

# Determination of Free Fatty Acids in Palm Oil Samples by Non-Aqueous Flow Injection Using Salicylaldehyde-2,4-Dinitrophenylhydrazone as Colorimetric Reagent

Abdussalam Salhin Mohamed Ali<sup>1,\*</sup>, Abdurrrhman Moussa Abdurrrhman<sup>2</sup>

<sup>1</sup>School of Chemical Science, Universiti Sains Malaysia, 11800, Penang, Malaysia

<sup>2</sup>Chemistry Department, Faculty of Science, Sirt University, Sirt, PO. 674, Libya

\*Corresponding Author: [salam\\_salhin@yahoo.com](mailto:salam_salhin@yahoo.com)

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**Abstract** The free fatty acids (FFA) in palm oil were determined by flow injection titrimetric method using salicylaldehyde-2,4-dinitrophenylhydrazone (SDPH) as a new coloring reagent. The compound was synthesized and its structure was established using different spectroscopic data. It exhibited sensitive colour changes in basic medium and absorb at 482 nm. Single-line manifold using SDPH as indicator was developed. Flow injection operating parameters such as carrier, reagent concentration, flow rate, size of mixing chamber and injection volume were optimized. The method is recommended for the determination of oil samples with acidity degree (a.d.) higher than 0.5 a.d. Twelve oil samples were tested using the appropriate FIA manifold and the obtained results were compared to the standard PORIM method. Good correlation ( $r^2 = 0.99$ ) between the two methods was obtained

**Keywords** Palm oil, FIA analysis, Free Fatty Acid, Hydrazone.

## 1. Introduction

Palm oil is one of the major oils and fats produced and traded in the world today. Within the span of four decades, palm oil has emerged as the fastest growing oil in the world. Currently, most of the world's production of palm oil comes from south-east Asia, in particular Malaysia and Indonesia. Malaysia is one of the largest producers in the world [1], accounting for 32.7% of the world oils and fats production in 2011 [2]. To meet the ever changing needs and for the industry to remain competitive, innovations in many forms are necessary. The major fatty acids (FFA) in palm oil are myristic, palmitic, stearic, oleic and linoleic. The minor constituents can be divided into two groups. The first group

consists of fatty acid derivatives, such as partial glycerides (mono- and diacyl glycerols), phosphatides, esters and sterols. The second group includes classes of compounds not related chemically to fatty acids called nonglyceride constituents. The non-glyceride fraction of palm oil consists of sterols, triterpene alcohols, tocopherols, phospholipids, chlorophylls, carotenoids volatile flavour components such as aldehydes and ketones. There are also some hydrocarbons, aliphatic alcohols, free sterols, tocopherols, pigments, partial glycerides and phosphatides, and trace metals [3].

FFA content is one of the most frequently determined quality indices during edible oils production, storage and marketing. It is a measure of the extent to which hydrolysis has liberated fatty acids from their ester linkage with the parent triglyceride molecule [4]. Edible oils undergo various processing steps, resulting in low FFA content [5]. Palmitic acid is the major saturated fatty acid in palm oil and this balanced by almost 39% monounsaturated oleic acid and 11% polyunsaturated linoleic acid. The remainder is largely stearic acid (5%) and myristic acid (1%). Palm oil has saturated and unsaturated fatty acids in approximately equal amounts [3]. Linoleic acid and palmitic acid are usually used as indicators to measure the extent of fat deterioration because linoleic acid is more susceptible to oxidation whereas palmitic acid is more stable to oxidation [6].

There are many analytical methods used for FFA determination such as the manual titration official method, standard method based on the PORIM method [7], capillary gas chromatography, and to a lesser extent, HPLC and capillary electrophoresis (CE), where the analyte is first isolated using liquid-liquid extraction or solid-phase extraction prior to the analytical separation. Derivatization methods are necessary for these methods either to increase the volatility of analytes or to improve the sensitivity as in HPLC and CE methods [8]. Accordingly, several papers have been published for the determination of FFA in

biological fluids [9,10], milk [11], fruit juices [12] and oil and fats [13-16]. Some of the methods used for the determination of oil acidity are potentiometry [17], sequential injection analysis using lab-on-valve system [4], biosensors based either on purified enzymes [18,19] or intact enzymes (e.g. butyrate kinase from *E. coli*) in bacteria [20].

