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# CHARACTERISATION OF SYNTHESISED PROTECTED FAT FROM USED COOKING PALM OIL AND PALM OLEIN FOR ANIMAL FEED APPLICATION

**MOHAMAD ASROL, K[1]; SUYUB, I B[1]*; PAK DEK, M S[2]; SUMITA, S[1] and JUSOH, S[1]**

**ABSTRACT**

*In the present work, a new protected fat was produced from used cooking palm oil (UCO) by using modified fusion method. Used cooking oil protected fat (UCOPF) was compared with produced palm olein protected fat (POPF) and commercial protected fat (CPF). The quality of protected fat produce was investigated using Fourier transform infrared (FTIR) and X-ray diffraction (XRD) studies. FTIR and XRD results confirmed the well-formation of protected fat from UCO and the XRD analysis revealed fatty acid from UCO bind well with calcium ion. FTIR spectroscopy indicated carboxylate bands at 1542 and 1575 $cm^{-1}$ showed the calcium ions associated with the COO- ions in the monodendate and bidendate structures in PF. Differential scanning calorimetry (DSC) study was conducted to find out the thermal behaviour of the produced UCOPF and the results clearly indicated good thermal stability. DSC indicated onset of melting at 151°C and 148°C for samples prepared UCOPF and POPF, respectively and it was slightly lower than CPF (157°C). Smooth and porous surfaces morphology of UCOPF and POPF was confirmed using scanning electron microscope (SEM) analysis. From the results it was evident that UCO can be utilised to produce protected fat as a safe animal feed.*

**Keywords:** animal supplement, oil quality, palm oil, rumen bypass fat, saponification.

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## INTRODUCTION

Protected fat (PF), also known as rumen bypass fat or inert fat, play an essential role in ruminant diets, particularly dairy diets (Lounglawan *et al.*, 2008). PF is an important source of energy in dairy diets due to their high calorific density (Jenkins and Harvatine, 2014). This is especially important in the initial stages of lactation when dry matter intake (DMI) is low, and cows are in a state of negative energy balance (Purushothaman *et al.*, 2008). Utilisations of PF in the dairy diets can be balanced with the proper quantity of concentrates and forages to promote a healthy functional rumen condition while providing the energy needs of the lactating cow for the production of high-quality milk (Naik, 2013).

PF is fat that is manufactured by various methods such as hydrogenation of tallow, prilled in spray drying system (Suksombat, 2009), encapsulation method (Shelke *et al.*, 2012) and calcium salts production of fatty acid (FA) (Naik *et al.*, 2007). Calcium salt is generally produced using the single fusion method and double

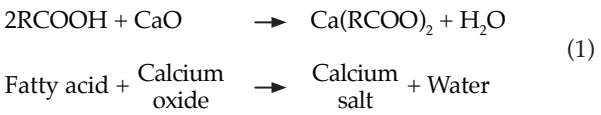
[1] Department of Animal Science,
Faculty of Agriculture,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia.

[2] Department of Food Science,
Faculty of Food Science and Technology,
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia.

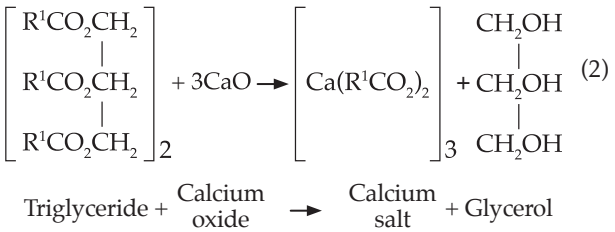
* *Corresponding author e-mail: iswan@upm.edu.my*

***** Disclaimer from the Editor in Chief of JOPR: This is a scientific study only. The utilisation of used cooking oil in the production of animal feed is not advised due to its substandard quality. From an ethical standpoint, such usage is not recommended.

precipitation method (Handojo *et al.*, 2019a). Usually, in commercial industry single fusion method is used to produce PF of calcium salts because it requires fewer materials, is more economically friendly and is environment viable (Shelke *et al.*, 2012; Suksombat, 2009). The process of single fusion for fatty acid is shown in Equation (1) and triglyceride in Equation (2) below.




