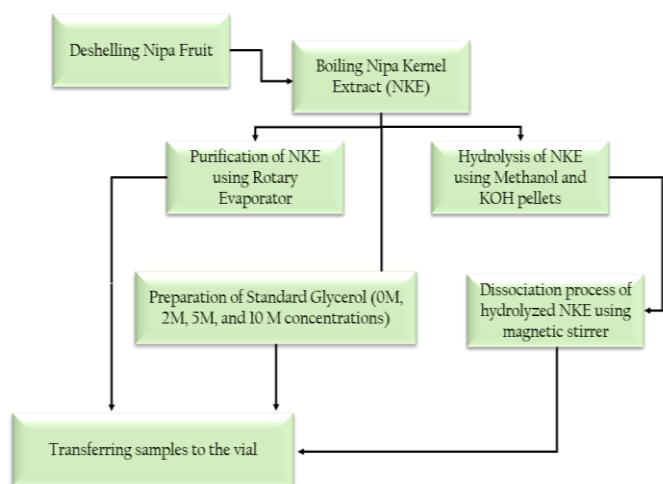


**Figure 1.** Site of nipa fruit collection

Oil extraction, purification, hydrolysis of oil extract, and instrumental analytical methods for characterizing glycerol content are some techniques used in this study.

### 2.2. Extraction of Nipa Kernel

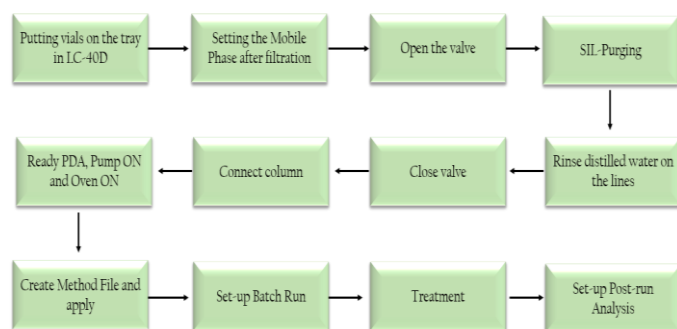
Nipa kernel was deshelled from the original fruit and subjected to boiling. To achieve the extract, 1360 grams of nipa kernel with 1360 mL distilled water is boiled for 2 hours in the first phase. The extract was transferred and settled overnight for 5.2°C – 5.4°C. It was then boiled for the same parameters for the second phase, which lasted for 60 minutes—using a soxhlet apparatus and rotary evaporator to separate the solvent on the extract. The Nipa Kernel Extract (NKE) is purified by warming it to 100°C – 65°C (100 rpm) for 45 minutes and allowing it to stand for 2 hours for impurities to settle [25].



**Figure 2.** Preparation of Standard and NKE Samples

### 2.3. Hydrolysis of Nipa Kernel Extract

Glycerol will be synthesized from NKE using hydrolysis/transesterification following the stoichiometric equation of the reaction of triglyceride and methanol.



**Figure 3.** LC Process

About 70 mL of 20% methanol is transferred into a beaker afterward, and a 2.3 g KOH catalyst is added to the NKE. The mixture is subjected to the magnetic stirrer for about 70°C for the methanol and KOH to dissolve properly. The NKE was transferred to a 1000-mL round bottom distillation flask to separate the water for the extract.

**Table 1.** Treatment Formulation of Standard Glycerol

No.	Treatment	Formulation of Glycerol
1	Std0	0 M
2	Std1	2 M
3	Std2	5 M
4	Std3	10 M

### 2.4. Analysis of the Samples by High-Performance Liquid Chromatography (HPLC)

Glycerol with 99.9% purity is used as the standard reagent for High-Performance Liquid Chromatography -

Hydrophilic-Interaction Chromatography (HPLC-HILIC) Shimadzu Nexera Lite with quaternary low-pressure gradient pump (LC-40D), SPD-M40 (wavelength range of 190 to 800 nm UV detector), the Autosampler SIL-40C X3 with 0.01-50 µL loop. The column measured 300mm for the concentration of glycerol in NKE. The mobile phase was 100% prefiltered distilled 65% water (line A-C-D) and methanol 35% (line B-R) solution using isocratic elution. The total analysis time in each sample is 5 minutes with a constant flow rate of 1 mL *min*<sup>-1</sup> with an injection volume of 10µL. Pump-A Pressure is set at a minimum of 2MPa, and Pump-A Degassing Line of -93kPa. Each sample was injected in triplicate to evaluate the repeatability precision of the calibration curve.