The above mentioned approaches, although interesting, are plagued by several problems, such as short lifetime, long response times and frequently poisoned by sample components. Accordingly, alternative analytical methods for the determination of palm oil quality parameters have been developed. An FTIR method for the determination of FFA in olive oil, fats and oils by measuring the carbonyl group (C=O) band at  $1711\text{ cm}^{-1}$  have been reported [15]. Although this method does not require solvents and short analysis time (2 min), it was not considered due to the higher cost in the instrumentation involved. Therefore, it was the interest of our group is to find an alternative analytical method for the determination of palm oil quality parameters. In our approach, low cost and one that can potentially be adopted for process control are given special consideration. Flow injection analysis (FIA) methods, in combination with suitable flow through detectors seem more attractive as they can be easily automated and are able to meet the objectives outlined above.

However, a few reports on the FIA determination of FFA in food samples were reported. The earlier FIA methods contain undesirable features such as involving on-line extractions uses toxic organic solvents, complex flow lines (multiple channels), and involving phase separators. A single- and two-line manifolds were developed using phenolphthalein (PHT) and bromothymol blue (BTB) as indicators [8]. Another alternative two-line manifold non-aqueous titimetric method for the determination of palm oil acidity was also developed using *N*'-(2,4-dinitrophenyl)acetohydrazide (NDA) as indicator [4]. The present study is developed based on the synthesized hydrazone reagent that exhibits colour change between acidic and basic medium using two-line manifold FIA method. Therefore, the synthesized compound will be applied as indicator for the determination of FFA in palm oil. The present method offered an interesting alternative method for the determination of various types of palm oil samples

## 2. Experimental

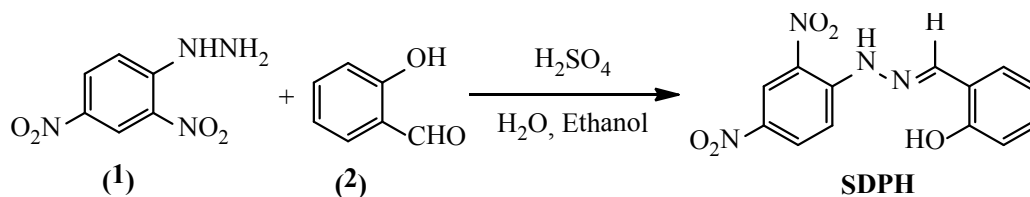


Figure 1. The synthetic route of SDPH.

### 2.1. Instruments

A Hitachi U-2000 double beam spectrophotometer was used to obtain UV spectra of the new reagent and palm oil in 1-propanol. A Hitachi U-1000 spectrophotometer was used for FIA analysis. Fourier transfer infrared analysis was performed using a Perkin Elmer 2000 FT-IR spectrophotometer. The  $^1\text{H}$ NMR spectra were recorded at  $25^\circ\text{C}$  using a Bruker 400 MHz spectrophotometer.

### 2.2. Chemicals

2,4-Dinitrophenylhydrazine, 98% (Aldrich), 1-propanol and ethanol (SYSTEMM), potassium hydroxide (R & M Chemicals), phenolphthalein (Ajax Chemicals), palmitic acid, 99% (BDH Chemicals), and salicylaldehyde (Fluka).

### 2.3. Synthesis of salicylaldehyde-2,4-dinitrophenylhydrazone (SDPH)

The present compound (SDPH) was prepared according to the reported method given in the literature [21-24]. A 2,4-dinitrophenyl hydrazine (1) (1.56 g, 10 mmol) was dissolved in 4 mL of concentrated sulfuric acid followed by addition of 20 mL distilled water and 50 mL ethanol. The mixture was thoroughly mixed and filtered if necessary. An equimolar (1.4 g, 10 mmol) salicylaldehyde (2) in 10 mL ethanol was added dropwise to the above solution with continuous stirring. An orange-yellow precipitate was formed almost immediately. The precipitate was filtered, washed with cold ethanol and dried in the oven at  $60^\circ\text{C}$ . Yield: 97 %. The synthetic route of the preparation of dye compound (SDPH) is depicted in Figure 1.