(1)




(2)

Calcium salts, one of the forms of PF used in the ruminant feed additive, is more desirable than feeding raw oil directly to the animal, known as unprotected fat (Palmquist and Jenkins, 2017). Contrasting, directly feeding raw oil to ruminants that hindered the fermentation of cows in the rumen, PF has an insignificant impact on the rumen fermentation (Ibrahim *et al.*, 2021; Naik, 2013). Ruminant consumption of PF calcium salts improves the quality and quantity of milk produced. It has been documented that feeding 0.45 kg of PF to the cow daily increases milk production by 3.0%-8.0% (Mishra *et al.*, 2005). Additionally, the milk fat content rises by 0.2%-0.3%, and the fertilisation of cows' success rate increases by 20.0% (McNamara *et al.*, 2003).

Used cooking oil (UCO) is a category of domestic wastes produced when edible vegetable oil is used for cooking and frying food (Iglesias *et al.*, 2012). The surge in demand for edible oil (EO) due to the increase of population growth, UCO was generated from frying activities in food sectors, residences, hotels, and restaurants (Yacob *et al.*, 2015; Yee *et al.*, 2018). This causes environmental problems due to the contamination of land and drinking water resources and ecosystems that are home to a variety of species (Martins *et al.*, 2021). In these cases, UCO must be properly disposed off, recycled, or recovered for sustainable purposes. It is not an appealing prospect since warehousing UCO is costly (Uz and Gökalp, 2020). Alternatively, recycling or recovering for an innovative use or utilising these resources to make an oil-based product are the most prominent strategies. Numerous studies have been conducted to assess the feasibility of utilising UCO in various applications such as biodiesel, soap, animal feed and so on (Azahar *et al.*, 2016; Fangfang *et al.*, 2021; Nanda *et al.*, 2019; Rincón *et al.*, 2019; Supple *et al.*, 2002; Tres *et al.*, 2013). Such UCO, if recovered, will obviously save resources, bring economic profit and relieve environmental pressure (Wei *et al.*, 2011). Moreover, UCO has the potential to satisfy the need for low-cost materials that cannot be fulfilled by other food crops (Panadare and Rathod, 2015).

Production of PF in the market utilises vegetable fat such as palm oil as the source of raw materials (Suksombat, 2009). Using vegetable fat as raw materials for animal feed will compete with human food (Panadare and Rathod, 2015). So, another source of making PF calcium salts is by using UCO (Wei *et al.*, 2011).

A variety of reasons, such as legal enforcement, financial issues, the adoption of non-harmful materials, the assurance of consistency in quality and so on, necessitate the ability to validate the characteristics and properties of feed fats. Traditional methods of feed and food analysis, such as wet chemistry for the determination of a feed or food macro-element or the detection of spoilage and quality assurance, were based on the evaluation of the amount of an indicator molecule or compound and the corresponding correlation with those identified in the technical information (Karoui and de Baerdemaeker, 2007).

For the most part, until recently, the fats utilised in feeding applications were primarily defined by a few fundamental caloric value parameters, such as overall fat percentage, moisture content, contaminants and unsaponifiable fats (Gasperini *et al.*, 2007). Approaches used predominately to determine the consistency of vegetable oils have emphasised measurements of physicochemical properties such as FFA, acid number, iodine, saponification value, density and refractive index, among others (van Ruth *et al.*, 2010).

To our knowledge, limited data is currently available on the production and characterisation of used cooking oil protected fat (UCOPF), and palm olein protected fat (POPF) using complex technologies such as Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM) and differential scanning calorimetry (DSC) to analyse quality of protected fat. As a first step toward additional research into UCO's potential as a calcium salt in dairy diet supplements, this work could serve as a springboard for future research. The production and characterisation of UCO and POPF of calcium salts were the focus of the current study, which utilised FTIR spectroscopy, XRD, SEM and DSC to accomplish its objectives.

## MATERIALS AND METHODS

### Materials

Calcium oxide (CaO), activated carbon and monosodium glutamate were purchased from Malaysia Fisher Scientific Co. UCO was purchased from Bangi Mosque UCO collection centre Selangor, Malaysia and palm olein (Vesawit) was purchased from local Boss Family Grocer in Serdang, Selangor. The fatty acid characteristics of used cooking palm oil and palm olein are illustrated in *Table 1*.

