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REVIEW ARTICLES

- Prevalence of Work-Related Musculoskeletal Disorders (WMSDs) and Recent Initiatives to Address Them in the Oil Palm Industry: A Systematic Review
- Greening the Palm Oil Industry: Prospects and Barriers of Supercritical CO₂ Extraction



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PREVALENCE OF WORK-RELATED MUSCULOSKELETAL DISORDERS (WMSDs) AND RECENT INITIATIVES TO ADDRESS THEM IN THE OIL PALM INDUSTRY: A SYSTEMATIC REVIEW

‘UMAR MOHAMAD NOOR¹ and MOHAMAD IKHWAN ZAINI RIDZWAN^{1*}

ABSTRACT

This systematic review investigates the prevalence of work-related musculoskeletal disorders (WMSDs) within the oil palm industry, where the incidence is high due to the widespread use of manual tools, despite the availability of intervention tools in the market. The objectives of this review are twofold: To identify and summarise studies focusing on WMSDs prevalence in oil palm plantation work, and to examine research related to biomechanical evaluations and the design of intervention tools aimed at addressing WMSDs. A comprehensive search of databases including Web of Science, Scopus, PubMed, and ScienceDirect was conducted, covering articles published from 2013 to 2023. From the initial pool of 245 articles identified, a rigorous selection process yielded 27 studies meeting the inclusion criteria for this review. The majority of these studies were conducted in Malaysia (22 studies), with additional contributions from Thailand (2 studies), Colombia (2 studies) and Indonesia (1 study). In conclusion, WMSDs prevalence remains alarmingly high in oil palm plantation works. However, promising approaches utilising modern tools such as inertial measurement units (IMU) and electromyography (EMG) for direct measurement, as well as sophisticated human musculoskeletal model simulation in software like AnyBody, have been explored in some studies, indicating avenues for future intervention strategies.

Keywords: ergonomics, harvesters, intervention tools, MSDs, occupational health.

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INTRODUCTION

The oil palm industry is one of the most promising agricultural industries. It serves as the source of many important products ranging from palm oil, palm kernel oil and is also used in the making of various products such as soap, margarine and ice cream. Since 2005, Indonesia has emerged as the largest producer of palm oil, with oil production reaching 15.6 million tonnes and increasing up to 28.5 million tonnes by 2012 (Murphy, 2014). In 2017,

Indonesia contributed about 50% of the worldwide palm oil production (Shigetomi et al., 2020). By 2020, Indonesia followed by Malaysia were the two highest producers of palm oil (Shigetomi et al., 2020). When compared to soybean and rapeseed, oil palm stands out as the most efficient crop, yielding the highest oil-based hydrocarbons (Murphy, 2014; Sibhatu, 2023). In short, in terms of oil produced per hectare planted, it is the most productive crop (Rahman et al., 2016; Sibhatu, 2023). Oil palm cultivation is therefore profitable to plantation owners, but this may be different for oil palm workers who do not have farms (Shigetomi et al., 2020).

Comparing oil palm planted area in Malaysia from 2021 to 2023, a decrease of 1.1% and 0.4% respectively in two consecutive years resulted in the reduction of the oil palm planted area from

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5.74-5.65 million hectares (Malaysian Palm Oil board [MPOB], 2023, 2024). The reduction of 1.1% planted area from 2021-2022 was attributed to the MPOB license renewal procedures upgrade and adherence to the Malaysian Standard for Sustainable Palm Oil Production (MSPO) (Parveez et al., 2023). The slight decrease in the planted area (0.4%) from 2022-2023 was, to a certain extent, due to replanting preparation (MPOB, 2024). In managing such a vast area, the sustainability aspect of the oil palm industry should be a focus to ensure the industry's survival and prosperity. This effort should begin at the oil palm plantation itself, by taking care of the manual workers involved in oil palm fronds pruning, oil palm fresh fruit bunches (FFB) harvesting, FFB loading, oil palm loose fruits (LF) collection and many other tasks, as they are vulnerable to the harsh physical and mental demands of the work. Malaysia had been dependent on foreign labour, hence the post-effect of COVID-19 which resulted in the reduction of imported labour had seriously affected productivity (Parveez et al., 2023), with the lasting consequences still experienced by the industry, as indicated by only a slight growth in FFB yield (1.9%) in 2023 compared to 2022, despite the recovering of manpower supply (Parveez et al., 2024). This signalled the need to hasten mechanisation and automation, to reduce labour requirements (Parveez et al., 2023).

Traditionally, to harvest FFB, harvesters must adopt a stooping posture and use a chisel during early harvesting when the oil palm tree is under 3 m (Ng et al., 2013a). Stooping posture was characterised by trunk flexion adopted by the harvesters, to avoid contact with the oil palm fronds, as the fronds were shorter in height compared to the harvesters and hence acted as obstacles during harvesting activity. On the other hand, for tall oil palm trees, harvesters must tilt their heads upward in search of ripe FFBs and maintain this position throughout the harvesting motion (Ng et al., 2013a). Coupled with heavy tools such as traditional sickles/poles and chisels, the repetitive motions involved in these activities can result in injury or disorder to the musculoskeletal system of oil palm workers. The manual methods of FFB harvesting, FFB loading, and LF collection using traditional tools such as a sickle, chisel, and sharp metal skew are still used nowadays, as indicated in a study by Bhuanantanondh et al., (2021). This was due to the new technology not being widely adopted (Saibani et al., 2015).

Some of the inventions made for the frond pruning and FFB cutting tasks were the motorised cutter, while FFB loading task was the mechanical grabber attached to a tractor-trailer, whereas for LF collection task was the use of a vacuum-suction machine. Based on a study by Mohiddin and Pebrian (2021), the degree and capacity of

mechanisation in oil palm plantations in Malaysia were explored, using 2013 survey data from the MPOB, which involved 4,541 estates. Mechanisation degree, expressed in percentage, is the ratio of plantation areas utilising machines, to the entirety of plantation areas (Mohiddin & Pebrian, 2021). On the other hand, mechanisation capacity, also expressed in percentage, is the ratio of machines availability multiplied with their working capacity and frequency of usage, to the total yearly yield from the whole operations (Mohiddin & Pebrian, 2021). Motorised cutters showed 2.36% of mechanisation degree and 18.52% of mechanisation capacity. Besides, the infield collection incorporating both FFB loading and LF collection; by using the motorcycle-trailer, tractor-trailer, tractor-trailer with grabber, three-wheel cart, vacuum (LF collection) and other tools, showed the mechanisation degree of 29.66% and mechanisation capacity of 12.81% (Mohiddin & Pebrian, 2021). This finding shed light on the opportunity and responsibility, to devise mechanised tools related to FFB harvesting activities in providing essential mechanisation needs for the oil palm plantation works. Considering the fact of the various tools' availability and usage, one of the focuses of this systematic review is to review studies on intervention tools, only if they incorporate biomechanics or ergonomics or risk evaluations of the human body. This will ensure the safety and suitability of the inventions to the targeted user, especially in prolonged usage.

The agriculture industry is synonymous with work-related musculoskeletal disorders (WMSDs) issues, showing 60%-80% WMSDs prevalence for farmers in Africa, America, East Asia and South Asia in previous studies, highlighted by Akbar et al. (2023). One of the effects of musculoskeletal disorders (MSDs) was the decrease in productivity of the oil palm plantation (Oktaviannoor et al., 2015). Thus, increasing working hours only resulted in decreasing work efficiency for farmers who suffered from MSDs (Prommawai et al., 2019). From the systematic review by Akbar et al. (2023), the prevalence of WMSDs was very high among farmers in the Southeast Asia Region (80.10%), with prevalence as high as 88.39% in Malaysia, 81.27% in Indonesia and 78.31% in Thailand. The only two included studies from Malaysia were related to oil palm workers (Akbar et al., 2023), hence it could be said that the prevalence of WMSDs for farmers in Malaysia were those of oil palm workers. Agriculture-related study was scarce in Indonesia, despite the importance of the agricultural sector to the country (Widyanti, 2018).

To the best of our knowledge, only two systematic reviews have closely addressed the prevalence of WMSDs in the oil palm industry; Akbar et al. (2023) and Myzabella et al. (2019). Firstly, Myzabella et al. (2019) conducted a systematic

review focusing on occupational health and safety in the oil palm industry, including WMSDs, albeit limited to studies up to 2018. However, they overlooked the technological aspect utilised by researchers to assess biomechanical aspects of oil palm works, for instance, the use of inertial measurement unit (IMU) and electromyography (EMG) as shown in *Figure 1*. Magnetic-inertial measurement units (MIMU) or IMUs have also been used in an industrial context, with 20 studies focusing on risk assessment (using MIMU/IMU) included, and four of them combining the usage of MIMU/IMU with EMG technology, as listed by Digo et al. (2022). The 20 studies were from the year 2014-2022 (Digo et al., 2022). Secondly, Akbar et al. (2023) specifically reviewed the prevalence of WMSDs among farmers of various agricultural backgrounds, also encompassing oil palm workers in some studies, in Southeast Asia, from 2015 to 2022, though excluding certain study types such as experimental, case-control and cohort studies from their analysis. Thus, there is a need for a comprehensive systematic review to identify and summarise current findings and efforts aimed at addressing oil palm work issues, particularly WMSDs, with a focus on enhancing the work environment for oil palm workers. This review encompasses a wide range of sources, including journal and conference articles, and considers various study designs, such as experimental, simulation, cross-sectional, observational and survey studies.

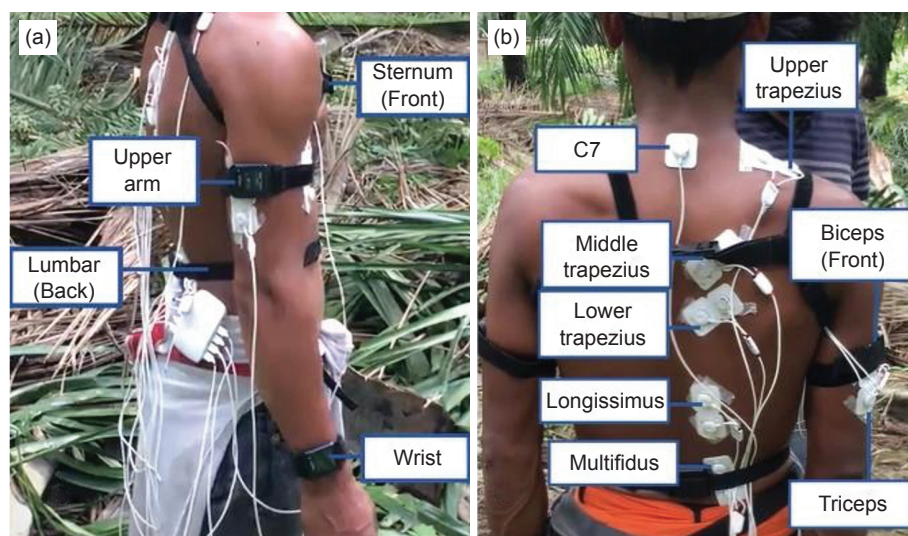
MATERIALS AND METHODS

This systematic review was performed based on Preferred Reporting Items for Systematic

Review and Meta-Analysis (PRISMA) guidelines. Relevant articles were identified by searching four databases, Web of Science, Scopus, PubMed and ScienceDirect. The search was restricted to articles published between 1 January 2013 and 31 December 2023. This time frame was selected because the articles of interest were primarily from the most recent 10 years. This was done to highlight current issues in WMSDs and the methods used to address them, with the anticipation of incorporating more modern tools.

The use of Boolean operators (OR, AND) had to be limited because ScienceDirect database only allowed a maximum of eight Boolean operators. Additionally, truncation of word searches was not available in certain databases. In light of these limitations, preliminary searches were conducted with various combinations of keywords. Ultimately, the final set of keywords was chosen for its effectiveness in retrieving relevant papers from all four databases. These keywords consisted of a single line of common words describing oil palm works, WMSD issues faced by the oil palm workers, and interventions designed to address these issues. The keywords used were (oil palm worker OR oil palm harvester OR oil palm harvesting OR oil palm FFB) AND (work-related musculoskeletal disorders OR WMSDs OR ergonomic OR intervention OR tool).

Additionally, the result from ScienceDirect was further refined to only four publication titles, namely: 1) *Procedia Engineering*, 2) *International Journal of Industrial Ergonomics*, 3) *Results in Engineering* and 4) *Applied Ergonomics*. The subject area was also refined to only Engineering. These were done to limit the search to only relevant studies.



Source: Teo et al. (2021).

Figure 1. (a) IMU Sensor placement, and (b) EMG sensor placement on the harvester's body.

Inclusion and Exclusion Criteria and Evaluation of Articles

Inclusion criteria encompassed quantitative data articles addressing WMSDs, biomechanics related to oil palm activities, or interventions with proper data. This review focused exclusively on oil palm plantation workers. Only articles published between 1 January 2013 to 31 December 2023, were considered. Exclusion criteria were applied to systematic review articles, qualitative data studies, time and motion studies, production studies, research on oil palm mill workers and studies not directly addressing WMSDs or biomechanical evaluation.

The process of article selection and elimination is outlined in *Figure 2*. Critical Appraisal Skills Programme Programme (CASP) (CASP, 2019) checklists were used to assess the quality of selected papers included in this systematic review. All developments including search terms, inclusion and exclusion criteria and quality appraisal of included papers were discussed with M.I.Z.R.

RESULTS AND DISCUSSION

The database search resulted in 245 papers, all of which underwent screening based on title and abstract as shown in *Figure 2*. Among these, 71 articles were selected by excluding 174 articles not related to oil palm or WMSDs. Duplicate articles, which were the same articles but retrieved from different databases, were then removed, resulting in the elimination of 26 papers. The removal process commenced after title and abstract screening, as this systematic review was conducted manually without specific systematic review software. A total of 45 full-text articles were evaluated, and after excluding 18 articles based on the exclusion criteria, 27 articles remained for analysis.

All articles included in this systematic review are listed in *Table 1* and 2. Out of the 27 articles, 22 were from Malaysia, two from Thailand, two from Colombia, and one from Indonesia. As stated in the inclusion criteria, only articles published between 2013 and 2023 were considered. The distribution of articles by publication year was as

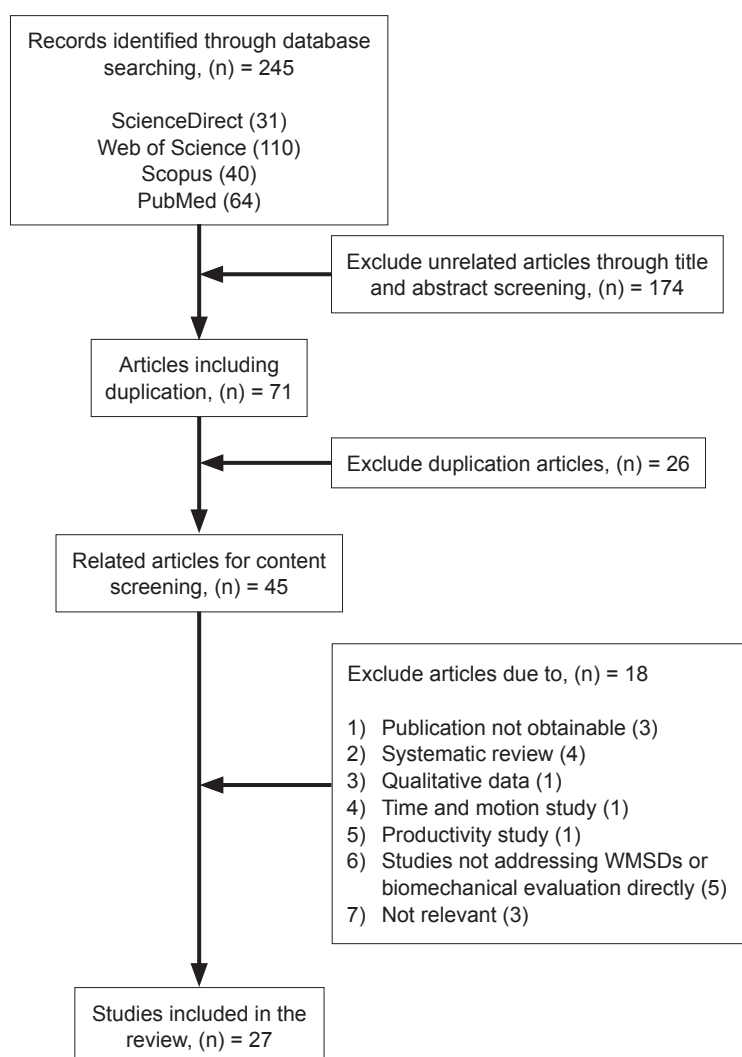


Figure 2. Systematic review study selection flowchart.

follows: Three in 2013, two in 2014, three in 2016, one in 2017, three in 2018, three in 2020, seven in 2021, one in 2022 and four in 2023. No articles from 2015 and 2019 were included in this systematic review.

The critical appraisal of selected articles using the CASP Randomised Controlled Trial (RCT) checklist (18 articles) is presented in *Table 1*. One article scored 7 (maximum score is 11), 12 articles scored between 4 and 6 and five articles scored between 1 and 3. Evaluation using the CASP Cohort checklist (nine articles) is depicted in *Table 2*, where one article scored 11 (maximum score is 12), one scored 8, two scored 7 and five ranged from 4-6. Low CASP scores were primarily attributed to

the strict criteria of the RCT checklist, especially for experimental (nine studies) and simulation (eight studies) studies, wherein none of the studies were randomised. Marks were also deducted as the RCT checklist was intended for intervention studies. Cross-sectional (six studies), observational (two studies), and survey (one study) studies were evaluated using the CASP Cohort checklist, given its similarity to cohort studies, which are observational. It is worth noting that, in contrast to a cross-sectional study where results were obtained instantly during that specific time of study, a cohort study was employed to study certain effects over time.