### 2.4. Carrier solutions

Stock carrier stream solutions was prepared by weighing 0.28 g (10 mmol) KOH and dissolved in 30 mL 1-propanol in 50 mL volumetric flask. The solution was first sonicated for 15 minutes and then diluted to the mark with 1-propanol, and then calibrated following a reported procedure [8]. A one mmol SDPH was prepared by dissolving 0.31 g of the dye compound in 1 liter 1-propanol after sonication for 15 minutes. Carrier solution containing a mixture of  $1.0 \times 10^{-3}$  M KOH and  $1.5 \times 10^{-4}$  M SDPH was prepared daily by diluting from the stock solutions of both.

## 2.5. Sample preparation

### 2.5.1. Stock oil (100 g L<sup>-1</sup>) Samples

Cooking oil (Vesawit, Yen Lee Edible Oils Sdn. Bhd) was purchased from local supermarket. Its acidity degree was tested before the preparation of stock solution. A stock sample was prepared by dissolving 25 g oil in 50 mL 1-propanol. This sample was used to prepare stock and standard palmitic acid solutions.

### 2.5.2. Stock Palmitic Acid Solution, 100 a.d

A 5.0 g palmitic acid was weighed in and dissolved in 50 mL stock oil solution prepared above. The solution was sonicated for 20 minutes and made up to the mark with stock oil, and stored at room temperature. This solution was used to prepare working calibration curve samples. Table 1 presents the standard solutions used for the preparation of the calibration curve of palmitic acid.

**Table 1.** Preparation of standard solutions of palmitic acid

Concentration required, a.d.	Volume of stock palmitic acid, mL	Volume of stock oil, mL
0.5	0.05	9.95
1.0	0.10	9.90
2.0	0.20	9.80
5.0	0.50	9.50
10.0	1.00	9.00
20.0	2.00	8.00
40.0	4.00	6.00
80.0	8.00	2.00
100.0	10.00	0.00

### 2.5.3. Palm Oil Samples

Samples were prepared following reported procedure with modifications [8,15]. Palm oil samples were firstly neutralized and then extracted four times with 0.01M KOH in a separating funnel to remove all FFAs. The aqueous layer was removed and the resulted neutralized oil was centrifuged for 60 minutes at 3600 rpm. Samples that were used in the

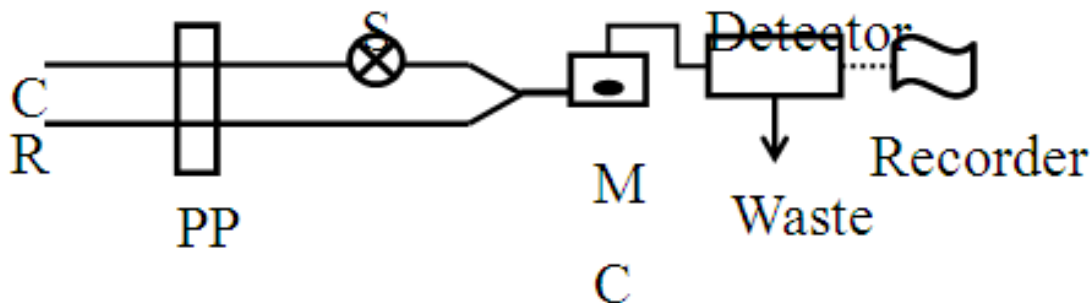
study consisted of RBD palm oil, RBD kernel palm kernel oil, crude palm oil and RBD palm stearin. All samples were heated to 60-70°C for homogenization before sampling. For refine oils (liquid form), 10 grams of sample was diluted to 50 mL with 1-propanol. For oil samples that have solid fraction (crude palm oil and stearin), free fatty acids were extracted as follow: 10 grams of heated oil samples was weighed and diluted with 25 mL of 1-propanol when sample was still hot. The sample was then stirred and allowed to cool and crystallized, filtered and washed with 10 mL 1-propanol. Finally, the filtrate was collected and top up to the 50 mL mark with 1-propanol.