**TABLE 1. FATTY ACID COMPOSITION OF USED COOKING PALM OIL AND PALM OLEIN**

| Fatty acid | Mean ± standard error (n=3) | |
|---|---|---|
| | Used cooking palm oil | Palm olein |
| C12; Lauric acid | 2.96 ± 0.02 | 1.41 ± 0.76 |
| C14; Mrystic acid | 2.33 ± 0.06 | 3.91 ± 0.83 |
| C16; Palmitic acid | 44.21 ± 0.17 | 43.99 ± 0.43 |
| C18; Stearic acid | 1.11 ± 0.06 | 1.76 ± 0.38 |
| C18:1; Oleic acid | 39.38 ± 0.20 | 39.64 ± 0.07 |
| C18:2; Linoleic acid | 8.25 ± 0.03 | 8.28 ± 0.00 |
| SFA | 50.61 ± 0.29 | 51.07 ± 0.44 |
| MUFA | 39.38 ± 0.20 | 39.64 ± 0.07 |
| PUFA | 8.25 ± 0.03 | 8.28 ± 0.01 |

### Production of Protected Fat

***UCO purification.*** The purifying procedure was followed through to completion by mixing raw UCO with activated carbon and monosodium glutamate (MSG) at a ratio of 30:1:1 according to Wei *et al*. (2011). After mixing the oil sample and adsorbent, they were heated for 30 min at a temperature of 70°C-80°C. Following this procedure, active carbon and MSG were removed from the oil using cellulose filtration No. 1 Whatman filter paper.

***UCO saponification.*** The saponification procedure was performed using the modified fusion method followed by the drying and milling process (Pablos Pérez, 2008). The procedure was described in detail below.

30 g of oil sample was heated to 80°C, then CaO powder with a 20% weight of oil was added. The mixture of oil and CaO was heated and stirred homogeneously. Immediately after homogeneous mixing was achieved, water equivalent to 20% of the oil weight at temperature 60°C was added to the mixture. The reaction mixture was stirred vigorously for about 10 min.

After the saponification process was completed, the mixture was dried for about 24 hr at 80°C in a forced air circulation oven (Memmert UF53) before being cooled at room temperature overnight. Then, the soap material was ground into flakes form.

### Characterisation of Protected Fats

***Surface chemistry.*** The attenuated total reflectance (ATR) method was used to obtain the FTIR of the oil sample and PF. The FTIR of the oil sample and PF had a spectrum in the region of 4000-650 $cm^{-1}$.

***X-ray diffraction (XRD) analysis***. The wide-angle X-ray diffraction pattern technique was used to evaluate the PF powder. The X-ray diffractograms were acquired using Cu K$\alpha$ ($\lambda$ = 1.5406 Å) radiation at a speed of 2° $min^{-1}$ from 20° to 70°. Throughout the operation, the voltage (30 kV) and current (30 mA) were maintained at the same levels. Bragg's law was used to compute the spacing between bilayers.

$$N\lambda=2d\sin\theta \quad (3)$$

where n is 1 and $\lambda$ is 1.5.

Using the method provided by Nara and Komiya (1983), the percentage of crystallinity Xc% was calculated as a ratio of the crystalline area to the total area of the peaks. Following is a description of the equation for percentage of crystallinity Xc%:

$$Xc\% = \frac{Ac}{Aa + Ac} \times 100 \quad (4)$$

where Ac is the area of crystalline phase, Aa is the area of amorphous phase and Xc is the percentage of crystallinity.

***Morphology analysis***. The surface morphologies were studied using scanning electron microscopy (SEM) with energy-dispersive X-ray spectroscopy (EDX) (SEM, Hitachi S-3400N, Japan) under vacuum. Prior to observation, the PF's surfaces were gold-coated for the 90s. The EDX technique was used to investigate the distribution of the elements on the surfaces.

***Differential scanning calorimetric (DSC) analysis***. A differential scanning calorimeter (Mettler Toledo, DSC823/700) was used to evaluate the thermal characteristics of PF. In an aluminium pan, approximately 5 mg of oil and PF samples were sealed. As a reference, an empty sealed pan was used. Just before the measurement, the lids of the aluminium pans were punctured, enabling desorbed water to escape. Heating the samples at a rate of

10°C $min^{-1}$ from 30°C to 200°C was performed. The samples were heated at a rate of 10°C $min^{-1}$ from 30°C to 200°C.