TABLE 1. ARTICLES RATED USING RCT CASP CHECKLIST

Article No.	Author	Location	Year	Participant(s)	Measurement tool(s)	Main result(s)	CASP score
1	Mohamaddan et al.	Malaysia	2021	30 male FFB harvesters	CATIA V5R20 and AnyBody software	RULA score of 7 for works using pole, chisel and loading spike	4
2	Abdullah et al.	Malaysia	2023	10 male harvesters	S-Type Load cell, force plate, IMU, and EMG	Maximum cutting force of 1,601.23 ± 424.26 and 420.80 ± 96.00 N for sickle and chisel respectively	6
3	Chan et al.	Malaysia	2022	6 male FFB harvesters	IMU, EMG, and OpenSim	The highest average peak joint moment was during back lateral bending to the right (19.02 Nm/kg), back flexion (13.69 Nm/kg) and dominant shoulder extension (5.98 Nm/kg)	4
4	Teo et al.	Malaysia	2021	8 FFB harvesters and 8 LF collectors	IMU, EMG	Peak muscle activities during FFB harvesting and LF collection were all higher than 50%, for all muscles (upper trapezius, middle trapezius, lower trapezius, longissimus, multifidus, biceps and triceps).	4
5	Ahmad et al.	Malaysia	2023	4 workers (only 2 harvesters)	Noise meter and vibration test equipment	Noise level of 60 dB. Magnitude of vibration at the pole and throttle of 1.3 and 0.8 m/s ² respectively	5
6	Jelani et al.	Malaysia	2018	6 workers (only 2 harvesters)	Vibration test equipment	Vibration level of 0.6 and 0.4 m/s ² at throttle and pole respectively	6
7	Tumit et al.	Malaysia	2021	20 male harvesters	Quintic biomechanics system	Greater range of harvesting movements at initial posture in the support shoulder by participants in the age group of 40-49 (90.4°) and 30-39 (86.0°) compared to 20-29 (63.6°)	5
8	Harith et al.	Malaysia	2021	10 non-experienced male participants	EMG and survey form	Mean %MVC showed significant reduction when using exoskeleton: Left and right deltoids for 43.2% and 31.3% respectively and left biceps for 51.8% reduction	6

TABLE 1. ARTICLES RATED USING RCT CASP CHECKLIST (continued)

Article No.	Author	Location	Year	Participant(s)	Measurement tool(s)	Main result(s)	CASP score
9	Ahmad et al.	Malaysia	2020	(unclear number*) oil palm harvester	Load cell	Maximum cutting force of 300.2 N using a magnetic actuator oil palm cutter, in a lab experiment	5
10	Parra-Ruiz et al.	Colombia	2018	4 oil palm harvesters	SolidWorks and Siemens PLM software	OWAS score of 1 using a new simulated tool and a score of 4 using traditional sickle	3
11	Rahman et al.	Malaysia	2016	(unclear number*) oil palm harvester	AnyBody Technology software	The maximum force of 15.12 N to triceps lateral head (LH), 11.30 N to extensor carpi ulnaris, 6.89 N to deltoideus scapular and 4.51 N to trapezius clavicular	1
12	Nawi et al.	Malaysia	2013	1 harvester, 1 loader	Video recorder	Highest REBA score of 13 for FFB harvesting and 10 for FFB loading	3
13	Rahman et al.	Malaysia	2017	(unclear number*) oil palm worker	AnyBody Technology software	Biceps muscle required 3.50×10^2 , 2.91×10^{-1} and 4.02×10^3 N, to move the pole, chisel and loading spike respectively	2
14	Sabri et al.	Indonesia	2020	(unclear number*) operator	Not stated	The maximum human energy needed to operate the intervention tool was 250.2 N.m	1
15	Parra et al.	Colombia	2018	(unclear number*) oil palm harvester	Video recorder, Kinovea, and JACK Siemens software	Maximum back compression of 8,385 and 440 N when using a conventional sickle and new intervention respectively	4
16	Teo et al.	Malaysia	2023	8 male harvesters	IMU and EMG	The first muscle synergy was contributed by the upper trapezius (activated during 20% to 60% harvesting movement), the second synergy was contributed by the longissimus and multifidus (activated during 10%-25% harvesting movement), and the third synergy was contributed by the biceps (activated during 20%-70% harvesting movement),	4
17	Chan et al.	Malaysia	2023	6 male harvesters	IMU and OpenSim software	Reduction in average peak muscle activation and peak muscle force for most muscles when exotendon was applied, by up to 49.22% and 82.00% respectively	5
18	Mohamaddan et al.	Malaysia	2021	30 male harvesters	Digital video camera, survey, CATIA and AnyBody software	The highest leg mean muscle force of 96.83 N at the left gastrocnemius medialis (loading spike) 82.19 N at the right gastrocnemius medialis (chisel) and 79.06 N at the left gastrocnemius medialis (pole)	4

Note: *Unclear number of participants, due to the author not stating it or due to participant was not necessary for a simulation-type or lab experimental study.

TABLE 2. ARTICLES RATED USING COHORT CASP CHECKLIST

Article No.	Author	Location	Year	Participant(s)	Measurement tool(s)	Main result(s)	CASP score
19	Bhuanantanondh et al.	Thailand	2021	334 oil palm harvesting workers	Questionnaire (Thai-JCQ-54 and modified standardized Nordic questionnaire)	The highest 12-month MSDs prevalence was lower back (59.0%), shoulder (37.1%) and neck (27.2%)	11
20	Deros et al.	Malaysia	2016	70 male oil palm workers	Ergo Fellow 2.0 software and Modified Nordic questionnaire	Upper and lower back pain was experienced by 87.1% and 94.3% of workers respectively	6
21	Ng et al.	Malaysia	2013b	143 harvesters	Questionnaire (Nordic musculoskeletal questionnaire)	The highest MSD prevalence for the past 12 months was for the lower back (58.0%), knee (45.5%) and shoulder (32.9%)	7
22	Yusoff et al.	Malaysia	2014	273 male harvesters	SolidWorks and CATIA software and questionnaire	RULA score of 7 for 51.6% of harvesters, a score of 5 or 6 for 45.1% of harvesters and a score of 3 or 4 for 3.3% of harvesters	5
23	Mongkonkansai et al.	Thailand	2020	50 workers	Questionnaire and REBA	The highest MSD prevalence after 1 year was lower right arm (76%), upper right arm (76%), right shoulder (74%), lower back (74%) and right hand (70%)	7
24	Mokhtar et al.	Malaysia	2013	6 FFB harvesters and 1 truck driver	Questionnaire and video recorder	RULA final score of 7 for 83% (5 out of 6) harvesters	5
25	Deros et al.	Malaysia	2014	3 FFB harvesters	Video camera and Digital Human Modelling Software (DHMS)	Final RULA score of 7 for all harvesters	4
26	Rozadi and Fatin	Malaysia	2021	40 oil palm workers	Questionnaire	Common pain faced by workers were shoulder (36 workers), neck (33 workers) and lower back (34 workers)	6
27	Nawi et al.	Malaysia	2016	88 male oil palm workers	Nordic musculoskeletal questionnaire and video camera	Body parts with the highest pain complaints were the lower back (99%), upper back (85%) and shoulder (77%)	8

WMSDs Prevalence

There were six articles included in this systematic review focusing on WMSDs prevalence among oil palm workers. The WMSDs prevalence was quantified using questionnaires, answered by the respondents. A study by Bhuanantanondh et al. (2021) found that the body region with the highest 12 months MSDs prevalence was the lower back (59.0%), shoulder (37.1%) and neck (27.2%). This finding was comparable with a study by Ng et al. (2013b) where the lower back (58.0%), shoulder (32.9%) and neck (32.2%) were three of the highest body regions affected over the past 12 months. This was supported by findings by Rozadi and Fatin (2021) and Nawi et al. (2016), with both studies showing a high prevalence of pain in the lower back, shoulder and neck (74.0% and higher). A study by Deros et al. (2016) only reported the prevalence of lower and upper back pain, at 87.1% and 94.3% respectively. The lower back pain prevalence of 87.1% was comparable to the finding of 85.0% by Rozadi and Fatin (2021). The reported 12 months MSDs prevalence for any body parts was very high, which ranged from 78.0%-93.0% (Bhuanantanondh et al., 2021; Mongkonkansai et al., 2020; Ng et al., 2013b).

Postural Risk Assessment

There were three types of postural risk assessment tools employed by 10 studies included in this systematic review, which were Rapid Upper Limb Assessment (RULA) (5 studies), Rapid Entire Body Assessment (REBA) (4 studies) and Ovako Working Posture Analysis System (OWAS) (1 study) as listed in Table 3.

RULA. Two studies (Deros et al., 2014; Mohamaddan et al., 2021b) used CATIA P3 and CATIA V5R20 software respectively for the RULA analysis. For

loading spike, a RULA score of 7 was obtained in the study by Mohamaddan et al. (2021b). Both Deros et al. (2014) and Mohamaddan et al. (2021a) obtained RULA scores of 7 for harvesting activities using a pole. Harvesting by using a chisel, on the other hand, also resulted in a RULA score of 7 (Mohamaddan et al., 2021b) and was similarly obtained for 51.6% of the respondents in a study by Yusoff et al. (2014). The other 45.1% obtained scores of 5 or 6 and 3.3% obtained scores of 3 or 4. Also, 5 out of 6 (83.0%) subjects obtained a RULA score of 7 when using a pole or a chisel (Mokhtar et al., 2013). As most of the findings showed a maximum final RULA score of 7, several investigations and changes were required immediately for all the tasks involving poles, chisels and loading spikes (Deros et al., 2014; Mohamaddan et al., 2021b; Yusoff et al., 2014). Finally, an intervention tool simulated design, which was a design of a chisel with added front and back handles for hand placement, managed to reduce the final RULA score to 3 (Yusoff et al., 2014).

In a study by Tumit et al. (2021), only Section A of RULA (arm and wrist) was used to analyse the risks associated with age and initial shoulder placement. Findings showed that during the tool placement phase in targeting a specific FFB, a higher risk of MSDs at the support shoulder was experienced by 40-49 years old harvesters, compared to those of 20-29 years old.

REBA. An automated REBA tool from Xsens was used by Abdullah et al. (2023), while the Ergo Fellow 2.0 software was used by Deros et al. (2016) in evaluating postures using the REBA risk assessment tool.

The average peak REBA scores obtained for the fronds pruning tasks using sickle and chisel were 10.30 ± 1.16 and 11.00 ± 1.05 respectively (Abdullah et al., 2023), whereas less score was obtained by

TABLE 3. RISK ASSESSMENT TOOL, METHOD AND EQUIPMENT/SOFTWARE USED

Article No.	Author (yr)	Risk assessment tool	Method	Equipment/software
7	Tumit et al. (2021)	RULA (Section A)	Manual	Quintic Biomechanics system
18	Mohamaddan et al. (2021b)	RULA	Simulation	CATIA V5R20
22	Yusoff et al. (2014)	RULA	Simulation	CATIA
24	Mokhtar et al. (2013)	RULA	Manual	Video recording
25	Deros et al. (2014)	RULA	Simulation	CATIA P3
2	Abdullah et al. (2023)	REBA	Simulation	Xsens automated REBA tool
12	Nawi et al. (2013)	REBA	Manual	Video recording
20	Deros et al. (2016)	REBA	Simulation	Ergo Fellow 2.0 software
23	Mongkonkansai et al. (2020)	REBA	Not stated / manual	Not stated / no
10	Parra-Ruiz et al. (2018)	OWAS	Simulation	Jack Siemens

Deros et al. (2016) when harvesting FFB (5) and highest score was obtained by Nawi et al. (2013) when harvesting FFB using sickle and chisel (both 13, respectively). The FFB loading task, on the other hand, showed a very high score of 12 (Deros et al., 2016) and a high score of 10 (Nawi et al., 2013) and was also labelled as high risk (8-10 score) by Mongkonkansai et al. (2020). Nevertheless, investigation and implementation of change should be done quickly for frond pruning, FFB harvesting and FFB loading tasks.

OWAS. Only one study implemented OWAS, which was a study by Parra-Ruiz et al. (2018). The maximum simulated OWAS score (4) was obtained when a traditional sickle was used, whereas, with the new simulated intervention cutting tool, a minimum OWAS score (1) could be obtained. Hence, immediate corrective actions were required for the traditional sickle.

Muscle Force and Joint Moment

AnyBody modelling software was used by four studies (Mohamaddan et al., 2021a, 2021b; Rahman et al., 2016, 2017) to obtain muscle force, while OpenSim was used by one study (Chan et al., 2022).

The highest simulated mean muscle force when the loading spike was used, in the upper part of the human body, was at the left triceps (16.36 N), left biceps (10.55 N), and right biceps (Mohamaddan et al., 2021b). For the chisel, the highest simulated mean muscle forces were at the left triceps (14.24 N), right triceps (14.23 N) and both right and left biceps (12.00 N). For the sickle, the highest simulated mean muscle forces were at the left triceps (15.06 N), both right and left biceps (13.00 N), and right triceps (9.25 N). Compared to the sickle from the simulated result by Rahman et al. (2016), the maximum reaction force of the left triceps lateral head, (15.12 N) and right triceps lateral head (9.93 N) were both close in magnitude. The highest reaction force was at flexor carpi ulnaris (15.61 N) (Rahman et al., 2016). For the lower part of the human body, the highest mean muscle forces were at the left gastrocnemius medialis (96.83 N) when using a loading spike, right gastrocnemius medialis (82.19 N) when using a chisel, and at the left gastrocnemius medialis (79.06 N) when using a pole (Mohamaddan et al., 2021a).

To move the pole vertically, the chisel horizontally and the loading spike vertically, the required biceps muscle forces were 350.000, 0.291, and 4,020.000 N respectively (Rahman et al., 2017). Finally, a simulation by Chan et al. (2022) showed the joint moments that would potentially lead to MSDs were back flexion and back rotation, with the average peak joint moments of 13.69 and 5.23 Nm/kg.

Muscle Activation

All muscles (biceps brachii, middle deltoid, upper and middle trapezius) were reported to be highly activated [$>50\%$ maximal voluntary contraction (MVC)] in tasks related to oil palm fronds pruning using sickle and chisel except for erector spinae in certain tasks (Abdullah et al., 2023). This was similar to a study by Chan et al. (2022) showing an average normalised peak muscle activation of more than 50% for every muscle (longissimus, multifidus, biceps, triceps, rectus abdominis, iliocostalis, external oblique, internal oblique and latissimus dorsi) for optimal forces of reserve actuator of 30 and 50 N. Also, similar findings by Teo et al. (2021), peak muscle activities during FFB harvesting and LF collection, for all muscles (upper trapezius, middle trapezius, lower trapezius, longissimus, multifidus, biceps and triceps) were all higher than 50%.

Muscle synergies in oil palm harvesting activity were studied by Teo et al. (2023), showing three muscle synergies, the first one being synergy contributed by upper trapezius, the second synergy contributed by longissimus and multifidus (both are lower back muscles) and the third and last synergy was contributed by biceps.

Next, to test for the efficacy of an intervention tool prototype, an upper limb exoskeleton tailored for oil palm FFB harvesting activity in a preliminary study by Harith et al. (2021), a comparison of muscle activity was done in a simulated harvesting environment to mimic the real-life FFB harvesting experience. By wearing the exoskeleton, muscle activity for all simulated tasks of pole raising, tugging, and carrying decreased. A significant reduction was observed for mean %MVC in left and right deltoids and left biceps (43.2%, 31.3% and 51.8% respectively) during pole tugging. Besides, a significant reduction of mean %MVC was also found in the left and right biceps and right deltoids (25.8%, 35.6% and 47.0% respectively).

Besides, a simulated intervention, which was a passive exotendon assistive device to support rectus abdominis, longissimus and iliocostalis was studied in a simulation by Chan et al. (2023), showing reduction of the most value of average peak muscle activation, average peak muscle force and average normalised peak joint moment. However, the relationship between the longissimus and rectus abdominis should be addressed cautiously, as some workload from the longissimus would be transferred to the rectus abdominis (Chan et al. 2023).

Spinal Loadings

The limits proposed by the National Institute of Occupational Safety and Health (NIOSH) for

compressive and shear forces at L5-S1 disc were 3,400 and 1,000 N respectively. The compressive and shear forces to L5-S1 disc during harvesting activities were estimated by Abdullah et al. (2023) and Parra et al. (2018) using 3D Static Strength Prediction Programme (3DSSPP) and JACK Siemens software respectively. Estimated $2,981.40 \pm 761.20$ N of compressive force and 505.30 ± 90.22 N of shear force were reported for pruning activity using a chisel while $2,493.20 \pm 1,349.48$ N of compressive force and $1,446.10 \pm 411.00$ N of shear force were reported for pruning activity using sickle (Abdullah et al., 2023). The simulated result by Parra et al. (2018) for compressive force was a maximum of 8,385 N when using sickle, which far exceeded the 3,400 N safety limit. The proposed simulated intervention Cutting System however managed to reduce the compressive force to 440 N, which was far below the safety limit.

Noise and Vibration

Noise and vibration of mechanised harvesting tools should be quantified and made sure to fall below the safe limit to prevent oil palm workers from developing WMSDs.

Studies on the design of mechanised intervention tools such as experiments done on the Cantas Evo (Jelani et al., 2018), Cantas Elektro (Ahmad et al., 2023) and magnetic oil palm cutter prototype (Ahmad et al., 2020) incorporated important measurements such as hand-arm vibration (HAV). Cantas Evo was a superior version of Cantas (the earlier version of the mechanised cutting tool) with a new design of cutting head, pole, pole gripper and shaft guider. Cantas Elektro, on the other hand, is a battery-powered version of Cantas. The magnetic oil palm cutter was a prototype of a cutting tool using a magnetic actuator and incorporating a counter shear attachment to the design. All three interventions had a vibration level below the threshold level of 2.50 m/s^2 . The vibration at the pole and throttle for Cantas Evo was 0.60 and 0.40 m/s^2 (Jelani et al., 2018), Cantas Elektro was 1.30 and 0.80 m/s^2 (Ahmad et al., 2023), while the magnetic oil palm cutter was at 1.20 m/s^2 (Ahmad et al., 2020). Also, the noise level of Cantas Elektro was measured to be 60 dB, lower than the maximum noise limit of 85 dB recommended by NIOSH (Ahmad et al., 2023).

Cutting Force and Human Energy

In a lab experiment done by Ahmad et al. (2020), the maximum cutting force required by a magnetic actuator cutting tool to cut frond samples was 300.2 N. On the other hand, the maximum cutting force of 1601.23 ± 424.26 and 420.80 ± 96.00 N were

obtained by using a sickle and chisel respectively, to cut oil palm fronds in the field (Abdullah et al., 2023).

The calculated human energy to operate a mechanised intervention tool in a study by Sabri et al. (2020) was 250.2 N.m, which was categorised as a very light job. Besides, the proper distance between the operator and the oil palm tree was suggested, whereby for a 5 m tall tree, a 4 m distance between the operator and the tree was considered ideal to moderate the force, tension, and deflection exerted on the tools.

Intervention Tools

In this systematic review, there were nine studies related to interventions in oil palm activities, with a total of eight different interventions (two articles referred to the same intervention (Parra et al., 2018; Parra-Ruiz et al., 2018). One study was on the simulated design of a hand handle for the chisel (Yusoff et al., 2014), one preliminary study on an exoskeleton prototype (Harith et al., 2021), one study on a simulated new Cutting System (Parra et al., 2018; Parra-Ruiz et al. 2018), one study on simulated passive exotendon assistive device (Chan et al., 2023) and four studies on mechanised tools (Ahmad et al., 2020, 2023; Jelani et al., 2018; Sabri et al., 2020).

Extensive Review

This study aimed to conduct a thorough review of research on the prevalence of WMSDs among workers in oil palm plantations and to assess interventions designed to alleviate these issues through biomechanical evaluation or the design of intervention tools. By synthesising current study efforts, tools, and interventions, this study aimed to provide valuable insights into addressing WMSDs in the occupational setting.

The findings of the six studies highlighted the high prevalence of WMSDs among oil palm workers, emphasising the substantial risk posed by these tasks (Bhuanantanondh et al., 2021; Deros et al., 2016; Mongkonkansai et al., 2020; Nawi et al., 2016; Ng et al., 2013b; Rozadi & Fatin, 2021). The lower back, shoulder and neck were identified as the most commonly affected body parts. However, the specific body regions experiencing pain and WMSDs risk varied among workers, depending on their tasks, such as FFB harvesting, collecting, loading, LF collecting, or other plantation activities. For example, FFB and LF collectors faced a 3.98 times higher risk compared to FFB cutters in developing lower back MSDs (Bhuanantanondh et al., 2021). This was due to the need for FFB collectors to lift heavy FFBs, weighing up to 50 kg, in a forward bending and twisted posture, while

LF collectors had to adopt a stooping posture with arms to gather the scattered LF (Ng et al., 2013a).

Regarding shoulder MSDs, FFB cutters, and collectors faced a 2.51 times higher risk compared to LF collectors (Bhuanantanondh et al., 2021). This is because FFB cutters are required to lift their arm above the shoulder level to harvest from tall trees using a sickle, and employing a forceful pulling motion to cut the FFB. In contrast, the FFB collectors lift the FFB using a loading spike and then transfer the load onto their shoulders before transporting it to a wheelbarrow. Furthermore, the risk of developing neck MSDs was 4.82 times higher for FFB cutters compared to LF collectors (Bhuanantanondh et al., 2021). This discrepancy arises from the fact that FFB cutters must frequently tilt their heads upwards during the search for ripe FFB and throughout the cutting activity (Ng et al., 2013b).