### 2.5.4. Manual Titrimetry (Standard Method)

Palm oil samples were determined for their acidity degree using the official method [8]. The free acids are frequently expressed in terms of acid value. The acid value is defined as number of milligram of KOH necessary to neutralize 1.0 g of the sample. Sample was weighed according to the expected acidity and diluted with neutralized 1-propanol to the 50 mL mark. The sample was transferred into 250 mL conical flask, followed by the addition 0.5 mL 1.0 % (w/v) phenolphthalein in 1-propanol. The sample was then titrated against 0.1 M KOH till the first pink colour appears and was persistent for 30 seconds.

## 2.6. FIA Setup and Procedure

The flow injection manifold used in this study is depicted in Figure 2. It consists of a Gilson Miniplus 3 peristaltic pump, UV-Vis spectrophotometer equipped with an Unonic ultramicroflow cell (20  $\mu$ L and 1.0 mm path length), mixing chamber [8], a Rheodyne type 500 rotary injection valve and recorder. The FIA procedure is the same for both standards and samples. The oil is directly injected (0.5 mL) into the stream. In the mixing chamber, it reacted with the carrier, increasing the acidity along the sample zone and changing the colour from red to yellow. Therefore, a negative absorbance peak, which has a linear relationship with the logarithm of the acidity of the sample as reported by Saad et al., [8] and Karlberg and Pacey [25] was recorded.



**Figure 2.** Two-line FIA manifold. C, Carrier stream; R, reagent system; S, Sample injector; PP, peristaltic pump; MC, Mixing chamber; and W, Waste.

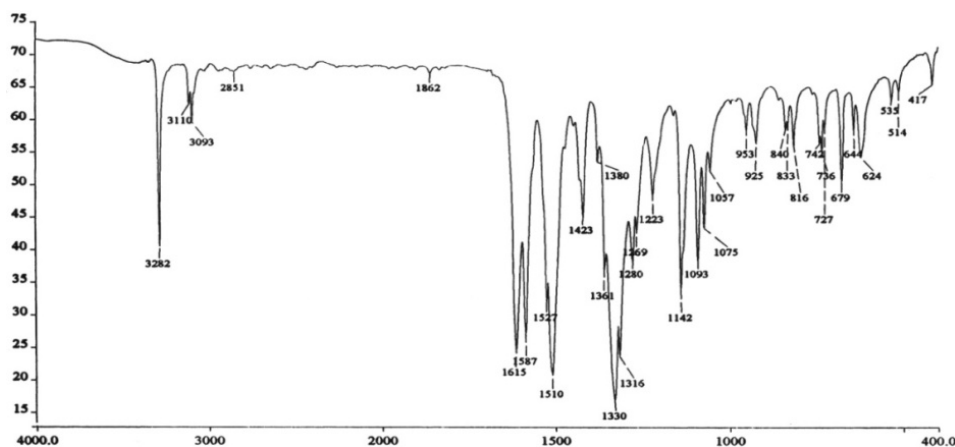


Figure 3. FTIR spectra of salicylaldehyde-2,4-dinitrophenylhydrazone (SAPH).

### 3. Result and Discussion

#### 3.1. Characterization of SDPH

**IR analysis:** The IR measurements were recorded over the range 4000–400  $\text{cm}^{-1}$ . The IR spectrum of SDPH (Figure 3) shows a strong shift in the absorption peaks compared with the free DNPH as reported by Chis *et al.*, [26]. C=N (stretching) was assigned at 1615  $\text{cm}^{-1}$ .  $\nu = 3326 \text{ cm}^{-1}$  (br, NH), 3282 (m, OH), 3101 (w), 1512 (s,  $\text{NO}_2$ ), 1419 (m), 1334 (s,  $\text{NO}_2$ ), 1273 (vs, C–O), 1142 (s), 833 (w), 764 (w), 578 (m)  $\text{cm}^{-1}$ . Nitro groups vibrations are shifted to 1587–1423 and 1380–1330  $\text{cm}^{-1}$ , respectively. Hydrogen bonding has little effect on the  $\text{NO}_2$  asymmetric stretching vibrations. The bands at 1609, 1571 and 1371  $\text{cm}^{-1}$  for SDPH are corresponded to asymmetric and symmetric stretching of  $\text{NO}_2$  group, respectively