## RESULTS AND DISCUSSION

### FTIR of Raw Materials and Protected Fats

According to Gorey and Escobar (2011), IR spectroscopy allows for the assessment of a substance's chemical properties, including chemical bonds, molecule alignments, molecular energy levels and molecular interconnections (Qu *et al.*, 2010). Therefore, FTIR analysis was performed on each sample, and the potential relationships between the functional groups in a PF were investigated.

*Table 2* displays the prominent peaks and their functional categories. Absorption peaks in the spectra of all samples at 3440-3390 $cm^{-1}$ indicate the existence of a hydroxyl group. The absorption bands in the spectra of all samples were found to be between 2925 and 2852 $cm^{-1}$, as determined by C-H stretching. The peaks between 2925 $cm^{-1}$ and 2855 $cm^{-1}$ are likewise indicative of scissoring of the $CH_2$ and $CH_3$ scissoring in all samples. Identified functional groups of esterified carbonyl C=O are allocated to sharp absorption bands of high intensity in the 1740-1746 $cm^{-1}$ range for all samples. This ester indicates the presence of triglycerides (Faridah *et al.*, 2015). The absorption band identified near 1574-1580 and 1540-1542 $cm^{-1}$ corresponded to the FAs' carboxyl group.

*Figures 1a-1e* depicts the FTIR spectra of UCO, palm olein (PO), UCOPF, POPF and commercial protected fat (CPF). The distinctive peaks of PF (*Figure 1c-1e*) were found at approximately 1542 and 1575 $cm^{-1}$ which the intensity (transmittance %) is strong compared to oil group (*Figure 1a-1b*). These bands are caused by antisymmetric stretching bands associated with the interaction of unidendate and bidentate with calcium ions (Sakai and Umemura, 2002). Antisymmetric and symmetric methylene stretching, as well as methylene scissoring bands (νaCH2, νsCH2, and $\delta sCH_2$), were identified in PF samples at around 2920, 2852 and 1467 $cm^{-1}$, respectively. These bands are caused by the presence of an alkyl chain in the structure of PF calcium salts (Gönen *et al.*, 2010). Every single PF sample shows strong similarities to the spectra of calcium stearate powders (Gönen *et al.*, 2010) and calcium carboxylates (Lu and Miller, 2002) that have been described in prior studies.

### XRD of Protected Fats

The shift in the crystalline nature was discovered using the XRD study. Insights on the crystallographic structure, chemical composition and physical characteristics of materials may be obtained using XRD method, which is frequently employed for crystalline phase characterisation (Jayakrishnan and Ramesan, 2016; Subbu *et al.*, 2014). Since the crystalline/amorphous ratio of the component material in a mixture provides a key function in comprehending the formation of the PF during saponification, the XRD structures for the produced UCOPF and POPF were investigated.

*Figure 2* illustrates the XRD peaks characteristic of UCOPF, POPF and CPF. The UCO and PO samples were omitted from the analysis due to their liquid state at room temperature. XRD data values can be used to identify a powder's crystalline nature. The pattern revealed that every PF sample possessed a highly distinctive peak at 20.6°. In all samples, another phase peak was detected at 21.8°, 26.7°, 36.5° and 51.2°.

**TABLE 2. FTIR FUNCTIONAL GROUPS OF USED COOKING OIL, PALM OLEIN, USED COOKING OIL PROTECTED FAT, PALM OLEIN PROTECTED FAT AND COMMERCIAL PROTECTED FAT**

| Functional group | Wavelength ($cm^{-1}$) | | | | |
|---|---|---|---|---|---|
| | UCO | PO | UCOPF | POPF | CPF |
| -OH stretching | 3 448.33 | 3 424.06 | 3 398.62 | 3 396.10 | 3 424.19 |
| CH stretching vibration (aliphatic) | 2 925.73 | 2 925.07 | 2 920.99 | 2 920.80 | 2 921.80 |
| | 2 855.27 | 2 854.89 | 2 852.21 | 2 852.08 | 2 852.78 |
| C=O stretching vibration (ester) | 1 746.49 | 1 746.61 | 1 741.43 | 1 741.84 | 1 740.82 |
| ($COO^-$) asymmetric stretching vibration | 1 577.01 | 1 576.69 | 1 579.95 | 1 574.89 | 1 575.72 |
| | 1 541.88 | 1 542.04 | 1 541.46 | 1 540.97 | 1 540.76 |
| C– H scissoring and bending vibration (aliphatic) | 1 462.92 | 1 463.73 | 1 467.76 | 1 467.99 | 1 468.03 |
| ($COO^-$) symmetric stretching vibration | ND | ND | 1 424.85 | 1 426.75 | 1 430.12 |