A cross-sectional study by Ng et al. (2015) involving 446 male respondents showed that the FFB cutters and collectors had to work daily for about 7 hr and 50 min including resting time, with 75% of them normally working overtime. Hence, it was not a surprise that during the 12 months time the prevalence of WMSDs was high for oil palm harvesters (86%) (Ng et al., 2015). The harvesters in the study by Mokhtar et al. (2013) on the other hand, only worked for 6 hr, with a break-time of 1 hr.

By identifying the underlying reasons for absence from work, such as physical and psychological job demands and financial conditions (for instance sick pay availableness), effective policies can further be introduced (Coggon et al., 2013). The prevalence rate ratio (PRR) for the risk factors associated with extended sickness leave by office workers, nurses and other workers was 1.43 [95% confidence interval (CI) 1.24 to 1.65] for pressure at work (Coggon et al., 2013).

In the broader sense of the agriculture sector, WMSDs were experienced by workers in this sector due to the similar working conditions such as working manually, repetitive movements, the need for a certain level of force to execute the task, design of the agriculture tools and vibration produced from the tools (Kaewdok et al., 2021). In a study by Nankongnab et al. (2019) involving vegetable, rice, sugarcane and fruit farmers, the 3-month time MSDs showed more pain experienced by organic farmers than conventional farmers. The reason was speculated due to the organic farmers had to spend more time taking care of the plants, such as manual weeding (Nankongnab et al., 2019). Besides, rice farmers also experienced MSDs to the shoulder (76.1%) and lower back (74.9%) (Widyanti, 2018). The common practices similar to oil palm activities were the use of manual tools in manual plowing tasks (hoe for plowing and sickle for

FFB harvesting), and flexed trunk posture during grass cutting, rice planting and harvesting for rice farming; and LF collection for oil palm harvesting. Various assessment tools were also implemented in this study such as the use of ergonomic checkpoints, Nordic body map questionnaire, REBA, RULA and 3DSSPP (Widyanti, 2018). On the other hand, in a study assessing WMSDs among mango-harvesting farmers in Thailand, besides similar usage of assessment tools such as REBA and RULA, tools such as Rating of Perceived Exertion (RPE) with Borg CR-10 and Fatigue Effective Index (FEI) with Power Superposition Theorem were also used. Shoulder, upper arm and lower back were the three body regions most reported for WMSDs, with percentages of 100.00%, 100.00% and 71.43% respectively (total of 14 mango-harvesting farmers). Again, manual tools were used to harvest the mango, similar condition to FFB harvesting. Next, a study on sugarcane farmers in Thailand showed a 74.29% MSDs prevalence, with the highest prevalence being that of the shoulder (53.50%) and lower back MSDs (53.12%) (Prommawai et al., 2019). Finally, a study by Saksornngmuang et al. (2020) on rubber farmers in Thailand showed a 12-month MSD prevalence of 87.70%, also with the highest prevalence at the lower back (36.80%) and shoulder (28.90%). The rubber farmers also had to use manual tools, specifically a tapping knife, to harvest latex from the rubber tree.

RULA was used for the evaluation of risk to upper body parts (McAtamney & Corlett, 1993), REBA for whole body evaluation (Hignett & McAtamney, 2000), while OWAS (Karhu et al., 1977) focused on back and upper limbs and lower limbs posture in terms of posture loadings and external load (Kee, 2022). Also, OWAS does not categorise the body into right and left parts in the assessment, as opposed to REBA and RULA which assess one side at one particular moment (Kee, 2022). Nelfiyanti et al. (2022) used all three tools namely RULA, REBA and OWAS in assessing risk to chassis assembly workers and obtained similar risk level results between them. In a comparison study by Kee (2021), OWAS produced significantly lower results than RULA statistically ($p < 0.01$), from the Wilcoxon signed-rank test. Similarly, OWAS underestimated the result compared to REBA (Kee, 2021). Finally, Kee (2021, 2022) concluded that among the three tools, RULA might be the best to examine postural loading and MSDs due to work. In terms of oil palm plantation work studies, most researchers used RULA and REBA. Thus, both results could be used and compared for each specific task, as it was expected for RULA and REBA to have similar results, due to the rate of agreement between both (Kee, 2022).

High muscle activations have been observed across various muscle groups during frond pruning (Abdullah et al., 2023), FFB harvesting (Chan

et al., 2022) and LF collection (Teo et al., 2021), indicating increased energy requirements to meet the demanding physical demands tasks, ultimately elevating the risk of WMSDs (Kodom-Wiredu, 2019). Simulated human musculoskeletal model analysis (HMMA) assists in identifying muscles exerting higher forces during oil palm activities, enabling targeted interventions to alleviate the muscle load. Prolonged and repetitive tasks involving more than 20 motion patterns per minute with tools like poles, chisels and loading spikes can induce fatigue, underscoring the importance of adequate rest intervals between tasks for energy recovery (Mohamaddan et al., 2021b).

Both experienced and inexperienced harvesters face risks of WMSDs due to their harvesting techniques. Inexperienced harvesters often lack the necessary technique and strength required for oil palm activities, while experienced harvesters may not consistently apply the correct method of handling the cutting tools and executing harvesting motions (Tumit et al., 2021). A study by Deros et al. (2016), highlighted that despite 81.0% of respondents being aware of the proper techniques for handling and lifting, only 17.1% practised them. This discrepancy was attributed to the perceived inefficiency and discomfort associated with adhering to correct techniques, leading to prolonged task completion times. In terms of age, no correlation was found between the age of palm farmers with MSDs prevalence in Pt. X oil palm plantation in South Kalimantan (Oktavianoor et al., 2015). However, generally, MSDs prevalence increases among older participants (Palmer & Goodson, 2015).

The analysis of spinal loadings revealed that while the mean compressive and shear forces for the chisel and the compressive force for the sickle remained within safety limits, the peak compressive force for both tools exceeded the safety limit (Abdullah et al., 2023). Moreover, the mean shear force for the sickle surpassed the safety limit. These findings raise concerns regarding the potential for lower back pain, particularly among harvesters using the sickle, given the elevated mean shear force at the L5-S1 disc, exacerbated by the prevalent trunk twisting posture adopted during harvesting activities.

The use of traditional tools such as sickles, chisels and loading spikes poses a significant risk of WMSDs, necessitating the need for interventions. For instance, the redesigned chisel by Yusoff et al. (2014) reduced RULA scores by minimising bending posture, while the Cutting System by Parra-Ruiz et al. (2018) alleviated spinal loadings and lowering OWAS scores.

Among the four studies on mechanised tools, three of them (Ahmad et al., 2020, 2023; Jelani et al., 2018) assessed the vibration level of the cutting tools

namely Cantas Evo, Cantas Elektro and a prototype of magnetic oil palm cutter, with all of them were under the safety limit of 2.5 m/s^2 , meaning they were safe to be used in a working period of 8 hr. As the operators would use the mechanised tools throughout the day, the vibration level should be kept to a minimum, for a more comfortable usage and to preserve their endurance (Ahmad et al., 2023). Besides, as the cutting tool would be the load that the FFB harvesters had to carry throughout the day, the optimum weight should also be considered. For comparison, a 6.00 m traditional pole only weighed 4.0 kg, whereas a 6.21 m Cantas Evo weighed 9.0 kg, a 2.50 m Cantas Elektro weighed 7.0 kg and a 5.00 m magnetic oil palm cutter weighed 6.3 kg (Ahmad et al., 2020, 2023; Jelani et al., 2018). From a time-motion study by Saibani et al. (2015), the time taken to adjust the cutting pole, cut the frond and finally cut the FFB was 23.36, 51.30 and 60.26 s. Next, the time taken to load the FFB into a wheelbarrow was 34.25 s, while LF collection took 77.49 s (Saibani et al., 2015). Hence, any interventions aimed at increasing productivity should always take a shorter time. Next, to attract stakeholders, employers and employees to adopt the new mechanised tools compared to traditional tools, the cost-effectiveness analysis should be included in the study, for them to make calculated decisions, as done by Ahmad et al. (2023) and Jelani et al. (2018).

In terms of mechanisation, the early 1960s marked its earliest adoption into oil palm plantations in Malaysia (Azwan et al., 2016), with Malaysia leading the research and development (R&D) in the oil palm industry (as a whole), for over 100 years (Parveez et al., 2024). However, up to now, FFB harvesting, FFB loading and LF collection were still done manually using traditional tools. Only certain tasks such as transportation of FFB from plantations to oil palm mills for processing, were done using a large capacity tractor, due to the large amount of FFB needed to be moved out, whereby fertilising and herbicide/insecticide spraying were done with a smaller tractor (Azwan et al., 2016). The rejection of interventions by the workers was due to extra time needed to familiarise themselves with the new tools, fear due to injury experience, insufficient information and training on the tools and health risks imposed by the machines, while some of the concerns by the plantation managers were on the decreased productivity and significant upkeep expenses (Nawi et al., 2015).

The WMSDs prevalence in oil palm plantation tasks such as FFB harvesting, FFB loading and LF collection were very high, with lower back, shoulder and neck being the three most affected body parts. This condition would persist, as long as the use of manual tools such as sickle, chisel and sharp metal skewers are still prevalent. In terms of postural risk assessment, the use of

IMU would provide the best representation and a more accurate workers' posture, compared to manual determination from video recording by ergonomists. The postural angle obtained from IMU can then be used to evaluate risk, whether using REBA for whole body assessment or RULA, if the area of interest is only the upper part of the human body. EMG would provide data on muscle activity, hence the effort needed for a worker to complete a certain task can be determined. Hence, the types of interventions should be focused, when the intervention is intended to change the posture adopted by the workers, IMU should be used. If the new intervention was targeting to reduce muscle effort in accomplishing certain movements, EMG should be used. AnyBody and OpenSim software are two useful tools to estimate specific muscle forces, where the data can be used to design the exoskeleton to assist specific muscle locations. The intervention had to be devised specifically with regards to the nature of an oil palm tree and the location of FFB such as the tree height and canopy arrangement of fronds, that must be cleared to get access to the FFB which is hidden by the oil palm fronds for harvesting purpose (Ng et al., 2013a). Scattered loose fruits, that originated from the outer parts of FFBs had to be collected too, due to their higher level of oil content compared to those in the inner part (Khalid et al., 2021). Besides, the concern over anthropometric data, where the design of the tools was only ergonomically suited for users from the origin country and also not suited for women, was raised by Kaewdok et al. (2021). Hence, an intervention design should follow the anthropometric data of targeted workers, using data for male populations in works mostly done by males, and both male and female data for works done by both genders. Height and grip measurements should also be considered. Finally, a mechanised cutting tool should be robust enough, maintainable and follow NIOSH specifications, so as not to induce other harm to the users, such as risk of excessive noise and vibration. Cost analysis and return on investment should be calculated, for the new tool or intervention to become more attractive and plausible for adoption.

Limitations of this systematic review were the inclusion and exclusion criteria mainly focused on only systematic review and did not take into consideration the possibility of conducting meta-analysis. Hence, new inclusion and exclusion criteria had to be determined for meta-analysis. Besides, this systematic review only covered articles in a period of 10 years, from 2013-2023. Hence, earlier WMSDs prevalence and interventions made were not discussed. However, this systematic review managed to serve its purpose in highlighting the WMSDs prevalence

in oil palm workers, methods and tools used in biomechanical evaluations and interventions proposed to reduce the risk of WMSDs.

CONCLUSION

A high prevalence of WMSDs was observed in oil palm plantation works. However, the WMSDs prevalence could only be quantified for Malaysia and Thailand, as prevalence studies were only conducted in these countries. Modern tools such as SolidWorks, CATIA, AnyBody and OpenSim software were employed in designing, measuring postural risk assessment, and building and interacting with human musculoskeletal models, respectively. IMU and EMG were extensively used in biomechanical evaluation studies to examine postural risk and quantify muscle activations. Some of the less common studies focused on muscle synergies and exotendon. Many interventions were designed, with one of the first of its kind in the scene of oil palm works being the exoskeleton prototype. The research gap, which would be very beneficial once filled in is the lack of comparison between an intervention tool and a traditional manual tool in every aspect such as in terms of postural risk assessments, muscle activations, muscle forces needed, productivity, training needed and cost. Hence, the biggest concern would be to design a new intervention that would be attractive enough to be adopted, with its main purpose still intact, to reduce WMSDs risk in FFB harvesting activities.

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GREENING THE PALM OIL INDUSTRY: PROSPECTS AND BARRIERS OF SUPERCRITICAL CO₂ EXTRACTION

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ABSTRACT

The palm oil industry, a cornerstone of global agribusiness, has faced mounting environmental and social scrutiny due to its significant contributions to deforestation, biodiversity loss and greenhouse gas (GHG) emissions. In response to these challenges, this review explores the transformative potential of supercritical carbon dioxide (SC-CO₂) extraction as a sustainable alternative for palm oil production. This review provides an in-depth examination of SC-CO₂ extraction principles, processes and its inherent advantages, including minimal environmental impact, enhanced oil quality, and improved yields. While SC-CO₂ extraction holds promise as an eco-friendly solution, it has its challenges. Technical complexities, energy requirements, and economic considerations are among the hurdles that must be addressed to facilitate its widespread adoption. Furthermore, this review offers insights into real-world case studies and scientific research, shedding light on the practical implications of SC-CO₂ extraction in the palm oil industry. It delves into the regulatory and policy frameworks shaping sustainable palm oil production, emphasising SC-CO₂ extraction's role in achieving compliance with stringent sustainability standards.

Keywords: hurdles, palm oil, prospects, supercritical carbon dioxide, sustainability.

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INTRODUCTION

The palm oil industry has long played a pivotal role in the global agribusiness landscape, supplying a versatile and ubiquitous commodity that finds its way into an array of consumer products, from food items to cosmetics, biofuels and industrial applications. Its economic significance cannot be overstated, providing livelihoods for millions, particularly in tropical regions where oil palm cultivation thrives. However, alongside its economic prosperity, the palm oil industry has garnered increasing attention.

The production of palm oil has been inextricably linked to a litany of environmental and social challenges, sparking international concern and calls for reform. The conventional extraction method for palm oil, though widely practised in the industry, presents significant drawbacks that warrant attention. Firstly, the process typically involves the use of large quantities of water. Therefore, it will produce the palm oil mill effluent (POME). POME

pose environmental risks due to their potential for soil and water contamination during disposal. Moreover, their volatile nature contributes to air pollution, impacting both human health and ecosystem balance.

Furthermore, the high energy consumption associated with conventional extraction methods exacerbates environmental concerns. The need for heating and mechanical processes consumes substantial amounts of energy, often derived from non-renewable sources, leading to increased greenhouse gas (GHG) emissions and further exacerbating climate change (Siikamäki et al., 2012, Singh & Singh, 2017).

In this context, one potential solution that has gained momentum is the application of supercritical carbon dioxide (SC-CO₂) extraction for palm oil production. SC-CO₂ extraction represents a departure from traditional palm oil extraction methods, such as solvent-based and mechanical pressing, by offering a more environmentally friendly and efficient approach. The allure of SC-CO₂ extraction lies in its ability to reduce the industry's environmental footprint while enhancing oil quality and yield (Pensupa et al., 2018). This technique utilises carbon dioxide in a supercritical state where it exhibits both gas-like and liquid-like properties to extract oil from palm fruit efficiently and with fewer chemical solvents, a feature that aligns well with sustainability goals (New et al., 2022).

This review embarks on a comprehensive exploration of the prospects and hurdles associated with SC-CO₂ extraction in the context of greening the palm oil industry. It aims to provide a comprehensive overview of this innovative technology, highlighting its potential to revolutionise palm oil production by addressing key sustainability challenges. Through a critical examination of the principles, processes and advantages of SC-CO₂ extraction, this review elucidates how this method can contribute to mitigating deforestation, conserving biodiversity and reducing carbon emissions.

However, despite its promise, SC-CO₂ extraction has its challenges and limitations. This review will delve into the technical complexities, energy requirements, and economic considerations that present hurdles to its widespread adoption. This review also analyses real-world case studies and scientific research to gain insights into the practical implications of SC-CO₂ extraction within the palm oil industry. Moreover, this review explores the regulatory and policy frameworks that influence sustainable palm oil production, emphasising the role of SC-CO₂ extraction in aligning with and contributing to compliance with stringent sustainability standards and certifications.

As the palm oil industry stands at a crossroads, facing mounting pressure to transition toward more sustainable practices, this review aims to shed light on the potential of SC-CO₂ extraction as a transformative force. This review recognises the urgent need for innovative approaches that can address the industry's environmental and social challenges while acknowledging the complexities and considerations that must be navigated on the path to achieving a greener palm oil sector. In summary, this review sets the stage for a comprehensive exploration of SC-CO₂ extraction as a sustainable alternative in the palm oil industry. It underscores the urgency of adopting more environmentally friendly practices and the potential of SC-CO₂ extraction to play a pivotal role in reshaping the industry's future.

SC-CO₂ EXTRACTION: PRINCIPLES AND PROCESS

SC-CO₂ as a Green Extraction Method

Extraction, in the context of chemical or physical processes, involves the separation of various constituents from natural substances. This essential process plays a crucial role in a wide range of industries and applications. However, in recent times, there has been a growing global emphasis on environmental conservation and sustainable practices. This has led to the adoption of "green extraction" methodologies (Abou Elmaaty et al., 2022; Rizkiyah et al., 2023a; Uwineza & Waśkiewicz, 2020).

Green extraction methods, such as SC-CO₂ extraction, are characterised by their environmentally conscious approach. They are designed to minimise the ecological footprint of extraction processes while ensuring the production of high-quality extracts. These methods align with sustainable practices by consuming less energy, allowing for the use of alternative solvents, and making use of renewable resources. SC-CO₂ extraction, in particular, has emerged as a leading example of green extraction. It harnesses the unique properties of supercritical carbon dioxide, which is a phase of carbon dioxide that exhibits both gas-like and liquid-like characteristics under specific conditions of temperature and pressure.

SC-CO₂ extraction of palm oil can utilise various potential sources of carbon dioxide (CO₂). Firstly, industrial processes such as fermentation, combustion, and chemical manufacturing produce CO₂ as a byproduct. Capturing and purifying these emissions can provide a sustainable source for SC-CO₂ extraction. Additionally, biogas and biomass facilities generate CO₂ during the production of biogas from organic waste. This CO₂ can be

captured and repurposed for SC-CO₂ extraction, contributing to a circular economy by utilising waste streams. Furthermore, advancements in carbon capture and utilisation (CCU) technologies enable the integration of CO₂ emissions captured from power plants and industrial facilities into SC-CO₂ extraction processes.

This not only reduces GHG emissions but also provides a source of CO₂ for extraction. Natural underground reservoirs, such as geological formations, also contain CO₂ that can be accessed through carbon capture and storage (CCS) technologies or naturally occurring geological formations suitable for CO₂ extraction. Moreover, CO₂ captured from renewable energy facilities powered by sources like solar or wind energy can be utilised for SC-CO₂ extraction, enhancing the sustainability of the process. By leveraging these diverse sources of CO₂, the palm oil industry can adopt environmentally friendly practices for SC-CO₂ extraction while minimising its carbon footprint and promoting sustainability. Several distinct advantages of this method is elaborated below.

Reduced environmental impact. SC-CO₂ extraction eliminates the release of potentially hazardous substances into the environment (Arsad et al., 2023).

Energy efficiency. It is energy-efficient compared to some conventional extraction techniques, contributing to reduced energy consumption and lower GHG emissions (Chemat et al., 2012).

Alternative solvents. SC-CO₂ can serve as a safe and sustainable alternative to conventional organic solvents, making it suitable for applications where chemical residues must be minimised (Putra et al., 2023a).

High-quality extracts. SC-CO₂ is highly selective, allowing for the extraction of specific compounds of interest while preserving their quality and bioactivity (Carmen et al., 2023).

Safety. It provides a safe and non-toxic extraction environment, ensuring the purity of extracted compounds (Putra et al., 2023b).