**NMR analysis:** The  $^1\text{H}$ NMR spectrum (prepared in DMSO) of the SDPH displayed 10 signals, at 10.02 ppm (–NH, s), 6.94 (ArH, t), 7.19 (ArH, s), 7.24 (ArH, d), 7.34 (ArH, t), 7.61 (ArH, d), 8.25, (ArH, s), 8.36 (ArH, q), 9.13 (ArH, d), 10.02 (H, s) and 11.29 (H, s).

Table 2. Characteristics of solvents studied as FIA carrier stream.

Solvents	Solubility		
	SDPH	KOH	palm oil
Water	Insoluble	Soluble	Insoluble
Methanol	Moderate	Moderate	Insoluble
Ethanol	Low	Soluble	Insoluble
Acetone	Soluble	Moderate	Soluble
1-propanol	Soluble	Soluble	Soluble
THF	Insoluble	Insoluble	Soluble
2-propanol	Moderate	Soluble	Soluble
DMSO	Soluble	Soluble	Soluble

#### 3.2. Choice of Solvent

This information is vital because a suitable solvent has to

be chosen to dissolve the indicator, KOH, and palm oil as well to be used as the FIA carrier stream. The characteristics of the eight solvents studied are summarized in Table 2. Data showed that only DMSO and 1-propanol were able to dissolve all components (SDPH, KOH, palm oil). Therefore, 1-propanol was chosen for further studies, because of it is non-volatile and cheaper.

#### 3.3. UV Characteristics

SDPH react rapidly in 1-propanol with KOH, forming intensively coloured red-brownish product. The maximum absorption takes place at 482 nm (Figure 4). Oil samples containing free fatty acid is directly injected (without dilution) into the carrier stream with 1-propanol as carrier solution contained potassium hydroxide as titrant and SDPH as indicator. In the mixing chamber, the sample mixes and reacts with the carrier stream, increasing the acidity along the sample zone and changing the colour of the SDPH from red-brownish to yellow.

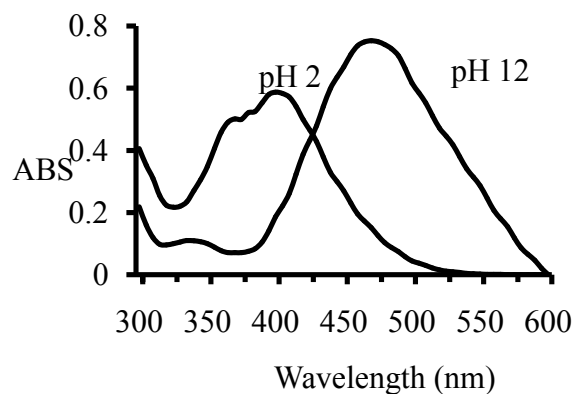


Figure 4. UV-Vis absorption spectra of SDPH ( $1.5 \times 10^{-4}$  M) in 1-propanol at different pH

A spectrophotometer set at  $\lambda_{\text{max}} = 482 \text{ nm}$  was used to monitor continuously the decrease of reagent absorbance and the FIA titration peak formed. The area of the peak ( $\Delta \text{Abs vs. time}$ ) is linearly correlated with the logarithm of the

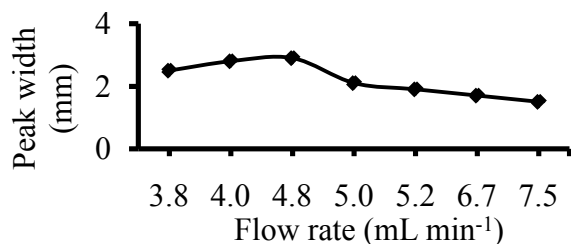
sample acidity [25]. The calculation of peak area (instead of peak height), enables a larger range of linearity between the value measured (the peak area) and the logarithm of sample acidity [27].