Note: UCO - used cooking oil; PO - palm olein; UCOPF - used cooking oil protected fat; POPF - palm olein protected fat; CPF - commercial protected fat; ND - not detected.

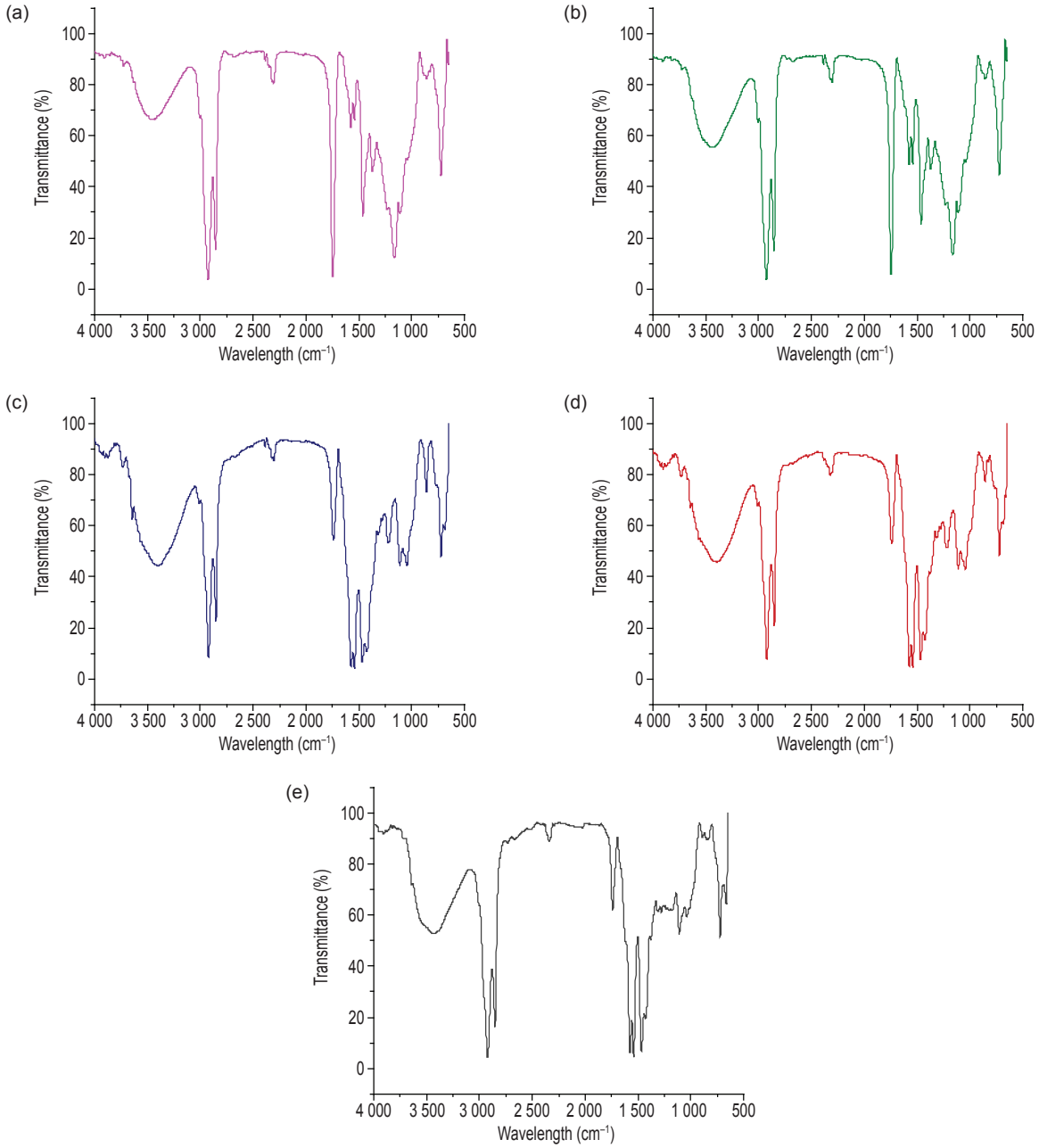



*Figure 1. FTIR spectra of (a) used cooking oil, (b) palm olein, (c) used cooking oil protected fat, (d) palm olein protected fat and (e) commercial protected fat.*