Peaks in the chromatogram measuring the maximum intensity of glycerol at (205 nm at α=0.99 UV Absorbance) and expressed in mAU (Milli absorbance-unit).

### 2.5. Validation of the Analytical Method

The purpose of validating the proposed analytical method is to verify the reliability of the results. This validation is partial in that it must conform to the requirements and standards. The partial validation of the analytical method will be performed in this study. The parameters are linearity, precision (intermediate and repeatability precision), accuracy (recovery), the limit of detection (LOD), and the limit of quantification (LOQ) [26].

#### 2.5.1. Linearity

The linearity validation is performed through triplicate injections of standard solution corresponding to every point of the analytical curve. The coefficient of determination (R<sup>2</sup>) is used to check the linearity. The range of values to make it linear falls above 0.99, and the angular coefficient of the line is constant [26].

#### 2.5.2. Precision (Repeatability and Intermediate Precision)

The repeatability and intermediate precision were used to evaluate the accuracy. The repeatability was examined for four standard treatment formulations in triplicate for the analytical curve. The repeatability precision is also called the coefficient of variation and can be expressed as the relative standard deviation (%RSD) and calculated using the equation.

$$\%RSD = \frac{\sigma}{\bar{X}} \times 100 \tag{1}$$

Where:  $\sigma$  is the standard deviation of the data sample and  $\bar{X}$  is the mean data sample. The intermediate precision was evaluated using the analysis of variance (ANOVA) with different analytical curves.

### 2.5.3. Accuracy (Recovery)

The accuracy was measured as recovery indicated by (%R) and verified using three different concentrations. The accuracy was calculated using the formula.

$$\%R = \frac{C_m}{C_e} \times 100 \quad (2)$$

Where:  $C_m$  is the measured concentration and  $C_e$  is the expected concentration.

### 2.5.4. Limit of Detection and Limit of Quantification

The analytical curve's standard deviation and slope were used in an equation to derive the limit of detection (LOD).

$$LOD = \frac{3s}{m} \quad (3)$$

where:  $s$  is the standard deviation of the analytical curve, and  $m$  is the slope of the analytical curve. The limit of quantification (LOQ) was calculated by multiplying 3.3 by the value of LOD [17].

## 3. Result and Discussions

### 3.1. Determination of concentration using Analytical Curve

**Table 2.** Linear Regression Table of Standard Glycerol and Absorbance in LC Method.

Concentration (M)	Absorbance (mAU)	Predicted Y	Residual
0	0	24.3795	-24.3795
2	151.89	141.6109	10.2794
5	349.77	317.458	32.312
10	592.32	610.5365	-18.2119

**Table 3.** Linear Regression

$\beta_1$	$\beta_0$	$R^2$	Correlation Coefficient	F	P-value
54.39	56.47	0.9928	0.9964	138.2272	0.0540

$$\hat{Y} = 54.3866X + 56.4709$$

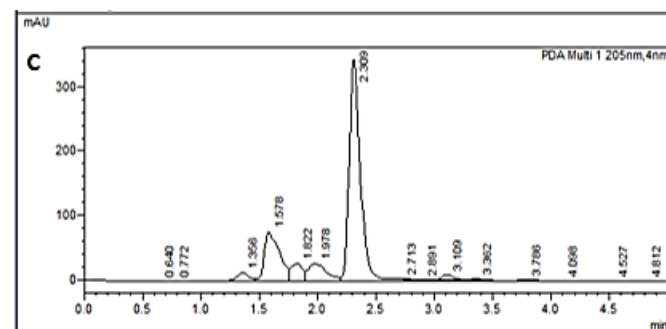
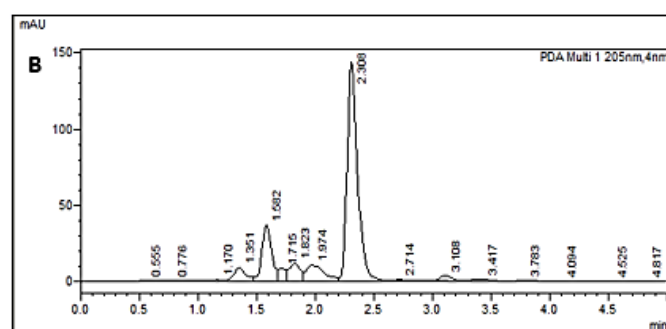
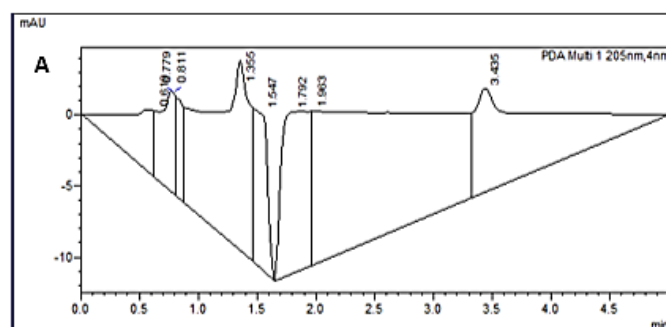
The ( $R^2$ ) is equal to 0.9928, meaning that the standard glycerol concentration explains 99.28% of the variability of absorbance. The correlation coefficient (R) represented by 0.9964 shows a solid direct relationship between absorbance and concentration. The overall regression is right-tailed, where the F (1,2) equals 138.2272, with a p-value of 0.0540. Since the p-value is more significant than  $\alpha$  (0.05), we reject the null hypothesis, indicating a significant relationship between concentration and