### 3.4. FIA Method Development Using Two-Line Manifold

A two-line manifold designed (Figure 2) for direct injection of sample into a carrier stream without prior dilution with the 1-propanol as carrier was investigated. The injection sample was merged with carrier stream, mixed in the mixing chamber, before finally passing through the flow cell of the spectrophotometer. The mixing chamber allowed the mixing of oil sample with 1-propanol before neutralized by KOH. Without the mixing chamber, peaks of poor reproducibility were obtained. Several parameters that affect the FIA signal were investigated. These include sample loop, mixing chamber, flow rate and carrier and reagent concentrations.

#### 3.4.1. Effect of Flow Rate

The effect of flow rate was tested by adjusting the peristaltic pump to different flow rates of 3.8, 4.0, 4.8, 5.0, 5.5, 6.6 and 7.5 mL min<sup>-1</sup>. The concentration of KOH and SDPH were 1.0 × 10<sup>-3</sup> M and 1.5 × 10<sup>-4</sup> M, respectively. It was found that at lower flow rate, the FIA signal tends to have a wider peak and peak shape tends to be asymmetry. Analysis time was found to be increased and thus consumed more solvent. However, as the flow rate continued to be increased, the produced FIA signal showed sharper peaks, making it difficult to integrate its area. However, increasing the flow rate of carrier stream from 3.8 to 7.5 mL min<sup>-1</sup> decreased the width of the FIA signal till a point when the peak was most satisfactory. When the flow rate was further increased, the peak width decreased slightly till a plateau was reached as shown in Figure 5. The choice of flow rate was a compromise between sample throughput and sensitivity. Flow rate of 4.8 mL min<sup>-1</sup> was then chosen.

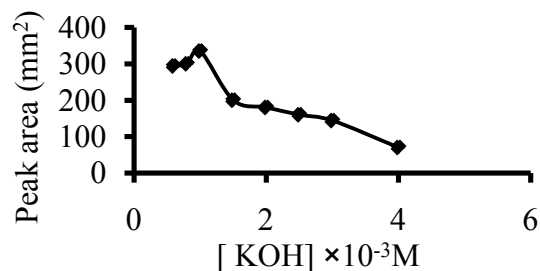


**Figure 5.** Effect of flow rate of carrier stream on FIA peak width with injection volume 70 μL

#### 3.4.2. Effect of KOH Concentration

The effect of KOH concentration was studied by varying its concentration from 6.0 × 10<sup>-4</sup> M to 4.0 × 10<sup>-3</sup> M. The concentration of SDPH was fixed at 1.5 × 10<sup>-4</sup> M. A 4.0 mL mixing chamber and 70 μL injection volumes with a flow rate of 4.8 mL min<sup>-1</sup> were used. The peak area becomes smaller while the KOH concentration increased from 6.0 ×

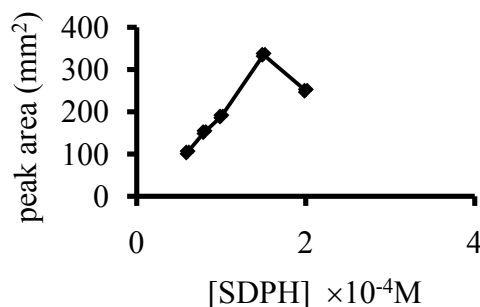
10<sup>-4</sup> M to 4.0 × 10<sup>-3</sup> M (Figure 6). The suitable KOH concentration was 1.0 × 10<sup>-3</sup> M as larger peak area was obtained.



**Figure 6.** The effect of KOH concentration on peak area.

#### 3.4.3. Effect of SDPH Concentration

The effect of the SDPH concentration on the peak area was also investigated. In this study, the KOH concentration was fixed at 1.0 × 10<sup>-3</sup> M as well other parameters while SDPH concentration was varied from 2.0 × 10<sup>-4</sup> M to 6.0 × 10<sup>-5</sup> M. SDPH concentration of 1.5 × 10<sup>-4</sup> M gives the highest peak area (Figure 7). The peak shape becomes smaller when concentration is increased.