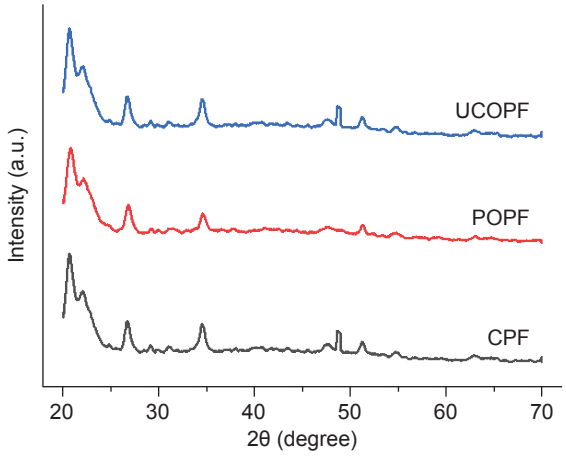



*Figure 2. X-ray diffraction of used cooking oil protected fat (UCOPF), palm olein protected fat (POPF) and commercial protected fat (CPF).*

The bilayer distance of all PF was calculated using Braggs law and higher-order reflection's 2θ, and it was determined to be approximately 4.3 nm (*Table 3*). According to prior research, the bilayer distance between the calcium stearate powder formed via precipitation was 4.8 nm (Gönen *et al.*, 2010). The d-spacing is described as the distance between planes of atoms that give rise to diffraction peaks. Each crystalline solid has a distinctive pattern that may be used as a "fingerprint" for identification using the XRD technique (Raval *et al.*, 2019). On the lattice properties, XRD may readily present errors of less than 0.01 angle. However, the current study had different value of d spacing compared to previous study. This was due to the lattice properties can change for the same material due to composition differences, gaps, and contaminants. It is entirely dependent on the type of material that had been utilised (Chauhan and Chauhan, 2014). The different d-spacing value may be due to different fatty acid in this study. The current study used a mixture of fatty acid whereas Gönen *et al.* (2010) used specific single fatty acid.

UCO contain high free fatty acid that may hinder the formation of protected fat during saponification. (Handojo *et al.*, 2019b) suggested that high concentration of calcium oxide is needed to convert excess free fatty acid to complete the saponification process. On comparing the percentage of crystallinity of the prepared UCOPF and POPF with CPF in *Table 3*, it is obvious that all three PF were found to show almost same crystallinity value than CPF. This proved that fatty acid from UCO and PO combined well with calcium ion from calcium oxide during the process of saponification to form protected fat.

**TABLE 3. D SPACING VALUE FOR USED COOKING OIL PROTECTED FAT, PALM OLEIN PROTECTED FAT AND COMMERCIAL PROTECTED FAT**

| Samples | d-spacing (nm) | Crystallinity (%) |
|---|---|---|
| UCOPF | 4.307 | 65.18 |
| POPF | 4.286 | 68.45 |
| CPF | 4.305 | 66.84 |

Note: UCOPF - used cooking oil protected fat; POPF - palm olein protected fat; CPF - commercial protected fat.

**SEM with EDX of Protected Fats**

Scanning electron microscopy (SEM) is a very beneficial instrument for describing the surface structure of the material and in understanding the theoretical and practical aspect of structure and behaviour of the produced material (Zeng *et al.*, 2013). Furthermore, SEM or EDX-equipped environmental SEM can recognise the substantial atomic component of material that are helpful for distinguishing carbon compounds from inorganic compounds (Abd Mutalib *et al.*, 2017).

SEM spectra, as shown in *Figure 3*, revealed the UCOPF and POPF had smooth and porous surfaces while CPF showed dense and irregular surfaces.