absorbance of glycerol in the standard solution. The linear regression model,  $\hat{Y} = 56.4709 + 54.3866X$ , is created, providing a better fit than the model without the independent variable. This model is used to compute glycerol concentration in the unknown pure and hydrolyzed sample of NKE. The  $\hat{Y}$  is the absorbance of the sample in mAU, while  $X$  is the concentration of the model.

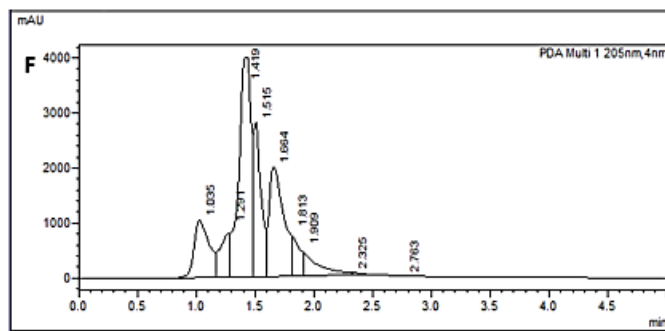
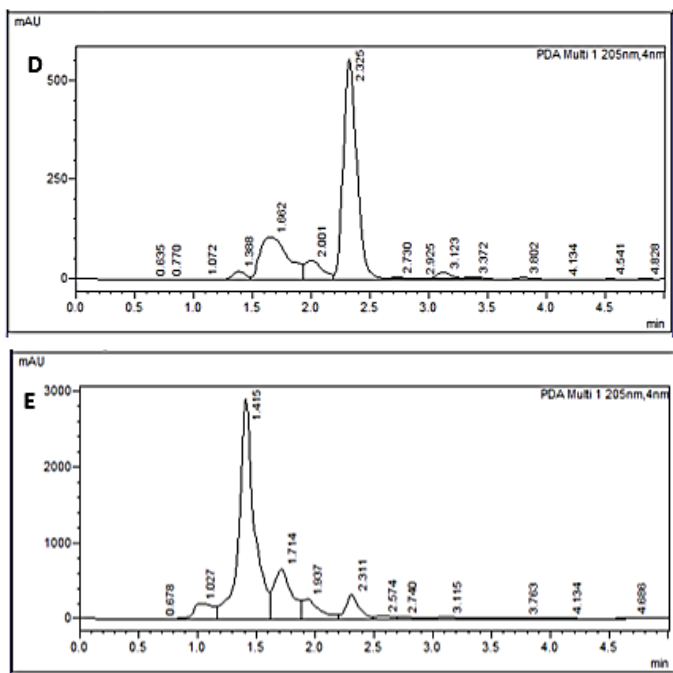
**Table 4.** The Comparison of Percent Concentration of Glycerol from Pure and Hydrolyzed NKE

	Pure NKE	Hydrolyzed NKE
Absorbance (mAU)	309.628	1585.961
Concentration (ppm)	6.556	40.704
Measured Volume (L)	0.232 L	0.273 L
Mass of Glycerol (g)	0.00152	0.0111
%(w/v)	0.655%	4.06%

The glycerol concentration from pure NKE is 6.556 ppm, while in the hydrolyzed NKE, the concentration is 40.704. The glycerol concentration from hydrolyzed NKE is higher in yield than pure NKE, as manifested by the % concentration in (g/L) of 4.06% in every 0.273 L sample.