**Figure 7.** Effect of SDPH concentration on the peak area.

#### 3.4.4. Effect of Injection Volume

The effect of injection volume was studied using injection loops of 10 to 125 μL. A flow rate of 4.8 mL min<sup>-1</sup> was used. Tailing peaks when 125 μL of injection volume was used were obtained. The injection volume poses some effect on the sensitivity of the system due to dispersion of sample in carrier stream and thus sample volume of 70 μL was used.

#### 3.4.5. Effect of Mixing Chamber

The present FIA titration system is based on peak width measurement. Therefore, large dispersion can be obtained by means of a mixing chamber. The effect of mixing chamber was studied by varying the size of mixing chamber from 1.0 to 6.0 mL. At fixed concentrations of KOH (1.0 × 10<sup>-3</sup> M) and SDPH (1.5 × 10<sup>-4</sup> M), injection volume (70 μL) and flow-rate (4.8 mL min<sup>-1</sup>), the peak area was increased with the increase of the size of mixing chamber (Figure 8). Therefore, 4.0 mL size of mixing chamber was chosen.

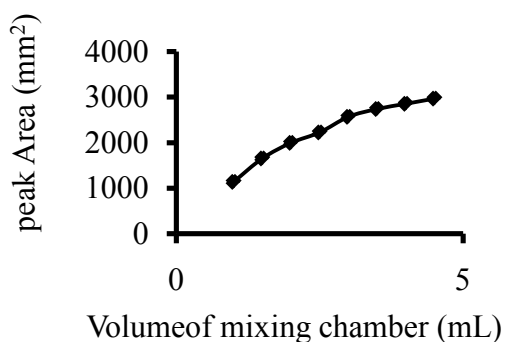


Figure 8. Effect of mixing chamber on peak area.

### 3.4.6. Adopted Parameter

The adopted FIA conditions for the two-line manifold employing the new indicator (SDPH) are summarized in Table 3. The sample volume needed in this system is smaller (70  $\mu\text{L}$ ), when compared to previous work done by Nourous *et al.*, [28] (175  $\mu\text{L}$ ) and Mariotti and Mascini (2001) (280  $\mu\text{L}$ ) using phenolphthalein (PHT) as indicator. The mixing chamber used was found four times larger than that used by Saad *et al.*, [8] and Teoh [4] on their FIA studies using PHT and NDA as indicators, respectively.

Table 3. Comparison between FIA condition used for two-line manifold employing SDPH, PHT, BTB and NDA as indicators.

Parameters	SDPH	PHT	BTB	NDA
KOH concentration /M	$1 \times 10^{-3}$	$5.0 \times 10^{-4}$	$2.5 \times 10^{-4}$	$5.0 \times 10^{-5}$
Indicator concentration /M	$1.5 \times 10^{-4}$	$2.5 \times 10^{-5}$	$2.5 \times 10^{-5}$	$1.4 \times 10^{-5}$
Volume of mixing chamber (mL)	4.0	1.0	1.0	1.0
Injection volume / $\mu\text{L}$	70	25	25	25
Flow rate / $\text{mL min}^{-1}$	4.8	4.2	4.2	3.5
Reagent consumption per sample/mL	3.75 -7.5	6.0-16. 0	11.0-16 .0	6.8 -13.6
Detection wavelength/nm	482	562	627	435
Reference	Present work	[8]	[8]	[4]

Table 3 shows a comparison between the FIA conditions applied for two-line manifold using SDPH, PHT, BTB and NDA as indicators for the determination of FFA. The solvent consumption in FIA is always less than the normal titration method, which consumes approximately 50 mL of solvent in an analysis. Therefore, even though the present method exhibits lesser sensitivity (higher detection limit, 0.5 a.d.) it still has the larger linear range (0.5 – 100 a.d.) when compared to the other studies.