The pores formation could be the effect of the saponification process and natural stress (Galván-Ruiz *et al.*, 2009). According to the literature, viscosity had a significant impact on increasing solvent and nonsolvent diffusion rates throughout the porous texture development process (Ekambaram and Doraisamy, 2016).

The EDX spectrum clearly demonstrated the existence of distinct peaks for carbon, calcium, magnesium, and oxygen, indicating the purity of the produced PF (*Table 4*). The carbon element represents the percentage of fatty acid and calcium element represent the calcium percentage in the material. The produced UCOPF and POPF element percentage indicating the saponification process shown possible element percentage same as the stoichiometric empirical formula (Handojo *et al.*, 2018). The presence of magnesium in the PF was derived from a magnesium compound found in calcium oxide powder.

**Differential Scanning Calorimetry (DSC) of Protected Fat**

Differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), thermomechanical analysis (TMA), and dynamic mechanical analysis (DMA) are the methods used most often to characterise phase transitions. The DSC method is the most popular among them

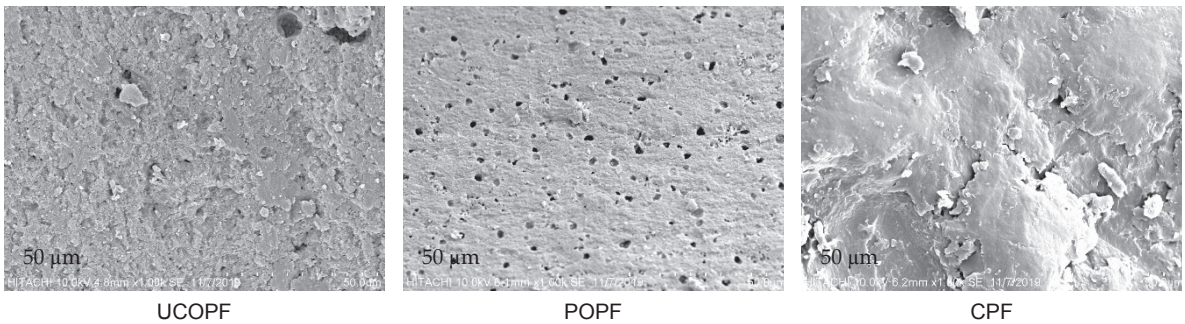



*Figure 3. SEM of used cooking oil protected fat (UCOPF), palm olein protected fat (POPF) and commercial protected fat (CPF).*

because it makes it simple to conduct both quantitative and qualitative analyses of the transitions and enables for the identification of transitions throughout a large temperature range (-90°C to 550°C) (Byrn *et al.*, 2017).

**TABLE 4. ELEMENTAL COMPOSITION OF USED COOKING OIL PROTECTED FAT, PALM OLEIN PROTECTED FAT AND COMMERCIAL PROTECTED FAT**

| Element (%) | UCOPF | POPF | CPF |
|---|---|---|---|
| Ca | 18.25 | 15.85 | 12.27 |
| C | 71.68 | 75.79 | 81.32 |
| O | 8.39 | 7.13 | 5.50 |
| Mg | 1.67 | 1.22 | 0.92 |

Note: UCOPF - used cooking oil protected fat; POPF - palm olein protected fat; CPF - commercial protected fat.

DSC is a renowned thermal analysis method that aids in comprehending the thermal behaviour of materials. Melting temperature (Tm), glass transition temperature (Tg), and crystallisation temperature (Tc) of a substance can be determined using DSC (Guirguis and Moselhey, 2011). DSC was discovered to be the most practical method for evaluating glass and melting temperature despite the availability of other methods including dilatometry, diffraction, rheological and dielectric approaches (Utracki and Favis, 1989). Additionally, it is generally recognised that one crucial factor determining a protected fat's inertness is its Tm (°C).

Though having a TGA analysis would offer further insight, DSC offers suitable characterisation of important thermal parameters, such as the Tg, Tc and Tm, which establish important protected fat physical and chemical properties, stability and processibility (Leyva-Porras *et al.*, 2019).

UCOPF, POPF and CPF have shown some interesting effects when heated upon heating, as shown in *Figure 4* and *5*. All PF samples were dehydrated and started to soften at temperatures between 104°C and 160°C. DSC measurement indicated the separation of water crystallisation by first endothermic.