As a result of these advantages, SC-CO₂ extraction has gained recognition as an effective and environmentally friendly alternative to conventional solvent extraction techniques, particularly in the extraction of bioactive compounds from natural sources. Its adoption represents a significant step toward achieving sustainable and eco-friendly extraction processes in various industries (Abou Elmaaty et al., 2022). The schematic diagram of SC-CO₂ extraction that can be applied in palm oil extraction is shown in

Figure 1. However, it is essential to consider the energy balance in extraction conditions, as noted by Meireles (2006). When evaluating whether the increased efficiency in extraction justifies the use of higher temperature and pressure, one must also account for the associated costs of cooling and pressurisation.

The SC-CO₂ extraction is a sophisticated method used for extracting palm oil, offering several advantages over traditional extraction techniques. The process begins with the preparation of the palm fruit, which is harvested and processed to obtain the oil-bearing fruit bunches. These bunches undergo sterilisation, threshing and pressing to extract crude palm oil. Once the crude oil is obtained, it is loaded into the extraction vessel of the SC-CO₂ extraction system.

In the SC-CO₂ extraction system, the crude palm oil is subjected to high pressure and temperature conditions to achieve supercritical state conditions for the CO₂. Typically, SC-CO₂ extraction occurs at temperatures around 31°C and pressures of approximately 73 atm (Putra et al., 2020, 2023d). At these conditions, CO₂ behaves as a solvent, penetrating the oil matrix and selectively extracting desired components, such as triglycerides, which are the primary constituents of palm oil. This selective extraction process ensures that only the desired compounds are extracted, leaving behind unwanted substances.

After the extraction process, the SC-CO₂ containing the dissolved palm oil components is depressurised and passed into a separator. The pressure is reduced, causing the SC-CO₂ to revert to its gaseous state, leaving behind the extracted palm oil. The CO₂ is then recycled for reuse in the extraction process, making the method environmentally friendly and sustainable. The extracted palm oil may undergo further purification steps, such as filtration or refining, to remove any remaining impurities and improve its quality. The final product is high-quality palm oil ready for use in various applications, including food, cosmetics, and biofuels.

In summary, SC-CO₂ extraction offers an efficient and environmentally friendly alternative to conventional palm oil extraction methods. By utilising SC-CO₂ as solvent, this method ensures the extraction of high-quality palm oil while minimising environmental impact and maintaining food safety standards.

SC-CO₂ as a Solvent for Palm Oil Extraction

Indeed, CO₂ has a long history of use as a supercritical fluid, and its selection is primarily based on its remarkable properties. One of the key advantages of SC-CO₂ extraction is its unique combination of characteristics, which make

Parameter Effects on SC-CO₂ Extraction

Optimising the conditions for SC-CO₂ extraction is a critical initial step in the procedure, aiming to achieve the efficient extraction of bioactive compounds while minimising the extraction of undesired components. This optimisation of operational parameters is fundamental in the development of SC-CO₂ technology, given the multitude of process factors that can influence extraction efficiency. Various parameters associated with supercritical fluid extraction (SFE) need careful adjustment to ensure the successful extraction of bioactive components from plant materials. *Table 1* shows the optimal conditions for extracting palm oil.

Several factors have been identified as having significant effects on the efficiency of SC-CO₂ extraction. These factors include the type of sample being processed, the choice of supercritical fluid, operating pressure, flow rate, temperature and extraction time. As previously reported (Reverchon, 1997), all of these parameters can influence the rate and success of the extraction process. However, it's worth noting that the primary parameters that play a pivotal role in determining extraction performance are pressure and temperature. These parameters are typically fine-tuned to achieve the desired outcomes in terms of both yield and selectivity.

Additionally, factors such as the moisture content in the sample matrix, the size of the sample and its particles and the flow rate of the solvent can also have a substantial impact on the overall extraction performance. Therefore, comprehensive optimisation of these parameters is essential to achieve the most efficient and effective SC-CO₂ extraction process, ensuring that the desired bioactive compounds are extracted while minimising the unwanted components.

Effect of Pressure at Constant Temperature in SC-CO₂ Extraction for Palm Oil

Pressure plays a pivotal role in the SC-CO₂ extraction process, exerting a significant influence on various aspects of the extraction procedure.

Several studies, including those by Li et al. (2023) and Putra et al. (2023e), have underscored the importance of pressure in SC-CO₂ extraction. One of the primary effects of pressure in SC-CO₂ extraction is its impact on the solubility of compounds and the diffusivity of the solvent within the matrix. By utilising a back pressure regulator to maintain the desired pressure level, precise control over the SC-CO₂ process can be achieved. At a fixed temperature, increasing the pressure is known to enhance the solubility of bioactive compounds and subsequently increase the extraction yield (Sodeifian et al., 2022). This occurs because elevated pressure boosts the solvent power and extraction efficiency of SC-CO₂. Additionally, higher pressure leads to an increase in the density of the SC-CO₂, further improving its solubilising capabilities, which is especially relevant in the recovery of compounds like carotene and tocopherol (Arsad et al., 2023).

However, it is important to note that raising the pressure above a certain point may have diminishing returns. Excessive pressure can potentially lead to reduced solvent diffusivity, meaning that the SC-CO₂ may have more difficulty penetrating the pores of the raw material, resulting in decreased solute dissolution (Putra et al., 2023b). Furthermore, excessively high-pressure conditions are not universally recommended for all target compounds, as they may cause compression of the raw material, potentially decreasing the overall extraction yield (Putra et al., 2018).

The effect of pressure on SC-CO₂ extraction is closely associated with changes in solvent power, which are influenced by the density of the solvent and the strengthening of intermolecular physical interactions. These factors collectively contribute to the complex relationship between pressure and extraction efficiency in SC-CO₂ processes (Farobie & Hartulistiyoso, 2021). In summary, pressure control is a crucial parameter in extraction, impacting the solubility of compounds, solvent diffusivity, and the overall extraction yield. Achieving the optimal pressure conditions is essential to maximise the efficiency and selectivity of SC-CO₂ extraction processes for specific target compounds and raw materials.

TABLE 1. THE CRITICAL PARAMETERS IN THE SC-CO₂ EXTRACTION IN THE PALM OIL RECOVERY

Parameter	The optimal range	References
Temperature	40°C-70°C	Bezerra, et al. (2018), Ooi et al. (1996), Tan et al. (2017)
Pressure	10-30 MPa	
Co-solvent	Ethanol (10%)	
Time	Less than 3 hr	
Flow rate	1-3 mL/min	
Particle size	0.25 dp < 1.80 mm	

Effect of Temperature at Constant Pressure in SC-CO₂ Extraction for Palm Oil

Temperature is a critical parameter in SC-CO₂ extraction, and its effects are multifaceted, especially when maintained at constant pressure. Temperature exerts dual influences on the SC-CO₂ extraction process, which are essential to consider in optimising the conditions.

Density and solvating capacity. As temperature increases, one of the primary effects is a reduction in the density of CO₂. This decrease in density can lead to a decrease in the solvating capacity of SC-CO₂. In other words, higher temperatures can reduce the ability of SC-CO₂ to dissolve and carry compounds from the sample matrix (Sodeifian et al., 2016).

Solubility and extraction yield. On the other hand, raising the temperature has a positive impact on the solubility of target compounds and, consequently, on the overall extraction yield. This increase in solubility is attributed to the rise in the vapour pressure of the target compounds, making them more amenable to extraction by SC-CO₂. However, this can also lead to a phenomenon known as “retrogradation,” where the isotherms cross. In simpler terms, higher temperatures may result in lower yields, while lower temperatures can lead to higher yields, depending on the specific compounds and conditions (Putra et al., 2023c; Sodeifian & Usefi, 2023).

Competing influences. The inversion of yield isotherms is a consequence of the competing influences of temperature on the extraction process. The dominant factor at play depends on the pressure conditions. Below a certain crossover pressure, the density effect tends to dominate, whereas, at higher pressures, it is the solute vapour pressure that controls the extraction process (Cockrell et al., 2020).

Impact on heat-sensitive compounds. It's essential to recognise that while raising the temperature can enhance extraction efficiency, it may not be suitable for all compounds, particularly those that are heat-sensitive. Many bioactive compounds can break down or undergo oxidation at elevated extraction temperatures, leading to a loss of their biological activity (Dhakane-Lad & Kar, 2021). Therefore, temperature conditions must be carefully adjusted, especially for thermolabile compounds like carotene and tocopherol, which are typically extracted at temperatures ranging from 35°C-70°C (Mustapa et al., 2011).

Temperature-pressure relationship. The effects of temperature and pressure are interconnected.

An increase in temperature at constant pressure will decrease the density of the solvent (SC-CO₂) and its solvation power. Conversely, higher temperatures will increase the vapour pressure of the solute, enhancing the solubility of the solute in SC-CO₂ (Kamaruddin et al., 2022).

In summary, temperature control is a vital aspect of SC-CO₂ extraction, with its impact on density, solvating capacity, solubility and extraction yield. Optimising temperature conditions is essential to balance the competing influences and achieve efficient extraction while preserving the integrity of heat-sensitive compounds when necessary. Additionally, understanding the interplay between temperature and pressure is key to fine-tuning the SC-CO₂ extraction process for specific applications.

Effect of Co-solvent/modifier in SC-CO₂ Extraction for Palm Oil

Co-solvents, organic solvents that can be mixed with SC-CO₂ at various ratios, are instrumental in the extraction processes, mainly when targeting hydrophilic or polar compounds. SC-CO₂, by its nature, is a nonpolar solvent and excels in extracting nonpolar compounds. However, it needs to extract hydrophilic substances efficiently. Co-solvents are introduced to modify the polarity of SC-CO₂, enhancing its ability to dissolve and interact with hydrophilic molecules. This addition expands the versatility of SC-CO₂ extraction, allowing for the effective extraction of a broader range of compounds (Marcus, 2018). Co-solvents bring about a crucial enhancement in solvent power through their intrinsic polarity, facilitating interactions like hydrogen bonding and dipole-dipole interactions with the target compounds. These interactions substantially elevate extraction yields, making SC-CO₂ an effective method for a wide array of compounds (Pimentel-Moral et al., 2019).

The choice of co-solvent depends on several factors, including the nature of the material being extracted, the specific compounds of interest, and the outcomes of preparatory tests. Commonly used co-solvents in SC-CO₂ extraction processes include ethanol and methanol, with ethanol often recommended for use in food-related applications due to its safety credentials according to the Food and Drug Administration (FDA) in the United States (Rizkiyah et al., 2023b). Nonetheless, the use of methanol is limited in food or oil manufacturing due to its toxicity concerns. In summary, co-solvents play a pivotal role in SC-CO₂ extraction, allowing for the efficient extraction of hydrophilic and polar compounds that would be challenging to extract using pure SC-CO₂. The choice of co-solvent is made based on the specific extraction requirements, safety considerations, and regulatory guidelines for the intended application.

Effect of Particle Size in SC-CO₂ Extraction for Palm Oil

Particle size is a crucial factor that significantly influences the success of SC-CO₂ extraction. The size and form of particles in the sample matrix have direct implications for the extraction process, especially regarding mass transfer kinetics and the interaction between SC-CO₂ and soluble compounds. Smaller particle sizes play a pivotal role in enhancing extraction efficiency and mass transfer kinetics. When the sample matrix has a small physical morphology, the diffusion path lengths for the extracted solutes to move into the fluid phase become shorter. This results in higher extraction efficiencies and increased mass transfer surface area, as noted by Putra et al. (2023c). The particle size typically varies depending on the nature of the natural product being extracted, falling within the range of 0.25-1.80 mm. However, it's essential to evaluate the optimal particle size on case-to-case basis.

Particle form is another crucial consideration. During the grinding process, particles can take on various shapes, including spherical, flaky, plate-like or irregular forms. These different forms can impact the diffusivity of the solvent as it penetrates the raw material. For instance, smaller particle sizes have been observed to increase the diffusivity coefficient of extracts (Kim & Lim, 2020). However, there is a limit to how small the particles should be, as the smallest sizes, such as 0.2 mm, can lead to reduced diffusivity coefficients ($0.72 \times 10^{-13} \text{ m/s}^2$). This reduction is often attributed to compaction and channelling within the particle bed, which can hinder mass transfer. Fine particle sizes may also lead to bed caking and the formation of preferential SC-CO₂ flow paths, further reducing mass transfer efficiency.

In the realm of palm oil extraction using SC-CO₂, an important consideration is the particle size of the palm oil material. The particle size plays a crucial role in the efficiency and effectiveness of the extraction process. If the particle size becomes too small, it can lead to issues such as clogging in the extraction equipment and difficulty in handling the material during the extraction process.

To address these concerns, Putra et al. (2020) have suggested an optimal particle size for palm oil extraction using SC-CO₂. Their research recommends that the average particle size should be around 1 cm. This size ensures that the palm oil material is large enough to prevent clogging while still being small enough to facilitate efficient extraction by SC-CO₂. By maintaining an average particle size of 1 cm, several benefits can be realised. Firstly, it helps to prevent clogging in the extraction equipment, ensuring smooth operation and minimising downtime. Additionally, it allows

for easier handling of the palm oil material during the extraction process, reducing the risk of material loss or inefficiencies.

Moreover, optimising the particle size for SC-CO₂ extraction contributes to the overall effectiveness of the process. It promotes better contact between the CO₂ solvent and the palm oil material, enhancing the extraction efficiency and maximising the yield of high-quality palm oil. In summary, controlling the particle size of the palm oil material is crucial for successful extraction using SC-CO₂. By adhering to the recommended average particle size of 1 cm, operators can mitigate potential challenges such as clogging and ensure optimal extraction performance, leading to improved efficiency and yield in the palm oil extraction process.

In summary, particle size and form are critical considerations in SC-CO₂ extraction processes. While smaller particle sizes generally enhance extraction efficiency and mass transfer, an optimal size must be determined based on the specific material and compounds being targeted. The form of the particles also influences diffusivity and should be taken into account to maximise extraction yields while avoiding compaction and channelling issues that can hinder the process.

Effect of Flow Rate in SC-CO₂ Extraction for Palm Oil

The flow rate of carbon dioxide (CO₂) plays a pivotal role in SC-CO₂ extraction experiments, significantly influencing the efficiency of the process. A higher CO₂ flow rate effectively reduces the residence time of CO₂ within the extraction vessel, facilitating increased interactions between CO₂ and the target compounds, such as carotene and tocopherol in palm oil. This enhanced contact leads to improved extraction efficiency, as observed by Peng et al. (2020). Furthermore, the SC-CO₂ flow rate has a profound impact on the mass transfer during extraction, encompassing the solubility-controlled and diffusion-controlled phases. Lowering the solvent flow rate, specifically CO₂, can optimise the efficiency of the extraction process, particularly within the solubility-controlled zone. In this phase, the rate of solute dissolution in CO₂ governs the process, and lower flow rates can be advantageous. They not only enhance efficiency but also reduce the overall volume of solvent needed to extract a specific quantity of target compounds.

The production of compounds of interest, such as carotene and tocopherol in palm oil, is intricately linked to the quantity of CO₂ required for extraction within the solubility-controlled zone, particularly at lower pressures. Controlling the CO₂ flow rate provides a means to fine-tune the extraction process, achieving the desired yield of bioactive

compounds, as noted by Idham et al. (2020). In summary, the CO₂ flow rate is a critical parameter in SC-CO₂ extraction, impacting residence time, mass transfer, and overall process efficiency. By carefully adjusting the flow rate, researchers can optimise interactions between CO₂ and target compounds, improve solubility-controlled extraction phases, and minimise solvent usage while achieving the desired yields of bioactive compounds in the extraction processes.

Recent Studies on Palm Oil Extraction by SC-CO₂

A wealth of research has been devoted to exploring various techniques for palm oil recovery, with a particular focus on methods like SC-CO₂ extraction. The parameters governing SC-CO₂ extraction have been extensively studied, aiming to optimise the extraction of valuable compounds such as carotenes and tocopherols as shown in Table 2. These results have yielded valuable insights for improving palm oil processing. Ooi et al. (1996)

conducted experiments involving the continuous SC-CO₂ processing of crude palm oil, revealing its effectiveness in reducing levels of free fatty acids, monoglycerides, diglycerides, specific triglycerides and certain carotenes. The resulting refined palm oil exhibited reduced free fatty acid content and elevated carotene levels. The study highlighted how increasing extraction pressure enhanced the solvent power of the supercritical fluid.

Tan and Zhao (2017) introduced a unique approach combining SC-CO₂ and n-hexane to extract palm oil and enrich natural β -carotene. This two-step procedure effectively preconcentrated β -carotene using SC-CO₂, followed by solvent extraction, significantly increasing β -carotene concentrations. Bezerra et al. (2018) investigated palm fibre oil extraction using SC-CO₂, showing that oil yields reached a maximum at specific temperature and pressure conditions. Notably, SC-CO₂ extraction resulted in oils with higher saturation levels and lower carotenoid content compared to traditional solvent-based extraction.

TABLE 2. PREVIOUS STUDIES OF PALM OIL EXTRACTION BY SC-CO₂

Authors	Objective	Findings
Ooi et al. (1996)	Investigate the effectiveness of continuous SC-CO ₂ processing for refining crude palm oil.	- Reduction of free fatty acids, monoglycerides, diglycerides, specific triglycerides, and carotenes in palm oil. - Refined palm oil with less than 0.1% free fatty acid content. Introduction of a co-solvent, ethanol, notably lowered free fatty acid levels to below 0.1%. - Adjusting parameters like pressure, temperature, and solvent-to-feed ratio is feasible for refining palm oil with SC-CO₂.
Tan and Zhao (2017)	Combine SC-CO ₂ extraction and n-hexane to extract and enrich natural β -carotene from palm oil.	- β-carotene concentration increased from 10 g/100 g to 16.7 g/100 g. - Combination with n-hexane solvent further increased β-carotene concentration to 58.7 g/100 g. - SC-CO₂ selectively removed contaminants, allowing purification of β-carotene through hexane extraction.
Bezerra et al. (2018)	Investigate SC-CO ₂ extraction of palm pressed-fibre oil at various temperatures and pressures.	- Oil yields reached a maximum of 6.09% at 40°C and 450 bar. - Oils obtained exhibited high saturation levels but relatively low carotenoid content.
De França and Meireles (2000)	Explore the kinetics of carotene and lipid extraction from crushed palm fibres using SC-CO ₂ .	- Extract from pressed palm fibres exhibited varying concentrations of free fatty acids and carotenoids. - Carotenoid concentrations increased while free fatty acid concentrations decreased over time. - Mathematical model effectively quantified the entire extraction curve, providing valuable insights into the extraction process.
Putra et al. (2020)	Investigate the impact of operational parameters on oil production, total tocopherol compounds, and total carotene compounds during SC-CO ₂ extraction.	- The highest oil yield reached 18.49% under specific conditions. - Total tocopherol compounds measured 2020.09 mg/kg. - Total carotene compounds reached 2076.96 mg/kg under specific conditions. - High-pressure conditions were ideal for extracting palm fibre oil due to increased extraction rates, diffusivity, and solubility. - Increased pressure led to higher CO₂ density, enhancing solvent-solute interactions.

De França and Meireles (2000) explored the kinetics of carotene and lipid extraction from crushed palm fibres using SC-CO₂, developing a mathematical model for mass transfer. This model effectively quantified the extraction process, offering insights into the dynamics of the extract composition. Putra et al. (2020) conducted a comprehensive investigation into the SC-CO₂ impact of extraction on oil production, total tocopherol compounds, and total carotene compounds. They used semi-empirical models to determine extraction rates and solubility, revealing that high-pressure conditions were ideal for extracting palm fibre oil due to enhanced extraction rates and solubility.