**Figure 4.** Titles placed below the image are then numbered sequentially. Chromatogram of the Standard Glycerol (A) 0M; (B) 2M; (C) 5M; (D) 10M; and NKE Samples (E) Pure; (F) Hydrolyzed Undertaken from the Post-Run Analysis of HPLC.

### 3.2. Linearity Test

Figure 4 presents the analytical curve obtained from the glycerol standards in 1.5 mL ranging from 0M, 2M, 5M, and 10 M. The determination coefficient ( $R^2=0.9928$ ) shows a linear relationship; the model  $\hat{Y} = 56.4709 + 54.3866X$  is

used to quantify glycerol concentrations from NKE. The determination coefficient of ( $R^2=0.9928$ ) is greater than the acceptance criteria of 0.990 from international standards for validating analytical methods. This indicates good linearity from the determined concentration ranges.

**Table 5.** Linear Regression Table of Standard Glycerol and Absorbance in LC Method.

	R1	R2	R3	X	$\sigma$	%RSD
2M	60.906	61.491	36.787	53.0613	14.0970	2.0526
5M	61.639	62.705	38.485	54.2763	13.6861	2.0067
10M	45.997	61.998	39.392	49.1290	11.6239	2.2169
Pure NKE	5.858	5.999	7.811	6.5560	1.0891	16.6130
Hydrolyzed NKE	45.714	35.708	40.689	40.7037	5.0030	2.6758

Acceptance criteria for %RSD = < 20%

### 3.3. Repeatability and Intermediate Precision

The %RSD describes the repeatability precision of the triplicate injections done from pure and hydrolyzed NKE from the post-run analysis. According to UN Validation of Analytical Methodology [27], for HPLC methods, the acceptance criteria for repeatability should fall below 20%, and the RSD values from pure and hydrolyzed NKE along the range of 16.6130% and 2.6758%, respectively. This shows that the concentrations measured by the chromatogram show good precision.

For intermediate precision of the chromatogram from the standard glycerol and NKE samples and verified using one-way ANOVA. The p-value (0.00119) is less than  $\alpha$ , which is 0.05; thus, the null hypothesis is rejected, which states that the concentration taken from the method is statistically significant. This shows that the glycerol

concentration from the hydrolyzed sample is evident in the standard sample.

### 3.4. Accuracy (Recovery)

Table 5 shows the %R (accuracy) of the measured concentration from the NKE samples compared to the concentrations of the standard glycerol at 2.30 min. Retention time. The recovery values range from 76.7105% to 82.8505%, showing strong agreement of experimental and expected concentrations of glycerol. The accuracy results fall within the acceptance criteria of international standards of analytical validation (70-120%) [17].

### 3.5. Limit of Detection and Quantification

The limit of detection (LOD) is defined as the 10% portion of %RSD. The requirement is to detect the analyte at a

minimum concentration. The calibration curve's standard deviation equals 26.44, and the slope is 54.39. The LOD and LOQ computed is 0.0001458% (w/w), and 0.0004182% (w/w), respectively.

**Table 6.** Accuracy Validation of the Concentration obtained from the Chromatogram

Concentration (ppm)	%R	
	Pure NKE	Hydrolyzed NKE
53.061	12.3555	76.7105
54.276	12.0789	74.9933
49.129	13.3444	82.8505

According to the UN Validation of Analytical Methodology [27], the accepted criteria conform to the range below the minimum concentration of the previous method undertaken in a laboratory. According to Muniz et al. [17], the computed LOD is lower than the value obtained from the spectrophotometric detection of 0.000941% glycerol.

## 4. Conclusion

Nipa kernel extract attributed by HPLC method to quantify the concentration of glycerol was compared to standard glycerol from p-value (0.00119) hence imposed the values to be statistically significant due to pure and hydrolyzed NKE concentration. This was strengthened by the concentration of glycerol in standard and hydrolyzed NKE to be comparable. The method reflects a detection limit of 0.0001458% (w/w) and much lower based on the literature on quantifying glycerol. The precision (16.6130% & 2.6758%) and accuracy (76.7105% to 82.8505%) were within acceptable criteria for validating the analytical methodology. The results obtained from the method used are adequate for determining glycerol concentration and can be an alternative procedure for chemical analysis. Further studies on the impurities and free fatty acids attached to glycerol also absorbed similar absorbance units. Quantifying the concentration of other unknown compounds in the matrix is expected to be analyzed and identified.

## Acknowledgments

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