As the temperature increased, there was a second endothermic peak of UCOPF, POPF and CPF at approximately 147°C, 146°C and 154°C, respectively, which correlates with the collapse of the crystal lattice that is associated with melting (Gregorova, 2013). This clearly showed that Tm was observed for all PF which indicated that the PF were high thermal stability. As evidenced by Ahmed *et al.* (2020), hydrogenated fat presented melting temperature of about 51°C-54°C thus showing that the high biohydrogenation of unsaturated fatty acid compared to calcium salts with Tm of more than 100°C. The higher melting

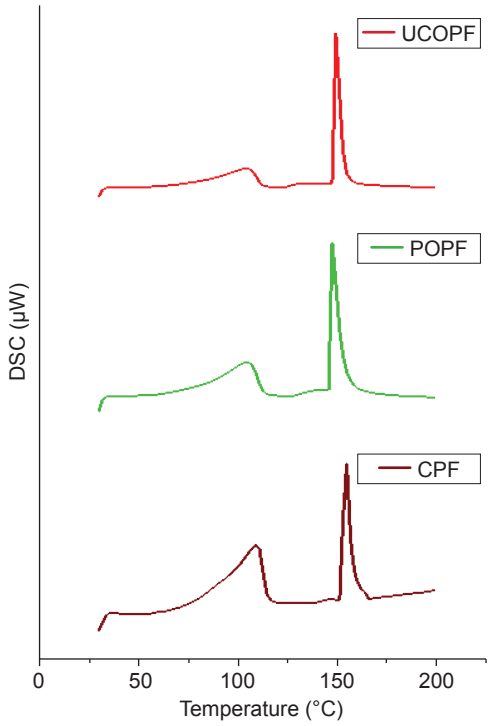



*Figure 4. Thermal behaviour for used cooking oil protected fat (UCOPF); palm olein protected fat (POPF) and commercial protected fat (CPF).*

point of PF is one of the important characteristics that can withstand digestion by the rumen microbes (Suksombat, 2009). Due to the PF form of the fat, which has the melting point above the rumen temperature (39°C), the PF can resist rumen hydrolysis, will only be digested in the small intestine, and avoid negative impact on rumen environment by protecting the unsaturated fatty acid from biohydrogenation process. Similar thermal behaviour has been described for calcium stearate (Ekambaram and Doraisamy, 2016), magnesium stearate and other metal salts (Delaney *et al.*, 2017).

**TABLE 5. MELTING POINT OF USED COOKING OIL PROTECTED FAT, PALM OLEIN PROTECTED FAT AND COMMERCIAL PROTECTED FAT**

| Sample | Temperature (°C) | |
|---|---|---|
| | Glass transition | Melting point |
| UCOPF | 103.52 ± 0.87$^{b}$ | 151.20 ± 2.10$^{b}$ |
| POPF | 103.16 ± 0.63$^{b}$ | 148.42 ± 0.99$^{b}$ |
| CPF | 109.19 ± 0.13$^{a}$ | 157.46 ± 1.91$^{a}$ |

Note: UCOPF - used cooking oil protected fat; POPF - palm olein protected fat; CPF - commercial protected fat.

## CONCLUSION

It could be concluded that the PF prepared using UCO and PO have been blended well which was confirmed by FTIR spectroscopy. The XRD

results showed that UCOPF an POPF containing same crystallinity percentage with CPF. DSC study revealed that all the PF samples showed good thermal stability. From SEM analysis, it was observed that the PF, especially UCOPF and POPF, were porous and showed uniformity and found to be smooth when compared to CPF, which has a denser surface. Thus, the UCOPF and POPF were very well supported by the analytical studies such as FTIR, XRD, DSC and SEM. Therefore, the prepared UCOPF and POPF showing properties at par with CPF and both UCO and PO can be employed as FA sources to create PF as a feed supplement for ruminants, which is accomplished through the fusion process. Consequently, the utilisation of FTIR, XRD, DSC and SEM should increase our understanding of the determinants of PF quality and may allow devising a structure engineering of animal feed. For future improvement, different type of UCO and its combination for PF production should be conducted and the physicochemical and *in vitro* analysis should be carried out before commercialisation

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