In summary, these studies collectively provide valuable insights into optimising and improving palm oil recovery through SC-CO₂ extraction methods. The results show the significance of parameter adjustments, co-solvent utilisation, and pretreatment processes to enhance the extraction of valuable compounds from palm-derived materials, contributing to more efficient and sustainable palm oil production.

ADVANTAGES OF SC-CO₂ EXTRACTION FOR PALM OIL EXTRACTION

SC-CO₂ extraction stands at the forefront of innovative technologies that hold immense promise for the palm oil industry. This advanced extraction method has garnered attention for its ability to provide numerous distinct advantages, ranging from environmental sustainability to improved product quality and economic viability. SC-CO₂ extraction offers several distinct advantages for the palm oil industry, as shown below.

Environmental Benefits

The environmental benefits of SC-CO₂ extraction are significant and make it an environmentally friendly choice for various industries, including the palm oil industry. One of the primary environmental benefits of SC-CO₂ extraction is that it utilises CO₂ as the solvent. CO₂ is non-toxic and non-flammable, making it inherently safer for both workers and the environment compared to traditional organic solvents often used in extraction processes (Abdul Aziz et al., 2022). This reduces the risk of accidents, spills and harmful exposure to chemicals in the workplace.

Traditional solvent-based extraction methods can leave behind residues of organic solvents in the extracted product (Fernando et al., 2021). These residues can be harmful to human health and the environment. In contrast, SC-CO₂ is a clean and pure solvent that does not leave any harmful residues or

chemical traces in the final product. This ensures that the extracted palm oil and its constituents are free from solvent contaminants. The use of organic solvents in conventional extraction processes can also lead to environmental pollution, as these solvents may be released into the air or water during extraction and purification steps. SC-CO₂, on the other hand, is a closed-loop system where the CO₂ can be easily captured, recycled, and reused, as shown in Figure 1. This closed-loop nature of SC-CO₂ extraction minimises emissions and prevents the release of harmful substances into the environment, contributing to a cleaner and healthier ecosystem.

SC-CO₂ extraction aligns with sustainability goals by reducing the overall environmental footprint of the palm oil industry (Fomo et al., 2020). The closed-loop system not only prevents CO₂ emissions but also minimises waste generation. Since SC-CO₂ can be recycled and reused, there is less need for the disposal of solvent waste, reducing the industry's impact on landfills and waste management systems. SC-CO₂ extraction also aligns with increasingly stringent environmental regulations and safety standards (National Academies of Sciences, Engineering and Medicine, 2017). As governments and regulatory bodies around the world place greater emphasis on environmentally friendly practices and worker safety, SC-CO₂ extraction provides a solution that helps companies meet these requirements while ensuring compliance with environmental regulations.

In summary, SC-CO₂ extraction offers a range of environmental benefits for the palm oil industry. Its non-toxic and non-flammable properties, along with the absence of harmful residues, make it a safe and sustainable choice. The closed-loop system minimises environmental pollution and waste generation, contributing to a greener and more environmentally responsible palm oil extraction process.

Improved Oil Quality and Yield

Improved oil quality and yield are critical advantages of SC-CO₂ extraction in the palm oil industry, owing to the precise control it offers over critical extraction parameters (Temelli, 2009). SC-CO₂ extraction provides a high degree of control over parameters such as temperature and pressure. This precision allows operators to tailor the extraction process to their specific requirements. In the context of palm oil extraction, this means that the process can be optimised to target certain compounds while leaving undesirable components behind selectively.

One of the notable improvements in oil quality achieved through SC-CO₂ extraction is the significant reduction in undesirable components, such as free

fatty acids (Friedrich & Pryde, 1984). Free fatty acids can negatively impact the quality and shelf life of palm oil. By using SC-CO₂ extraction, these unwanted compounds can be effectively removed, resulting in palm oil with a reduced free fatty acid content. SC-CO₂ extraction is also a gentle process that operates at moderate temperatures (Majid et al., 2019). This is crucial for preserving the quality of the extracted oil, especially in the case of heat-sensitive compounds. Unlike some conventional extraction methods that may expose the oil to high temperatures, SC-CO₂ extraction minimises the risk of degradation or alteration of the oil's chemical composition, ensuring that the extracted palm oil retains its desired characteristics.

SC-CO₂ extraction is known for its ability to yield higher quantities of valuable compounds found in palm oil, such as carotenes and tocopherols (Akanda et al., 2012; Lau et al., 2006). These compounds contribute not only to the nutritional value of the oil but also to its market value. The ability to extract more of these bioactive compounds enhances the overall quality and marketability of the palm oil product. The improved quality and higher yield of valuable compounds achieved through SC-CO₂ extraction translate into a more valuable end product. Palm oil producers can command better prices for high-quality, nutrient-rich palm oil in the market. This value addition can significantly impact the economic viability of palm oil extraction operations.

In summary, SC-CO₂ extraction's ability to offer precise control over extraction parameters, reduce undesirable components, preserve oil quality and enhance the yield of valuable compounds makes it an attractive choice in the palm oil industry. This technology not only results in higher-quality palm oil but also increases its overall value, contributing to improved product quality and economic advantages for palm oil producers.

Economic Advantages

The economic advantages of SC-CO₂ extraction in the palm oil industry are notable, offering long-term benefits that outweigh the initial investment in specialised equipment. SC-CO₂ extraction's ability to improve the quality of palm oil and yield higher quantities of valuable compounds directly contributes to increased profitability for palm oil producers. Higher-quality palm oil with reduced levels of undesirable components, such as free fatty acids, is often more marketable and can command better prices in the market (Bello-Bravo et al., 2015). This enhanced product quality translates into improved revenue streams for producers.

SC-CO₂ extraction allows for the selective extraction of valuable bioactive compounds like carotenes and tocopherols, which are in demand

due to their nutritional and health benefits. These compounds enhance the market value of the palm oil product. By diversifying their product offerings with value-added extracts, palm oil producers can tap into additional revenue streams, further bolstering their economic prospects. SC-CO₂ extraction's precise control over extraction parameters ensures efficient resource utilisation. This efficiency minimises the wastage of raw materials and reduces the need for reprocessing or additional treatments, which can be costly. The technology's ability to target specific compounds also ensures that valuable palm oil components are fully extracted, optimising resource utilisation and minimising losses.

Over the long term, SC-CO₂ extraction can lead to reduced production costs. The fine-tuned control over temperature, pressure and other parameters results in more efficient extraction processes (Knez et al., 2019). This efficiency can translate into energy savings, reduced solvent usage, and lower maintenance costs. Additionally, the elimination of the need for certain post-extraction treatments or additional refining steps can further reduce operational expenses. Palm oil producers adopting SC-CO₂ extraction can gain a competitive edge in the market. Higher-quality palm oil and the availability of value-added extracts not only command better prices but also attract discerning consumers and industries looking for premium, sustainable products. This competitive advantage can help palm oil producers secure long-term contracts and partnerships, contributing to financial stability.

The economic advantages of SC-CO₂ extraction extend to long-term sustainability. By investing in environmentally friendly and sustainable extraction practices, palm oil producers can align themselves with the growing consumer and regulatory demand for environmentally responsible products. This positions them for continued success in an evolving market landscape. In conclusion, while SC-CO₂ extraction may require initial investments in equipment, its economic advantages become apparent over time. Improved product quality, increased yield of valuable compounds, efficient resource utilisation, reduced production costs, and a competitive edge in the market all contribute to the long-term profitability and sustainability of palm oil production operations.

CHALLENGES AND BARRIERS IN SC-CO₂ EXTRACTION FOR PALM OIL EXTRACTION

While SC-CO₂ extraction holds immense promise for revolutionising the palm oil industry, it has its set of challenges and hurdles. These technical, economic, and safety considerations, among others, present complexities that must be navigated to

fully harness the potential of SC-CO₂ extraction in palm oil production. As this article explores the intricacies of SC-CO₂ extraction in the palm oil sector, it is imperative to shed light on the challenges that accompany this innovative technology. From the technical intricacies of equipment and process optimisation to energy consumption and regulatory compliance, these hurdles demand careful consideration.

In this section, we delve into the technical challenges and limitations that arise from the complexity of SC-CO₂ extraction equipment and the need for meticulous process optimisation. We also address the energy consumption and equipment costs that may pose economic challenges, particularly for smaller producers. Additionally, safety considerations and regulatory compliance are paramount to ensuring the responsible use of SC-CO₂ extraction technology. By understanding these challenges and actively seeking solutions, the palm oil industry can pave the way for a more sustainable and efficient future, harnessing the full potential of SC-CO₂ extraction while mitigating its inherent complexities.

Technical Challenges and Limitations

Technical challenges and limitations in SC-CO₂ extraction for palm oil extraction are multifaceted, encompassing equipment complexity, process optimisation, matrix effects and scale-up challenges. One of the primary technical challenges lies in the complexity of the equipment required for SC-CO₂ extraction (An et al., 2021). This process demands specialised high-pressure vessels capable of withstanding the elevated pressures necessary for supercritical conditions. Precise control systems are also essential to manage parameters such as pressure, temperature, and flow rates. However, the initial investment in such advanced equipment can be prohibitively high, particularly for smaller palm oil producers or those operating with limited financial resources. This complexity can pose a significant barrier to entry into SC-CO₂ extraction technology.

Attaining ideal extraction conditions represents a pivotal element in SC-CO₂ extraction, albeit often presenting a formidable task. Factors such as pressure, temperature, and flow rate necessitate meticulous calibration to achieve a delicate equilibrium between effective compound extraction and maintaining the integrity of the extracted oil (Dhara et al., 2022). Identifying the ideal set of parameters often involves extensive experimentation, testing, and iterative adjustments. The need for precise control further underscores the technical expertise required for successful SC-CO₂ extraction. The variability in the composition of palm fruit and its matrix presents a significant technical hindrance. Factors such as

moisture content, fruit ripeness, and the presence of impurities can substantially influence the efficiency of SC-CO₂ extraction (Srinivas & King, 2010). Variations in these matrices can lead to inconsistent yields and product quality. As a result, robust quality control measures and process adaptations may be necessary to accommodate the inherent matrix diversity of palm fruit.

Transitioning from laboratory-scale SC-CO₂ extraction to large-scale industrial operations is a complex undertaking. While laboratory experiments provide valuable insights, scaling up the process introduces engineering and logistical challenges. Maintaining the efficiency and consistency of the extraction process at an industrial scale requires careful consideration of equipment size, throughput rates, and overall process design. Scaling up without compromising product quality or yield demands a thorough understanding of both the technology and the specific characteristics of palm oil extraction. In summary, the technical challenges and limitations of SC-CO₂ palm oil extraction encompass the intricate nature of the equipment, the need for precise process optimisation, the impact of matrix variations and the complexities of scaling up from laboratory to industrial settings. Addressing these challenges requires a combination of technical expertise, innovative engineering solutions, and a commitment to optimising the process for efficient and high-quality palm oil extraction.

Energy Consumption and Equipment Costs

Energy consumption and equipment costs are pivotal considerations when evaluating SC-CO₂ palm oil extraction processes, as they significantly impact both the feasibility and economic viability of adopting this technology (Kayathi et al., 2021). SC-CO₂ extraction involves operating equipment under high pressures and temperatures, which can translate into substantial energy requirements. The compression and maintenance of CO₂ in its supercritical state demand energy-intensive processes. This energy consumption can be a notable concern, especially in regions with elevated energy prices or unreliable power sources. The cost of energy used during extraction directly influences the overall production costs and profitability of palm oil extraction via SC-CO₂. Therefore, optimising energy efficiency is a key challenge in the implementation of this technology.

The initial capital investment associated with SC-CO₂ extraction equipment is a significant factor to consider (Hussain & Wahab, 2018). Specialised high-pressure vessels, precise control systems, and safety features are essential components of SC-CO₂ extraction setups, making the technology capital-intensive. These upfront equipment costs may act as a deterrent for smaller palm oil producers or those

with limited financial resources. As a result, the potential benefits of SC-CO₂ extraction, including improved oil quality and higher yields, need to be carefully weighed against the initial investment. Its economic justification for this investment may become evident over time as the technology's advantages are realised.

In summary, the high energy consumption and substantial equipment costs associated with SC-CO₂ palm oil extraction are pivotal challenges and considerations for stakeholders in the palm oil industry. Balancing the need for energy-intensive operations with long-term economic benefits, improved product quality and increased yields will determine the feasibility and attractiveness of adopting SC-CO₂ extraction technology for palm oil production. Furthermore, ongoing research and technological advancements may lead to more energy-efficient and cost-effective SC-CO₂ extraction solutions, potentially mitigating some of these challenges in the future.

Safety Considerations

Safety considerations are of paramount importance in SC-CO₂ palm oil extraction due to the use of high-pressure equipment and the need to protect both personnel and the environment (Norhuda & Jusoff, 2009). SC-CO₂ extraction relies on high-pressure vessels to maintain CO₂ in its supercritical state. Although CO₂ itself is non-toxic and non-flammable, the elevated pressures involved can pose safety risks if not properly managed. To address this, rigorous safety protocols and training are essential. Operators and maintenance personnel must receive thorough training in the safe operation of high-pressure equipment, including recognising potential hazards, understanding emergency procedures and using protective gear. Regular equipment inspections and maintenance are also crucial to ensure the integrity of pressure vessels.

Ensuring the safety of individuals working with SC-CO₂ extraction equipment is of utmost importance. This includes not only those directly involved in the extraction process but also maintenance and support staff. Safety measures should encompass comprehensive training on the proper handling of SC-CO₂ equipment, safe operating procedures and emergency response protocols. The provision of personal protective equipment (PPE) is essential to minimise risks, and its correct use should be enforced. Additionally, equipment design should prioritise operator safety, with features that prevent accidental exposure to high pressures or temperatures.

While SC-CO₂ is generally considered environmentally friendly, there are environmental concerns related to potential CO₂ emissions during the extraction process. To mitigate this,

strict containment measures should be in place to prevent the release of SC-CO₂ into the atmosphere. Any potential leaks or emissions must be promptly identified and addressed. Regular monitoring and maintenance of the extraction system are crucial to minimise environmental impact. Furthermore, efforts should be made to capture and recycle any released CO₂ to minimise its contribution to GHG emissions, aligning with sustainability goals. In summary, safety considerations in SC-CO₂ palm oil extraction revolve around managing high-pressure equipment, ensuring worker safety through training and protective measures and addressing potential environmental impacts associated with CO₂ emissions. Stringent safety protocols, ongoing training and proactive maintenance are essential components of a safe and responsible SC-CO₂ extraction process.

Regulatory Compliance

Regulatory compliance is a critical aspect of adopting and operating SC-CO₂ extraction technology in the palm oil industry. Ensuring adherence to local and international regulations and standards is essential for legal, safety, and environmental reasons. SC-CO₂ extraction, while considered more environmentally friendly than some traditional extraction methods, still needs to comply with environmental regulations. This includes ensuring that emissions of CO₂ or other substances are within allowable limits and that waste disposal methods are in line with environmental standards. Operators may need to obtain permits or approvals from environmental agencies.

Operating SC-CO₂ extraction equipment involves high-pressure systems, which are subject to safety regulations. These regulations may vary depending on the jurisdiction and may cover the design, construction, inspection and operation of pressure vessels. Compliance with safety standards is crucial to prevent accidents and protect workers. Regular inspections and certifications may be required to demonstrate compliance. If the palm oil extracted using SC-CO₂ is intended for human consumption, it must meet food safety and quality standards. This includes compliance with regulations related to food additives, contaminants, and packaging. Ensuring the purity and safety of the extracted oil is essential to meet these standards and gain market acceptance.

The safety of workers involved in SC-CO₂ extraction is subject to occupational health and safety regulations. These regulations may specify requirements for training, protective equipment, emergency response plans and safe operating procedures. Complying with The Occupational Health and Safety (OHS) regulations is essential

to protect the well-being of employees. For palm oil producers involved in international trade, compliance with export and import regulations is crucial. These regulations may relate to customs documentation, tariffs, trade agreements and product specifications. Meeting these requirements ensures smooth international transactions.

Obtaining certifications related to sustainability, quality and safety can be beneficial for palm oil producers using SC-CO₂ extraction. Certifications like Roundtable on Sustainable Palm Oil (RSPO) and Malaysian Sustainable Palm Oil (MSPO) demonstrate a commitment to sustainable practices. Additionally, certifications related to food safety (e.g., ISO 22000) or organic production can enhance the marketability of palm oil products (Overbosch & Blanchard, 2023). Proper disposal of waste generated during the SC-CO₂ extraction process, such as spent palm fruit materials, may be subject to waste management regulations. Compliance with these regulations is essential to minimise environmental impact and legal liability.

Regulatory compliance often involves thorough record-keeping and reporting. Maintaining comprehensive documentation of processes, safety measures, emissions data, and other relevant information is essential for demonstrating compliance and responding to regulatory inquiries. Navigating the regulatory landscape can be complex, and it may require legal counsel or regulatory experts to ensure full compliance. While compliance adds complexity and costs to SC-CO₂ extraction operations, it is essential for legal and ethical operation, ensuring the safety of workers and the environment and gaining market access for palm oil products.

Maintenance and Upkeep

Maintenance and upkeep of SC-CO₂ extraction equipment are critical aspects of ensuring its safe and efficient operation in the palm oil industry. Regular inspections of the SC-CO₂ extraction equipment are essential to identify wear and tear, potential issues, or signs of malfunction. These inspections should encompass all components, including high-pressure vessels, pumps, valves and control systems (Patrignani & Lanciotti, 2016). The frequency of inspections may vary but should adhere to a predefined schedule to catch problems early. Maintenance schedules should include routine servicing of the equipment. This involves tasks such as lubricating moving parts, replacing worn seals, and cleaning critical components. Properly maintained equipment operates more efficiently and has an extended lifespan.

Instrumentation and control systems, such as pressure gauges and temperature sensors, should be regularly calibrated to ensure accurate

readings. Periodic testing of safety features and emergency shutdown systems is also vital to verify their functionality in case of an emergency. Over time, components of SC-CO₂ extraction equipment may wear out or become damaged. The timely replacement of these parts is crucial to prevent equipment breakdowns and maintain operational efficiency. Common replacement parts may include seals, gaskets and valves.

Safety components, such as pressure relief valves, should be inspected and tested to ensure they meet safety standards. These safety features are essential for preventing accidents in high-pressure systems (Dinh et al., 2012). Personnel responsible for equipment maintenance should receive proper training on the specific maintenance requirements of SC-CO₂ extraction equipment. They should be familiar with safety protocols, troubleshooting procedures, and the correct use of maintenance tools and equipment. Having a well-defined emergency response plan is essential in case of equipment failures or safety incidents. Personnel should be trained on how to respond to emergencies, including shutdown procedures, evacuation protocols, and first aid.

Maintaining detailed records of all maintenance activities is crucial for tracking the history of equipment maintenance and ensuring compliance with safety and regulatory requirements (Mobley, 2011). These records can also help identify recurring issues and inform decisions about equipment upgrades or replacements. Implementing a preventive maintenance programme can help identify potential issues before they lead to equipment failure. This proactive approach involves regular check-ups, cleaning, and adjustments to keep the equipment in optimal working condition. Periodic assessments of equipment performance and maintenance practices should be conducted to identify areas for improvement. This may involve upgrading equipment to more efficient models or implementing enhanced maintenance procedures. In summary, maintaining SC-CO₂ extraction equipment in the palm oil industry is a multifaceted process that includes routine inspections, scheduled servicing, part replacements, safety measures, training, and documentation. A well-maintained system not only operates efficiently but also enhances safety and minimises downtime, ultimately contributing to the overall success of palm oil extraction processes.

PROSPECT FOR SUSTAINABLE PALM OIL PRODUCTION

The prospects for sustainable palm oil production are closely tied to innovative technologies like SC-CO₂ extraction, which have the potential

to revolutionise the palm oil industry. SC-CO₂ extraction has emerged as a game-changing technology with the potential to transform the palm oil industry towards sustainability. By offering a cleaner and more efficient method for palm oil extraction, SC-CO₂ can significantly reduce the environmental footprint of palm oil production. Its ability to selectively extract valuable compounds while improving oil quality and yield positions it as a sustainable alternative to traditional extraction methods.