## 2.5 Calibration

A calibration curve was obtained for the present FIA system (Figure 9). The line was linear from 0.5-100 a.d. The correlation coefficients, y-intercepts, and slopes of the calibration curve were also obtained. The calibration equation is  $y = 413.1x + 26.655$  with  $r^2 = 0.9908$

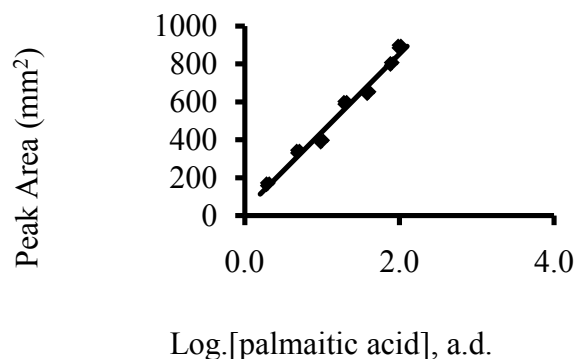


Figure 9. Calibration curve for different concentration of standard palmitic acid using SDPH under the optimum conditions (Table 3)

## 2.6 Analysis of Real Samples (Palm Oil)

Twelve palm oil samples were tested. Results obtained were compared to the results obtained by the PORIM standard method which involves manual titration. Results obtained for the twelve samples are shown in Table 4. The correlation of the FIA method with the standard PORIM method is shown in Figure 10, where good correlation was found between the two methods. The good correlation showed the proposed method is applicable for palm oil samples.

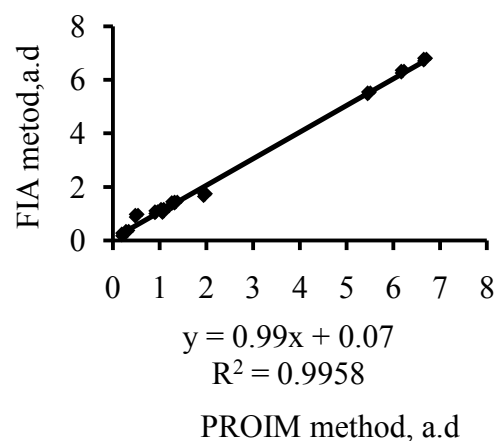


Figure 10. Correlation between FIA and standard PORIM methods.

**Table 4.** Determination of FFA in 12 palm oil samples

Sample No.	Sample Type	Palm oil acidity (FFA content, a.d.)	
		PORIM Official method	Present method
1	RBD palm oil	0.52 (0.21)	0.93 (0.13)
2	RBD palm oil	0.31 (0.01)	0.43 (0.22)
3	RBD palm oil	0.22 (0.11)	0.23 (0.23)
4	RBD palm stearin	0.93 (0.06)	1.06 (0.01)
5	RBD palm stearin	1.06 (0.02)	1.15 (0.02)
6	RBD palm stearin	1.09 (0.38)	1.07 (0.08)
7	RBD palm kernel oil	1.28 (0.07)	1.40 (0.38)
8	RBD palm kernel oil	1.97 (0.02)	1.79 (0.05)
9	RBD palm kernel oil	1.35 (0.41)	1.42 (0.06)
10	Crude palm oil	6.68 (0.61)	6.76 (0.03)
11	Crude palm oil	5.48 (0.18)	5.60 (0.09)
12	Crude palm oil	6.20 (0.21)	6.31 (0.17)

The values in parenthesis are the standard deviation ( $\pm$ SD) data

### 3. Conclusion

A simple, FIA method for the determination of FFA in palm oil samples using two lines FIA manifold employing SDPH hydrazone has been developed. The two-line manifold requires direct injection of oil samples resulting in good sample throughput, and consumes lesser amounts of reagent. The two-line manifold using SDPH as indicator is recommended for the determination of sample with FFA ( $>0.5$  a.d.). The FIA method is found suitable for higher acidity samples (such as Crude RBD palm). Good correlation between the present method and the official methods applied for the determination of FFA in palm oil samples.

### Acknowledgments

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