The adoption of SC-CO₂ extraction aligns with the industry's growing emphasis on reducing its environmental impact. Unlike conventional solvent-based extraction processes that employ toxic and environmentally harmful solvents, SC-CO₂ is non-toxic, non-flammable and does not produce hazardous residues. This inherently reduces pollution, making it a more sustainable choice. Sustainable palm oil production has become a global priority to address these concerns. SC-CO₂ extraction fits into this sustainability narrative by offering a greener alternative that minimises habitat destruction and ecosystem disruption.

Many sustainability certification standards, such as the RSPO and MSPO require adherence to environmentally friendly practices. SC-CO₂ extraction aligns with these standards and can help palm oil producers attain certification, thereby accessing premium markets and demonstrating their commitment to sustainability. Consumer demand for sustainable products is on the rise. Manufacturers and retailers are increasingly seeking sustainable palm oil sources to meet consumer expectations and enhance their brand image. Palm oil produced using SC-CO₂ extraction can be marketed as a sustainable and environmentally responsible choice, opening up opportunities for market growth.

Continued investment in research and development to refine SC-CO₂ extraction processes specific to palm oil can unlock even more excellent sustainability benefits. This may include optimising extraction conditions, reducing energy consumption and finding innovative uses for by-products, further enhancing the industry's sustainability profile. Collaboration among stakeholders in the palm oil industry, including producers, governments, NGOs and research institutions, can facilitate the adoption of sustainable practices like SC-CO₂ extraction. Knowledge sharing and best practice dissemination can accelerate the industry's transition towards sustainability. In conclusion, the prospects for sustainable palm oil production are promising, with SC-CO₂ extraction playing a pivotal role in this transformation. By reducing environmental impact, conserving biodiversity and aligning with sustainability goals, SC-CO₂

extraction technology offers a path towards a more sustainable and responsible palm oil industry, meeting the demands of consumers, certification standards and global environmental priorities.

FUTURE DIRECTIONS AND RESEARCH OPPORTUNITIES

Future directions and research opportunities in SC-CO₂ extraction technology for palm oil production offer exciting possibilities for enhancing sustainability, efficiency and product quality. Continued research into the optimisation of SC-CO₂ extraction parameters, such as pressure, temperature, flow rate and co-solvent use, can lead to improved extraction efficiency and higher yields of valuable compounds. Fine-tuning these parameters for different palm oil varieties and fruit maturation stages can enhance the versatility of the technology.

Developing scalable SC-CO₂ extraction systems for industrial use is essential. Research into cost-effective scaling techniques and equipment design will be crucial to making SC-CO₂ extraction accessible to a broader range of palm oil producers, from smallholders to large plantations. Exploring novel co-solvents and their effects on SC-CO₂ extraction can also improve the selectivity and efficiency of the process. Research into eco-friendly and food-grade co-solvents that enhance the solubility of specific compounds while maintaining product safety is an area of potential innovation. Investigating methods to preserve the nutrient and bioactive compound content of palm oil during SC-CO₂ extraction is also essential for maintaining product quality. Innovative techniques, such as encapsulation or controlled release, can help prevent oxidation and degradation.

Investigating ways to utilise by-products and waste streams generated during SC-CO₂ extraction can contribute to sustainability. Research can focus on converting these by-products into value-added products, such as biofuels, animal feed or fertilisers, reducing waste and increasing overall resource efficiency. Research efforts to reduce the energy consumption of SC-CO₂ extraction equipment can enhance its economic viability and environmental sustainability. Innovations in heat recovery, process integration, and energy-efficient designs can help lower operational costs. Furthermore, developing continuous SC-CO₂ extraction processes can improve productivity and reduce processing times. Research into continuous flow systems and their scalability for palm oil production can be a significant area of exploration. Advancements in automation and control systems can improve the precision and reproducibility of SC-CO₂ extraction processes.

Developing intelligent sensors and real-time monitoring tools can enhance process control and product consistency.

Conducting comprehensive life cycle assessment (LCA) to evaluate the environmental impact of SC-CO₂ extraction compared to traditional methods can provide valuable data for decision-makers. LCA studies can guide sustainability initiatives and inform stakeholders about the technology's eco-efficiency. Furthermore, encouraging collaboration among researchers, industry experts and policymakers is vital to drive innovation in SC-CO₂ extraction technology. Research consortia and knowledge-sharing platforms can facilitate the exchange of ideas and accelerate technology development.

Research should focus on aligning SC-CO₂ extraction practices with evolving sustainability standards and regulatory requirements. Staying informed about changing regulations and proactively adapting technology to meet compliance standards is crucial. Moreover, research efforts should extend to market expansion strategies and consumer education. Creating awareness about the benefits of palm oil produced using SC-CO₂ extraction can drive demand for sustainable palm oil products and incentivise industry-wide adoption. In summary, the future of SC-CO₂ extraction technology for palm oil production holds immense potential for sustainable, efficient, and high-quality oil extraction. Study in these areas can lead to innovations that not only benefit the palm oil industry but also contribute to broader sustainability and environmental conservation goals. Collaborative efforts and interdisciplinary research will be key drivers of progress in these directions.

CONCLUSION

In conclusion, SC-CO₂ extraction technology has emerged as a promising and environmentally friendly approach for revolutionising the palm oil industry. This manuscript has provided an in-depth exploration of the prospects, advantages, challenges, and research opportunities associated with SC-CO₂ extraction in palm oil production. The environmental benefits of SC-CO₂ extraction, characterised by its non-toxic nature, minimal waste generation and recyclability, position it as a sustainable alternative to traditional solvent-based extraction methods. This technology aligns with global sustainability goals and offers a pathway to reduce deforestation, conserve natural resources and produce palm oil of higher quality.

SC-CO₂ extraction empowers palm oil producers with precise control over extraction parameters,

enhancing oil quality while increasing yields of valuable compounds like carotenes and tocopherols. These improvements contribute to the economic viability of palm oil production, making it an attractive choice for both small-scale and large-scale operations. However, challenges and hurdles exist, including the complexity of equipment, energy consumption, safety considerations, regulatory compliance and maintenance costs. Addressing these challenges through continued research and innovation is essential to unlock the full potential of SC-CO₂ extraction in the palm oil industry. Prospects for sustainable palm oil production are promising, driven by the potential of SC-CO₂ extraction to align with sustainability goals, reduce environmental impact, and promote responsible practices. As palm oil remains a critical commodity in various industries, embracing this innovative technology can lead to a more sustainable and responsible palm oil sector.

Looking ahead, future research directions and opportunities in SC-CO₂ extraction technology include optimising process parameters, scaling up operations, exploring eco-friendly co-solvents, utilising waste streams, enhancing energy efficiency, and preserving nutrient and bioactive compounds. Collaboration, regulatory alignment and consumer awareness will play pivotal roles in realising the full potential of SC-CO₂ extraction in palm oil production. In conclusion, the adoption and further development of SC-CO₂ extraction technology hold great promise for reshaping the palm oil industry, fostering sustainability, and meeting the demands of a changing world while preserving our environment for future generations.

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EFFECT OF PLANTING DENSITY, PROGENY LINEAGE AND NITROGEN FERTILISER ON OIL PALM PERFORMANCE ON ALLUVIAL SOIL

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ABSTRACT

The primary objective for many oil palm growers has been to enhance crop yield, which can be achieved through the cultivation of more productive varieties. Therefore, there is a need to study new planting materials to find the optimum planting density in oil palm plantations. Oil palm growth and yield performance of two planting materials (PS1 and DxP) were determined using four planting densities and three nitrogen fertiliser rates. The analysis of variance for fresh fruit bunch (FFB) and its components over 17 years of recording show no significant interaction between planting density, progeny lineage and nitrogen (N). The N had a significant effect on FFB yield where N1 rate was a significant difference in average FFB yield by 0.89 t ha⁻¹ as compared to the control. However, a higher rate of N (N2) did not significantly increase FFB yield since the difference is only 0.09 t ha⁻¹. The standard rate of N fertiliser significantly produces maximum cumulative yields over 17 years of harvesting. Vegetative growth shows that higher planting density increased rachis length, frond length, height, leaf area and leaf area index. PS1 exhibited a significantly shorter rachis length measuring 3.08% less at 6.18 m, in comparison to DxP which measured 6.33 m. Over 17 years of harvesting, a planting density of 140 palms ha⁻¹ yielded the highest cumulative and average FFB yield amounting to 426.96 and 24.19 t ha⁻¹ yr⁻¹ respectively. However, it was not significantly different to the yield achieved at a density of 160 palms ha⁻¹, which yielded cumulatively at 425.68 t ha⁻¹ yr⁻¹ and average of 24.12 t ha⁻¹ yr⁻¹. The study shows that the standard practice of 140 palms ha⁻¹ continues to be the preferred agronomic optimum planting density.

Keywords: density, *Elaeis guineensis*, nitrogen, progeny, yield.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) maintains its position as the predominant commodity crop in Malaysia, leading in terms of planted area, production, and export earnings. In 2022, the overall oil palm planted area reached 5.67 million hectares, which slightly declined by 1.1% from the previous year due to the improvement in MPOB renewal licensing procedures for independent smallholders (Parveez et al., 2023). The palm oil sector contributed RM48 billion to the gross domestic product (GDP), accounting for 3.6% of Malaysia's total GDP in 2020, making it the foremost contributor among all

commodity crops (Ministry of Plantation Industries and Commodities, 2021).

Oil palm plantations are usually found on alluvial and inland soils (Sergieieva, 2023). In Malaysia, there are around 500 distinct soil types, with residual soils and alluvial soils emerging as the most common, including in Northern Borneo (Ashraf et al., 2017; Sellan et al., 2021). The alluvial soils originate from the weathering of hills composed of mudstone and sandstone and they are considered as types of soils which are suitable for oil palm plantations. These soils can vary from clayey and silty to sandy, and they are rich in organic matter with a pH range of 4.0-6.8 (Din et al., 2021; Sellan et al., 2021).

The fertilisation process plays a significant role throughout the majority of the oil palm's life cycle (Darras et al., 2019). Nitrogen (N) fertiliser is frequently employed in oil palm plantations as one

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of the commonly used chemical fertilisers (Azahari & Sukarman, 2023). The suggested application rate for N fertiliser in oil palm varies from 48-260 kg N ha⁻¹ yr⁻¹, with options for either split rates every six months or a single annual dose (Skiba et al., 2020).

Nitrogen fertiliser problems on alluvium soils in Malaysia are not clearly mentioned. However, N fertiliser management is a common issue in oil palm plantations. According to Sugianto et al. (2023), a substantial portion of the 973 fields, each averaging 2 ha, exhibited insufficient N levels, accounting for approximately two-thirds of the total. N losses are the most uncertain aspects of N fluxes. Hence, additional investigation into N losses is essential to enhance understanding of minimising environmental impacts and enhancing the economic and agro-ecological efficiency of management practices (Pardon et al., 2016).

Increasing crop yield has been the priority for most planters and can be accomplished by planting more productive varieties. Most breeding programmes are industry-driven, therefore aside from focusing on producing high oil-yielding planting materials, efforts are also channelled towards developing dwarfed planting materials to address harvesting issues. The new generation of oil palm planting materials based on MPOB-Nigerian *dura* × AVROS *pisifera* (PS1) was introduced to reduce the palm height of the standard (DxP) planting material from the current 45-75 cm yr⁻¹ to an average height increment of 40 cm yr⁻¹ (Kushairi et al., 2003; Rajanaidu et al., 1999). The PS1 was developed by MPOB to address harvesting issues that become more challenging as the palms mature. Despite the slow growth, PS1's FFB yields were not compromised as it is able to produce about 30.0-33.0 t ha⁻¹ yr⁻¹ with high oil content, high percentage of oil to bunch (O/B) and potentially increased oil yield of 7.7 t ha⁻¹ yr⁻¹ (Wahid et al., 2005). According to Hafiz and Rashid (2011), PS1 produces lighter fruitlets per bunch but records higher bunch weight compared to other standard planting materials.

Planting density in oil palm refers to the number of oil palm trees planted per hectare in a plantation. Typically, oil palm (*E. guineensis* Jacq.) is commonly planted in a triangular pattern (Bonneau et al., 2018; Van Leeuwen, 2019), at a density of 130-160 palms ha⁻¹ with a spacing of 8.50-9.42 m depending on the soil type and environmental conditions. Studies on planting density and its effect on oil palm yield have been extensively explored (Barcelos, et al., 2015; Bonneau et al., 2018; Korol et al., 2021; Woittiez et al., 2017). Increasing the number of productive oil palm trees per hectare is one feasible strategy to increase palm oil productivity. However, this is deemed a simplistic solution as there are other factors i.e., environmental, that need consideration.

There are several techniques to define the optimal oil palm planting density as follows:

- Current optimum: The oil palm planting density that provides the highest yield in any given year (Bonneau et al., 2018).
- Agronomic optimum: Oil palm planting density that produces the maximum cumulative FFB output over a specific period (Bonneau et al., 2018).
- Economics optimum: The density of oil palm plantations that produces the maximum accumulated discounted profit over a certain period (Latif et al., 2003).

In mineral soils, commercial DxP is routinely planted at 136-148 palms ha⁻¹. However, with the new and high-yielding variety, PS1, introduced in 1999, the feasibility of higher-density planting was explored. As with most agronomy studies, establishing the optimal planting density requires long-term evaluation. This article reports on the response of the progeny lineage towards higher planting densities and N fertiliser application on alluvial soil.

MATERIALS AND METHODS

The study was conducted at Lahad Datu, Sabah, 05°07'50"N latitude and 118°26'34"E longitude at an elevation of 50 m above sea level. The experimental site was located on the Bengawat family soil series and was classified as Typic Endoaquepts (USDA Soil Taxonomy) or Eutric Gleysols (FAO/UNESCO Legend). The trial was a randomised complete block design (RCBD) with split plot treatments. The RCBD was chosen for its practicality in the field, easy to implement and provide manageable experimental design. The design allows detection of treatment effects with a smaller sample size such as N. It was initiated in May 2000 with the following treatments: Main plot of four densities (D1: 140 palms ha⁻¹, 9.06 m triangular spacing; D2: 160 palms ha⁻¹, 8.50 m triangular spacing; D3: 180 palms ha⁻¹, 8.00 m triangular spacing; and D4: 200 palms ha⁻¹, 7.60 m triangular spacing), replicated thrice.

Two palm progenies (PS1 and commercial DxP) form the subplot treatments and three levels of N fertiliser application (N0 – control, N1 – 0.63 to 0.95 kg N palm⁻¹ yr⁻¹, and N2 – 1.26 to 1.89 kg N palm⁻¹ yr⁻¹) as the sub-plot treatments. Different varieties of oil palm may have distinct genetic characteristics, growth patterns, and nutrient requirements. The N fertiliser used was sulphate of ammonia (21% N). In addition, muriate of potash (MOP – 60% K₂O), rock phosphate (RP – 30% P₂O₅), kieserite (27% MgO) and borate 48 (48% B₂O) were applied as per normal practice (Nur Zuhaili et al., 2021).

Vegetative measurements were conducted on three to four recording palms per plot using conventional non-destructive techniques on frond 17 from the selected palms. Previous research (Foster, 2003) suggested that frond 17 be used as a reference for leaf analysis due to its midpoint location and the correlation between fruit bunch yield and leaf nutrient levels. Measurements were done once a year to monitor the oil palm vegetative growth in terms of frond production, leaf area, petiole cross-section, trunk height, and diameter. As for the leaf nutrient content, the central leaflets were sampled from frond 17. Calcium (Ca) and magnesium (Mg) content were determined using atomic absorption spectrophotometer (AAS). Colourimetry using the vanadomolybdate yellow method was used to measure phosphorus (P), while potassium (K) content was determined by a flame photometer. Nitrogen content in plant was analysed through wet digestion and titration by observing the colour change to red as endpoint was obtained. Soil samples were taken one year after planting by using a screw auger at three sampling points - the palm circle, frond pile and avenue and at three soil depths at each point, 0-15, 15-30 and 30-60 cm. The soil samples were analysed for pH, organic carbon (C), total N, available P, exchangeable K, Ca, Mg and soil texture.

Fresh fruit bunch (FFB) number and weight of the recording palms of each plot were taken at harvesting rounds every 10-15 days. The effects of the various treatments on palm growth and yield were analysed using analysis of variance (ANOVA) for split-plot design. Significant differences at $P \leq 0.05$ were determined followed by Duncan's test (DMRT) to compare the means of the parameters. All tests were accomplished using the SAS Statistics software program.

RESULTS AND DISCUSSION

Soil Physico-chemical Properties

Results of soil physical analysis of Bengawat Series are shown in *Table 1*. The soil texture analysis revealed that the clay content in the topsoil (0-15 cm) was about 14%, slightly higher to over 17% at 45 cm depth. The topsoil silt content was over 50%, marginally decreasing with depth whereas the fine and coarse sand increased slightly. These soils occurred on a level terrain and were characterised by the gleyic horizon occurring within 50 cm of the soil surface. The gleyic colour (2.5Y7/2-10.0YR7/2) horizon occurs within 50 cm due to poorly drained conditions. The soils were texturally classified as fine sandy clay to heavy clay with moderate medium subangular blocky and slightly sticky.

Most of the chemical parameters were found to be very high compared to the nutrients required by

the palms except for available P and organic C. In general, most soil nutrients decreased with depth, especially the mobile nutrients such as N and K. Available P was much higher in the topsoil (11.9 mg kg⁻¹) because P is relatively immobile. Exchangeable Ca and Mg did not change significantly with depth. The presence of high exchangeable Ca [13.68-16.02 cmol (+) kg⁻¹] and Mg [6.04-6.33 cmol (+) kg⁻¹] would most probably affect K and Mg uptake due to the dominance of Ca²⁺ ions in the soil as Ca constitutes over 65% of the total soil exchangeable cations. Exchangeable Ca and Mg in Bengawat family soils are generally higher, especially exchangeable Ca than in other soils.

TABLE 1. SOIL PHYSICAL ANALYSIS OF BENGAWAT SOIL

Soil texture (%)	Soil depth (cm)		
	0-15	15-30	30-45
	Mean ± SE	Mean ± SE	Mean ± SE
Clay	13.99 ± 1.18	15.51 ± 1.49	17.88 ± 1.46
Silt	51.26 ± 2.55	49.46 ± 2.73	45.27 ± 2.64
Fine sand	29.56 ± 1.28	29.35 ± 1.25	30.74 ± 1.31
Coarse sand	5.23 ± 0.72	5.68 ± 0.90	6.11 ± 0.89

Note: Values are the means from 48 samples; SE - standard error.

Effect of Nitrogen (N) and Vegetative Growth

The analysis of variance shows N had a significant effect on FFB yield as early as the sixth-year harvest. Over 17 years of harvest, there was a significant difference in average FFB yield by 0.89 t ha⁻¹ at the N1 rate as compared to the control plot (N0). Application of N at a higher rate (N2) did not significantly increase FFB yield and its components, the difference was only 0.09 t ha⁻¹. The results show that the N requirement for oil palm on alluvial soils was between 3-6 kg sulphate of ammonia (SOA) palm⁻¹ yr⁻¹ and it was in line with previous studies (Chang et al., 2022) which reported that the N requirement was about 4.2 kg SOA palm⁻¹ yr⁻¹ to meet the FFB yield of 30 t ha⁻¹ yr⁻¹. Meanwhile, the low level of leaf K in the entire plot was due to the imbalance effect of Mg and Ca on the soil cation exchange site. The leaf K ranged between 0.47% to 0.53% which was significantly low compared to the standard K values. The high values of exchangeable Mg and Ca in Bengawat soils contributed to the low uptake of K due to the dominance of Mg and Ca ion in the soil.

The summarised vegetative parameters in the ninth year of planting are in *Table 2*. Between the period of 7th-10th year after planting, increased planting density resulted in high leaf area index (LAI) values. The LAI greater than six indicates high inter-plant competition for light, water, and nutrients, subsequently affecting both bunch number and bunch weight. Aside from that, LAI

above six also contributed to higher total dry weight production caused solely by better vegetative growth, as yield ha^{-1} declined (Breure, 2003). Table 2 shows higher planting density has led to an increment in the rachis length, height, leaf area and LAI. Increasing planting density from 140 to 160, 180 and 200 palms ha^{-1} produced longer rachis length by 4.22%, 8.00% and 10.50%, respectively however no significant difference was detected between the density of 140 and 160 palms ha^{-1} . Planting at high density enhanced frond length, thus increasing the LAI values which benefits dry matter production for vegetative growth.

The LAI increased from 4.98-5.52 and 6.42-6.89 with increased planting density from 140-160 and 180-200 palms ha^{-1} , respectively with a decrease in annual frond production. Planting at high density resulted in low frond production and palm trunk diameter. Meanwhile, planting at 160 palms ha^{-1} with an index less than 5 affected the average bunch numbers. The LAI was expected to increase consistently with increasing palm age, especially at higher density. The frond production was about 4.30% and 8.12% lower at 180 and 200 palms ha^{-1} as compared to 140 palms ha^{-1} . The palm girth diameter was 5.97% and 10.45% lower at 180 and 200 palms ha^{-1} , respectively than 140 palms ha^{-1} . The palm height increment was between 0.44 to 0.46 m yr^{-1} without any significant difference at various densities. The results show that PS1 has a significantly shorter rachis length of 0.02 m or 3.08% compared to DxP. Indirectly, this variety is suitable for denser than the usual planting density. In the ninth year of planting, the height increment of PS1 in N1 plots ranged from 0.41-0.49 m yr^{-1} , while the DxP is between 0.44-0.47 m yr^{-1} , without significant difference.

Oil Palm Fresh Fruit Bunches (FFB) Yield and Bunch Components

The analysis of variance for FFB yield and components over 17 years of recording shows no significant interaction between planting density, progeny and N. FFB and its components were significantly affected by density, however, it was only FFB that varied with N. The application of fertiliser can affect FFB yield, but the relationship between density and fertiliser is not straightforward. According to Prabowo et al. (2023), the response of yield, nutrient uptake, and recovery efficiency (RE) to fertiliser application was significantly correlated with several factors, but the maximum yield response was negatively correlated with nutrient uptake. The mean and cumulative FFB yield and bunch components over 17 years were summarised in Table 3, except for density at 200 palms ha^{-1} . Data recording for the latter planting design was taken only for nine years because of a drastic decline in

yield in the 10th year of harvest due to the etiolation of the palms. Over 17 years of harvesting, it was the planting density at 140 palms ha^{-1} that gave the highest cumulative and average FFB yields but with no significant difference when compared to 160 palms ha^{-1} planting density.

An increase in planting density from 140 to 160 and 180 palms ha^{-1} had resulted in significantly decreased cumulative bunch production from 213.64 to 188.09 (11.95%) and 167.51 (21.59%) bunches palm^{-1} , respectively. These results were similar to previous studies. According to Bonneau et al. (2018) and Romero et al. (2022), higher planting density resulted in lower cumulative FFB production. The cumulative average bunch weight was slightly higher from 276.27-280.11 (1.37%) although negligible, at a density of 140-160 palms ha^{-1} , respectively. However, it significantly decreased from 276.27-262.59 (5.21%) with planting density from 140-180 palms ha^{-1} . The decrease in bunch weight is visible with the increase in density.

The optimum yield was obtained at a density of 140 palms ha^{-1} . Without any thinning practices at the early stages, planting density at 180 palms ha^{-1} was not advantageous for both progenies on alluvial soils. An increase in planting density from 140-180 and 160-180 palms ha^{-1} significantly decreased cumulative FFB yield by 17.49 t ha^{-1} (4.10%) and 16.21 $\text{t ha}^{-1} \text{yr}^{-1}$ (3.80%), respectively. Both progenies had no significant effect on FFB yield and bunch components. The PS1 produced a balanced cumulative bunch number and bunch weight; 171.99 bunches and 223.71 kg as compared to 165.32 bunches and 233.75 kg of DxP. Meanwhile, an increased rate of double N application from the recommended estate rate did not significantly increase the FFB yield and bunch components. A significant difference was found only in the control plot. The effects of planting density, progeny lineage and N on annual FFB yield $\text{t ha}^{-1} \text{yr}^{-1}$ were summarised in Table 4 and 5, average bunch weight (kg bunches^{-1}) (Table 6 and 7) and average bunch number ($\text{no palm}^{-1} \text{yr}^{-1}$) in Table 8 and 9. Table 4 and 5 show that the current optimum planting density for the first four years of cropping was 200 palms ha^{-1} , then reduced to between 160 and 140 palms ha^{-1} on the fifth year onwards. The results suggested that both lineages can be planted at a higher density for the first four years of cropping on alluvial soils. A planting density of 160-200 palms ha^{-1} gave an advantage of higher early yields which was similar to a study conducted by Latif et al. (2003).

As shown in Table 3, the cumulative yield of 140 and 160 palms ha^{-1} was not significantly different, whereby the extra palms in the latter planting gave no advantage on cumulative yields beyond the 10th year of harvesting onwards. The study showed a significant effect of progeny lineage on average bunch weight and bunch number. PS1 had a significantly

higher bunch number with a lower bunch weight compared to DxP. This difference was noticeable in the fourth year of harvest. However, over 17 years of harvesting, both bunch characters were not

significantly different. A similar trend was observed i.e., a higher bunch number with lighter bunch weight, which was the preferable bunch characteristic for the field workers.

TABLE 2. VEGETATIVE GROWTH OF OIL PALMS IN RESPONSE TO VARIOUS TREATMENTS

Planting density (palms ha ⁻¹)	Frond production	Rachis length (m)	Height (m)	Diameter (m)	Relative leaf area (m ²)	Frond dry weight (kg)	Petiole cross-section (cm ²)	Leaf area index	Height increment
140	24.11a	5.89c	3.13b	0.67a	16.18a	3.67a	33.89a	4.98d	0.45a
160	23.74a	6.15b	3.22b	0.67a	16.46a	3.85a	35.62a	5.52c	0.46a
180	23.07b	6.41a	3.08b	0.63b	16.20a	4.01a	37.19a	6.42b	0.44a
200	22.16c	6.58a	3.98a	0.60c	16.71a	3.62a	33.38a	6.89a	0.44a
LSD _(0.05)	0.6479	0.2042	0.2466	0.0185	0.7687	0.4490	4.3870	0.3429	0.0355
MSE	3.4943	0.3983	0.3566	0.0033	3.3134	0.7338	70.1861	1.9766	0.0072
CV (%)	4.13	4.84	11.81	4.29	7.07	17.59	18.59	8.55	11.91

Progenies	Frond production	Rachis length (m)	Height (m)	Diameter (m)	Relative leaf area (m ²)	Frond dry weight (kg)	Petiole cross-section (cm ²)	Leaf area index	Height increment
PS1	23.46a	6.18b	3.10a	0.65a	16.07a	3.58b	32.93b	5.92a	0.44a
DxP	23.08a	6.33a	3.09a	0.63b	16.20a	4.00a	37.11a	5.99a	0.44a
LSD _(0.05)	0.4582	0.1444	0.1744	0.0131	0.5435	0.3175	3.1020	0.2425	0.0251

Nitrogen level	Frond production	Rachis length (m)	Height (m)	Diameter (m)	Relative leaf area (m ²)	Frond dry weight (kg)	Petiole cross-section (cm ²)	Leaf area index	Height increment
N0	22.43b	6.23a	3.01a	0.64a	15.93a	3.76a	34.72a	5.83b	0.43a
N1	23.74a	6.26a	3.13a	0.64a	15.95a	3.78a	34.94a	5.83b	0.45a
N2	23.65a	6.27a	3.16a	0.65a	16.53a	3.83a	35.41a	6.20a	0.45a
LSD _(0.05)	0.5611	0.1768	0.2136	0.0161	0.6657	0.3888	3.7990	0.2970	0.0308

Note: Values are mean of three replications in the ninth year of recording. Means with different alphabets in the same column are significantly different at 5% level with DMRT (Duncan Multiple Range Test).

TABLE 3. MEAN AND CUMULATIVE OF FFB YIELD AND BUNCH COMPONENTS OVER 17 YEARS OF HARVESTING

Planting density (palms ha ⁻¹)	ABNO (palm ⁻¹)		ABWT (kg bunch ⁻¹)		FFB (t ha ⁻¹ yr ⁻¹)	
	Mean	Cumulative	Mean	Cumulative	Mean	Cumulative
140	12.80a	213.25a	15.55a	276.27a	24.19a	426.96a
160	11.31b	188.09b	15.75a	280.11a	24.12ab	425.68a
180	10.20c	167.51c	14.79b	262.59b	23.31b	409.47b
200*	12.79a	115.08d	10.43c	100.81c	23.38b	222.83c
LSD _(0.05)	0.644	9.35	0.729	11.34	0.747	10.83
CV (%)	8.12	7.95	7.66	8.94	4.68	4.51

Progenies	ABNO (palm ⁻¹)		ABWT (kg bunch ⁻¹)		FFB (t ha ⁻¹ yr ⁻¹)	
	Mean	Cumulative	Mean	Cumulative	Mean	Cumulative
PS1	10.45a	171.99a	12.62a	223.71a	20.91a	366.77a
DxP	10.09a	165.32a	13.18a	233.75a	20.97a	368.15a
LSD _(0.05)	0.455	6.614	0.515	8.022	0.528	7.655

Nitrogen level	ABNO (palm ⁻¹)		ABWT (kg bunch ⁻¹)		FFB (t ha ⁻¹ yr ⁻¹)	
	Mean	Cumulative	Mean	Cumulative	Mean	Cumulative
N0	10.26a	168.19a	12.39b	219.73b	20.32b	356.18b
N1	10.39a	171.61a	12.98ab	230.28ab	21.21a	372.80a
N2	10.16a	166.27a	13.32a	238.18a	21.30a	373.33a
LSD _(0.05)	0.558	8.101	0.631	9.82a	0.647	9.376

Note: Mean and cumulative values over ninth of years recording; ABNO - average bunch number; ABWT - average bunch weight; FFB - fresh fruit bunch. Figures are the mean and cumulative of three replications over 17 continuous years of recording. Means with different alphabets in the same columns are significantly different at a 5% level with DMRT (Duncan Multiple Range Test).

TABLE 4. THE EFFECT OF PLANTING DENSITY, PROGENY LINEAGE AND NITROGEN ON ANNUAL FFB YIELD (YEAR 1 TO 9)

Planting density (palms ha ⁻¹)	Year of harvesting									Average (t ha ⁻¹ yr ⁻¹)
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
140	8.52b	19.98b	27.94b	24.86b	27.96ab	30.21a	28.16a	29.97a	27.49a	25.01a
160	8.56b	20.48ab	30.69a	25.79ab	29.03a	29.02ab	27.64ab	27.96b	26.72a	25.09a
180	10.17a	20.02b	29.97ab	26.47a	28.52ab	28.20b	26.31b	26.54b	25.15ab	24.59a
200	10.93a	21.76a	29.97ab	26.67a	26.84b	25.11c	22.59c	23.31c	23.21b	23.38b
LSD _(0.05)	1.069	1.540	1.967	1.323	1.804	1.605	1.437	1.976	2.394	0.748
CV (%)	16.63	11.12	9.85	7.57	9.53	8.47	8.15	10.89	13.86	4.53
Current optimum density (palms ha ⁻¹)	200	200	160	200	160	140	140	140	140	

Progenies	Year of harvesting									Average (t ha ⁻¹ yr ⁻¹)
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
PS1	9.62a	20.89a	29.68a	26.07a	28.07a	28.00a	26.33a	27.45a	25.93a	24.67a
DxP	9.47a	20.24a	29.61a	25.83a	28.11a	28.27a	26.02a	26.44a	25.36a	24.37a
LSD _(0.05)	0.756	1.089	1.391	0.936	1.276	1.135	1.016	1.397	1.693	0.518

Nitrogen Level	Year of harvesting									Average (t ha ⁻¹ yr ⁻¹)
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
N0	9.53ab	20.71a	29.16a	26.01a	27.74a	27.40b	25.24b	25.53b	25.23a	24.06b
N1	9.04b	20.36a	30.29a	25.84a	28.78a	28.05ab	26.63a	28.00a	25.71a	24.74a
N2	10.08a	20.63a	29.48a	25.99a	27.75a	28.95a	26.65a	27.30a	25.99a	24.76a
LSD _(0.05)	0.926	1.334	1.703	1.146	1.562	1.390	1.245	1.711	2.073	0.648

Note: Values are mean of three replications in the ninth year of recording. Means with different alphabets in the same column are significantly different at a 5% level with DMRT (Duncan Multiple Range Test).

TABLE 5. THE EFFECT OF PLANTING DENSITY, PROGENY LINEAGE AND NITROGEN ON ANNUAL FFB YIELD (YEAR 10 TO 17)

Planting density (palms ha ⁻¹)	Year of harvesting								Average (t ha ⁻¹ yr ⁻¹)
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
140	20.52a	21.81a	24.04a	23.22a	24.35a	21.88ab	26.04a	24.34b	23.27a
160	18.67a	21.54ab	26.21a	23.40b	22.89a	22.82a	24.28b	24.42a	23.03ab
180	20.45a	21.00b	24.35a	18.12c	23.17a	20.23a	23.28b	24.38a	21.87b
LSD _(0.05)	2.183	1.606	2.390	1.805	1.787	1.917	1.540	1.713	1.231
CV (%)	21.73	15.05	19.02	17.36	15.07	17.53	12.42	13.91	8.16
Current optimum density (palms ha ⁻¹)	140	140	160	160	140	160	140	160	

Progenies	Year of harvesting								Average (t ha ⁻¹ yr ⁻¹)
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
PS1	14.69a	15.77a	18.51a	15.46a	17.20a	15.93a	18.33a	17.55b	16.68a
DxP	15.14a	15.91a	18.79a	15.41a	18.01a	16.54a	18.47a	19.02a	17.16a
LSD _(0.05)	1.543	1.135	1.690	1.276	1.264	1.356	1.089	1.211	1.018

Nitrogen level	Year of harvesting								Average (t ha ⁻¹ yr ⁻¹)
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
N0	14.29a	14.95a	16.42b	14.02b	17.18a	15.64a	18.25a	18.10a	16.10b
N1	15.12a	16.05ab	19.15a	15.89a	17.85a	16.50a	18.52a	18.85a	17.24a
N2	15.32a	16.52a	20.39a	16.39a	17.77a	16.56a	18.43a	17.91a	17.41a
LSD _(0.05)	1.890	1.391	2.070	1.563	1.548	1.660	1.334	1.484	1.244

Note: Values are mean of three replications in the eighth years of recording. Means with different alphabets in the same column are significantly different at a 5% level with DMRT (Duncan Multiple Range Test).

TABLE 6. THE EFFECT OF PLANTING DENSITY, PROGENY LINEAGE AND NITROGEN ON ANNUAL BUNCH WEIGHT (YEAR 1 TO 9)

Planting density (palms ha ⁻¹)	Year of harvesting									Average (kg bunch ⁻¹)
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
140	3.56a	6.84a	9.26a	9.88a	13.12a	12.77ab	14.08ab	17.27a	17.50a	11.59a
160	3.54a	6.60ab	9.21a	9.52ab	12.72a	12.69ab	14.20a	16.46ab	16.86a	11.31a
180	3.49a	6.51ab	9.14a	9.31ab	12.39ab	12.98a	13.50ab	15.59bc	16.61a	11.07a
200	3.40a	5.81b	8.43b	8.85b	11.45b	11.72b	12.93b	14.67c	16.53a	10.43b
LSD _(0.05)	0.299	0.797	0.585	0.714	1.029	1.042	1.137	1.269	1.212	0.618
CV (%)	12.72	18.39	9.63	11.28	12.3	12.33	12.34	11.77	10.66	8.32

Progenies	Year of harvesting									Average (kg bunch ⁻¹)
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
PS1	3.54a	6.38a	8.86a	9.09b	12.22a	11.95b	13.26b	15.45b	16.70a	10.83b
DxP	3.45a	6.49a	9.16a	9.69a	12.63a	13.14a	14.09a	16.55a	17.06a	11.36a
LSD _(0.05)	0.211	0.564	0.413	0.504	0.728	0.736	0.804	0.897	0.857	0.439

Nitrogen level	Year of harvesting									Average (kg bunch ⁻¹)
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
N0	3.41a	6.70a	8.83a	9.03b	12.06a	12.03b	12.90b	15.48b	16.49a	10.77b
N1	3.48a	6.28a	8.93a	9.21b	12.46a	12.33b	13.49b	15.72ab	16.94a	10.98b
N2	3.59a	6.34a	9.27a	9.93a	12.76a	13.27a	14.63a	16.80a	17.20a	11.53a
LSD _(0.05)	0.259	0.691	0.506	0.618	0.892	0.902	0.985	1.099	1.05	0.538

Note: Values are mean of three replications in the ninth year of recording. Means with different alphabets in the same column are significantly different at a 5% level with DMRT (Duncan Multiple Range Test).

TABLE 7. THE EFFECT OF PLANTING DENSITY, PROGENY LINEAGE AND NITROGEN ON ANNUAL BUNCH WEIGHT (YEAR 10 TO 17)

Planting density (palms ha ⁻¹)	Year of harvesting								Average (kg bunch ⁻¹)
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
140	17.94a	18.24b	20.22a	19.57ab	20.31a	21.80b	21.41ab	20.52a	20.00a
160	18.03a	19.34a	20.62a	20.53a	20.67a	23.11a	22.15a	21.64a	20.76a
180	16.72b	17.15c	18.60b	18.50b	19.44a	20.34c	20.49b	20.54a	18.97b
LSD _(0.05)	0.898	0.905	1.177	1.490	1.419	1.176	1.117	1.468	0.831
CV (%)	10.12	9.82	11.75	15.09	13.94	10.70	10.35	13.89	10.12

Progenies	Year of harvesting								Average (kg bunch ⁻¹)
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
PS1	12.97a	13.41a	14.58a	14.21a	14.73a	16.05a	15.96a	15.26a	14.65a
DxP	13.37a	13.95a	15.14a	15.09a	15.48a	16.57a	16.07a	16.09a	15.22a
LSD _(0.05)	0.635	0.640	0.832	1.054	1.004	0.831	0.790	1.038	0.760

Nitrogen level	Year of harvesting								Average (kg bunch ⁻¹)
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
N0	12.50b	12.65b	13.75b	13.99a	14.17b	15.60b	15.79a	15.37a	14.23b
N1	13.44a	14.29a	15.21a	15.20a	15.36ab	16.44ab	16.02a	15.97a	15.24a
N2	13.58a	14.11a	15.62a	14.75a	15.79a	16.90a	16.23a	15.68a	15.33a
LSD _(0.05)	0.778	0.784	1.019	1.291	1.229	1.018	0.968	1.271	1.008

Note: Values are mean of three replications in the eighth years of recording. Means with different alphabets in the same column are significantly different at a 5% level with DMRT (Duncan Multiple Range Test).

TABLE 8. THE EFFECT OF PLANTING DENSITY, PROGENY LINEAGE AND NITROGEN ON ANNUAL BUNCH NUMBER (YEAR 1 TO 9)

Planting density (palms ha ⁻¹)	Year of harvesting									Average
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
140	17.18a	21.93a	21.59a	18.27a	15.51a	17.25a	14.55a	12.60a	11.37a	16.69a
160	15.41a	20.51ab	21.15a	17.20ab	14.52a	14.57b	12.28b	10.70b	10.01b	15.15b
180	16.05a	17.43c	18.19b	16.16bc	12.89b	12.27c	10.99c	9.62c	8.53c	13.57c
200	16.15a	18.96bc	17.88b	15.22c	11.95b	10.80d	8.95d	8.06d	7.09d	12.79c
LSD _(0.05)	1.867	2.312	1.137	1.374	1.546	1.437	0.959	0.943	1.055	0.787
CV (%)	17.11	17.41	8.57	12.2	16.73	15.54	12.17	13.67	16.93	8.03

Progenies	Year of harvesting									Average
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
PS1	16.04a	19.53a	20.02a	17.32a	13.92a	14.39a	12.16a	10.78a	9.52a	14.86a
DxP	16.35a	19.88a	19.38a	16.11b	13.51a	13.05b	11.23b	9.71b	8.98a	14.24b
LSD _(0.05)	1.32	1.635	0.804	0.972	1.093	1.016	0.678	0.667	0.746	0.556

Nitrogen level	Year of harvesting									Average
	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	
N0	16.54a	19.35a	19.85ab	17.44a	13.94a	13.88a	11.84a	9.98b	9.32a	14.68a
N1	15.46a	19.93a	20.28a	17.01a	14.08a	13.86a	11.99a	10.81a	9.20a	14.73a
N2	16.60a	19.85a	18.97b	15.71b	13.12a	13.43a	11.25a	9.95b	9.23a	14.24a
LSD _(0.05)	1.617	2.002	0.985	1.19	1.339	1.245	0.83	0.817	0.913	0.682

Note: Values are mean of three replications in the ninth year of recording. Means with different alphabets in the same column are significantly different at a 5% level with DMRT (Duncan Multiple Range Test).

TABLE 9. THE EFFECT OF PLANTING DENSITY, PROGENY LINEAGE AND NITROGEN ON ANNUAL BUNCH NUMBER (YEAR 10 TO 17)

Planting density (palms ha ⁻¹)	Year of harvesting								Average
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
140	8.25a	9.02a	8.81a	8.79a	8.64a	7.06a	8.26a	8.55a	8.42a
160	6.72b	7.08b	8.21a	6.42b	6.96b	6.16b	6.97b	7.33b	6.99b
180	6.48b	6.06c	7.45b	5.93b	6.59b	5.68b	6.28b	6.76b	6.40c
LSD _(0.05)	0.701	0.756	0.698	0.786	0.714	0.593	0.767	0.737	0.531
CV (%)	19.40	20.19	16.93	22.09	19.10	18.63	21.17	19.33	10.72

Progenies	Year of harvesting								Average
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
PS1	5.38a	5.61a	6.14a	5.40a	5.65a	4.67a	5.47a	5.59a	5.48a
DxP	5.35a	5.52a	6.10a	5.17a	5.44a	4.78a	5.29a	5.73a	5.42a
LSD _(0.05)	0.495	0.535	0.493	0.556	0.504	0.419	0.542	0.521	0.412

Nitrogen level	Year of harvesting								Average
	Y10	Y11	Y12	Y13	Y14	Y15	Y16	Y17	
N0	5.13a	5.37a	5.69b	4.71b	5.71a	4.68a	5.35a	5.69a	5.29a
N1	5.51a	5.59a	6.22ab	5.43a	5.52a	4.72a	5.30a	5.77a	5.51a
N2	5.45a	5.73a	6.45a	5.72a	5.41a	4.78a	5.49a	5.53a	5.57a
LSD _(0.05)	0.607	0.655	0.604	0.681	0.618	0.514	0.664	0.638	0.438

Note: Values are mean of three replications in the eighth years of recording. Means with different alphabets in the same column are significantly different at a 5% level with DMRT (Duncan Multiple Range Test).

CONCLUSION

Non-destructive vegetative measurements, leaf nutrient content, soil sampling, and FFB counts are parameters that are commonly used for long-term study to gather reliable data without destroying the main palms. The FFB yield profile over 17 years of cropping was sufficient for a suitable recommendation towards an optimum agronomic planting density for PS1. Growth characteristics of PS1 such as shorter rachis length and lower height increment are ideal characteristics for planting at higher densities of 160 palms ha⁻¹. However, upon long-term evaluation, the FFB yield at 140 palms ha⁻¹ densities gave better cumulative results. The study shows that the standard practice of 140 palms ha⁻¹ is still the preferred agronomic optimum planting density. The finding of this study has demonstrated the importance of long-term evaluation of planting material testing at different planting densities for not less than the tenth year of harvest. The optimum agronomic density of 140 palms ha⁻¹ can be achieved without a need for thinning and the extra palms in 160 palms ha⁻¹ planting gave no advantage on cumulative yields.

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LIFE CYCLE, FOLIAR CONSUMPTION RATE, NATURAL ENEMIES AND POPULATION FLUCTUATION OF *Phobetrion hipparchia* CRAMER, 1777 (LEPIDOPTERA: LIMACODIDAE): AN EMERGING PEST OF OIL PALM IN COLOMBIA

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ABSTRACT

Phobetrion hipparchia is an occasional pest in oil palm plantations in the Colombian Caribbean zone. Its life cycle and foliar consumption rate were determined under laboratory conditions; additionally, the population fluctuation in an oil palm lot was recorded through sampling carried out every 20 days for two years. Subsequently, the population dynamics of *P. hipparchia* were correlated with the climatic variables: Precipitation, relative humidity (RH), temperature and natural biological control. The life cycle of *P. hipparchia* under laboratory conditions ($27.3 \pm 3.8^{\circ}\text{C}$, $85 \pm 15\%$ RH) lasted 74.3 ± 8.2 days, with the developmental phases distributed as follows: Egg: 6.3 ± 0.7 days, larva: 49.1 ± 5.3 days passing through nine instars, pupa: 14.4 ± 0.9 days and adult 4.5 ± 1.3 days. The larvae of *P. hipparchia* consumed 682.9 ± 103.6 cm² of the leaf blade. No correlation was found between the population dynamics and the climatic variables recorded. However, a correlation was found between the population dynamics of the larvae of *P. hipparchia* and the biological control caused by *Baryceros* sp. ($\rho = 0.685$; $p < 0.0001$) and *Cordyceps* sp. ($\rho = 0.505$; $p < 0.001$). This information enables the recognition of *P. hipparchia* in oil palm plots and emphasises the importance of natural control in regulating defoliating insect populations.

Keywords: *Baryceros*, climate, *Cordyceps*, herbivory, leaf-eating.

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INTRODUCTION

The cultivation of oil palm *Elaeis guineensis* Jacq., 1,763 is one of the main agro-industries in Colombia, with 595,722 ha currently planted, of which 111,790 ha are planted in the Colombian Caribbean, mainly in the departments of Magdalena and Cesar (Fedepalma, 2022). *Elaeis guineensis*, being an introduced species, is susceptible to pathogenic organisms or insect pests that initially fed on native palms and have migrated as well as adapted to

feeding on oil palms (Hernández-Lambrano et al., 2014; Mexzón et al., 1996; Plata-Rueda et al., 2020).

In Colombia, the main pests that attack oil palm crops belong to the order Lepidoptera, which are mostly defoliators for example *Opsiphanes cassina* Felder, 1862; *Stenoma impressella* Busck, 1914 and *Euprosterna elaeasa* Dyar, 1906 (Escalante et al., 2010; Hernández-Lambrano et al., 2014). The severity of the damage caused by defoliating insects depends on specific aspects of each insect pest species, such as the length of their life cycle, the rate of foliar consumption, and their association with pathogens that cause foliar necrosis (Calvache, 2004). Most of the defoliating insects that affect oil palm plantations in Colombia belong to the Limacodidae family, and their larvae are commonly known as

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slimy caterpillars; they are characterised because they are generally colourful, with a variety of shapes and sizes (Zaspel et al., 2016), they have an elastic abdomen, small thoracic legs, a retractile head capsule and usually have stinging hairs on the body (Epstein, 1996; Lill et al., 2006).

Most of the species in this family have a short life cycle (Syed, 1994); for example, the life cycle of *E. elaeasa* Dyar, 1906 is 63.0 days, *Sibine megasomoides* Walker, 1866 is 90.4 days and *Acharia fusca* Stoll, 1781 is 63.7 days (Alvarado et al., 2014; Martínez et al., 2014; Mexzón et al., 1996). However, in many species (e.g., *Phobetrion hipparchia* Cramer, 1777) belonging to this family, basic aspects such as their life cycle, natural enemies and foliar consumption rate are unknown. This species has been recorded to cause severe defoliation in oil palm plantations located on the Colombian Caribbean coast but also has been recorded to cause damage to eucalyptus (Pereira et al., 2001) and macadamia crops (Villegas, 2005). Due to the above and considering that the knowledge of the life cycle and the rate of foliar consumption is necessary to know the economic impact that a pest insect can have on its host (Pozo et al., 2005) and the establishment of integrated pest control management program, this study was carried out to determine the life cycle and foliar consumption rate of *P. hipparchia*, as well as its population fluctuation in oil palm plots and its natural enemies.

MATERIALS AND METHODS

Life Cycle and Morphology of *P. hipparchia*

During 2022, *P. hipparchia* larvae were collected in plots from the Palmas Sicarare plantation, located in the Agustín Codazzi municipality, Cesar, Colombia, 09°55'41"N, 73°18'17"W. The collected larvae were transferred in styrofoam cellars to the Entomology Laboratory located in the Palmar de la Sierra Experimental Field (Banana Zone, Magdalena, d Colombia). In the laboratory i.e. temperature and relative humidity (RH) ($27.3 \pm 3.8^{\circ}\text{C}$, $85 \pm 15\%$ RH), the larvae were individualised in Petri dishes, which contained a piece of absorbent paper moistened with sterile water and a 5 x 5 cm piece of palm leaflet collected from leaf 17 of oil palm cultivar IRHO. Every two days, the piece of leaflet and the piece of absorbent paper were replaced. The larvae were maintained under these conditions until they reached the pupal stage when the moistened paper and piece of palm leaflet were permanently removed. When the adults emerged from the pupae, they were transferred to an entomological cage (50 x 50 x 50 cm) made with a muslin cloth. Pieces of leaflets were placed inside the cage for the female's oviposition. The eggs were individualised in Petri dishes, and

the time from oviposition to the emergence of the larvae was recorded. The larvae were maintained using the same methodology as those from the field, their development was monitored daily with recordings on the date of instar change until the pupal stage, after which, the adults were placed in the oviposition cages to determine their longevity. The morphological description was made, and the length of 30 specimens of each stage of development was measured using an Olympus SZ61® stereoscope and an Olympus EP50® camera (Tokyo, Japan).

Foliar Consumption

The leaf area consumed by 30 *P. hipparchia* larvae from the first to the last instar was quantified. The larvae were kept individually in pieces of leaflets; for each leaflet piece, the area was measured through photography and image analysed with the Imagen® 1.47 program (National Institutes of Health, Maryland, USA). The leaf was replaced every two days, and consumption was estimated by calculating the difference between the leaf area before and after feeding. Data analysis was done using descriptive statistics.

Population Dynamics of *P. hipparchia*

In studying the population dynamics of *P. hipparchia*, the Casanare 1 lot belonging to the Palmas Sicarare plantation, located in the Agustín Codazzi municipality, Cesar, Colombia, (09°55'41"N, 73°18'17"W), was selected to determine its natural enemies as well as its interaction with its surroundings. The plot of 13.8 ha was planted in 2008 with a cultivar *Elaeis guineensis* (IRHO). In this lot, 5 x 5 sequential samplings were carried out (one palm every five palms every five lines) every 20 days from December 12, 2020 to February 7, 2023. At each sampling event for every selected palm, the total number of live larvae of *P. hipparchia* present on frond 17 was recorded. The parasitised specimens were brought to the laboratory and placed into individual Petri dishes until the emergence of parasitoids. Individuals with signs of infection by an entomopathogen were transferred to the Cenipalma Laboratory of Entomopathogenic Microorganisms, located in the Palmar de las Corocoras Experimental Field (Paratebueno, Cundinamarca, Colombia) for their isolation, identification, and storage. The climatic variables were recorded with the Vantage Pro 2® weather station (Davis Instruments, Haywards, CA, USA) located approximately 0.5 km from the lot, at coordinates 9°55'58"N, 73°15'46"W. The population dynamics of *P. hipparchia* were correlated with climatic factors: Precipitation, average temperature, and, as well as with the percentage of biological control registered on each sampling date through Spearman's correlation coefficient.

RESULTS AND DISCUSSION

Life Cycle and Morphology of *P. hipparchia*

The taxonomic identity of *P. hipparchia* was confirmed by Dr. Marc E. Epstein, a specialist in the taxonomy of the family Limacodidae of the California Department of Food and Agriculture. The taxonomic identification was made via images photographed of the larval and adult stages. The life cycle of *P. hipparchia* was 74.3 ± 8.2 days, including adult longevity under laboratory conditions ($27.3 \pm 3.8^\circ\text{C}$, $85 \pm 15\%$ RH). Eggs are circular, flattened, 1.2 ± 0.2 mm in diameter and yellow in colour (Figure 1a). The larvae are typical of the Limacodidae family, with a retractable hypognathous head, reduced thoracic legs, without the presence of pseudolegs (Epstein, 1996), recently emerged, measuring 1.1 ± 0.3 mm in length, presenting a large number of simple setae grouped

in 12 projections of the body of the larva. As the larva grows these setae increase in size, covering the larva and offering a similar appearance to a spider (Figure 1b), fully developed larvae measure 32.0 ± 7.0 mm in length and are brown. The total life cycle time of 66.10% is devoted to the larval stage, during which *P. hipparchia* goes through nine larval instars (Table 1).

The larvae that are about to pupate stop feeding and generate a brown cocoon, which remains attached to the palm leaves. It has a lid at its end through which the adult emerges. Cocoons measure, on average, 18.0 ± 3.3 mm. Adults present sexual dimorphism; females are larger than males with a wingspan of 28.1 ± 2.1 mm, while in males, it is 24.1 ± 2.7 mm. Additionally, females have forewings mottled with different shades of orange, yellow and red, while, males mottled are black, grey and yellow (Figure 2).

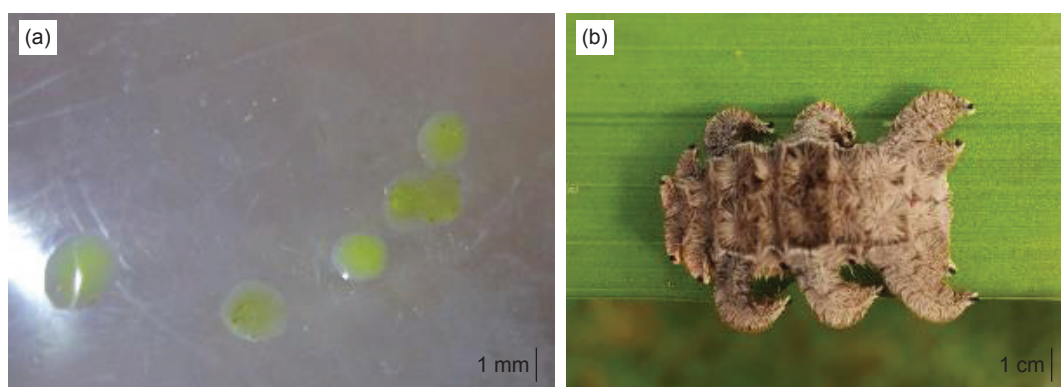


Figure 1. (a) Eggs, and (b) larvae of *P. hipparchia* on oil palm leaflets.

TABLE 1. THE LIFE CYCLE OF *P. hipparchia* UNDER LABORATORY CONDITIONS ($27.3 \pm 3.8^\circ\text{C}$, $85.0 \pm 15.0\%$ RH)

Stage	n	Average $\pm$ SD (days)	Min-Max (days)
Egg	91	6.3 ± 0.7	5-8
Larva I instar	71	4.9 ± 0.4	4-6
Larva II instar	67	4.8 ± 0.4	4-6
Larva III instar	67	4.3 ± 0.5	3-5
Larva IV instar	64	5.4 ± 0.9	4-6
Larva V instar	64	4.7 ± 0.8	4-6
Larva VI instar	63	5.1 ± 0.7	4-6
Larva VII instar	63	6.0 ± 0.6	5-7
Larva VIII instar	63	6.5 ± 0.5	6-7
Larva IX instar	63	7.4 ± 0.5	7-8
Total larva		49.1 ± 5.3	41-57
Pupa	72	14.4 ± 0.9	13-16
Adult	72	4.5 ± 1.3	3-9
Total life cycle		74.3 ± 8.2	62-90

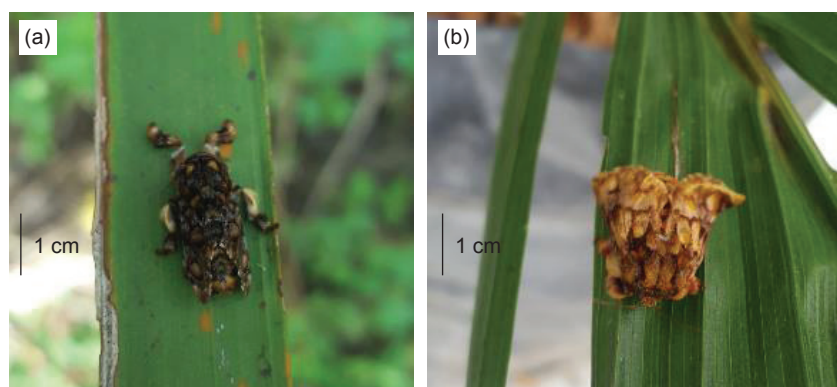


Figure 2. Adults of *P. hipparchia* on oil palm leaflets note the different colours corresponding to sexual dimorphism between adults: (a) Male, and (b) female.

Foliar Consumption

The average consumption of the leaf blade during the larval stage of *P. hipparchia* was 682.9 ± 103.6 cm² of leaf area; 69.0% was consumed in the last two instars (Table 2).

TABLE 2. CONSUMPTION RATE OF THE DIFFERENT LARVAL INSTARS OF *P. hipparchia* UNDER LABORATORY CONDITIONS ($27.3 \pm 3.8^{\circ}\text{C}$, $85 \pm 15\%$ RH)

Instar	Average $\pm$ SD (cm ²)	Intervals Min-Max (cm ²)
I Instar	1.4 ± 0.4	0.7-2.3
II Instar	2.1 ± 0.4	1.4-3.1
III Instar	3.8 ± 0.7	1.6-5.1
IV Instar	13.2 ± 1.9	9.7-17.0
V Instar	29.7 ± 3.7	23.4-37.6
VI Instar	48.9 ± 4.3	39.4-59.3
VII Instar	112.7 ± 14.6	86.0-141.0
VIII Instar	181.9 ± 37.3	134.0-301.0
IX Instar	289.1 ± 40.2	189.0-401.0
Total	682.9 ± 103.6	485.2-967.4

During the first three instars, the larvae of *P. hipparchia* only consume the superficial part of the leaf blade, and from the fourth instar onwards, they consume the leaf blade completely, leaving only the midrib (Figure 3).

Population Fluctuation of *P. hipparchia*

The monitoring of the population fluctuation of *P. hipparchia* shows that during 2021 there were two population peaks, the first in the months of January-February, when 131 larvae were recorded, and a second population peak in the months of September-October where 97 larvae were recorded, this population peak reappeared during 2022, registering on this occasion 162 larvae (Figure 4).

During the samplings, different natural enemies of *P. hipparchia* were recorded; among them, the wasp *Baryceros* sp. (Hymenoptera: Ichneumonidae) was observed parasitising 169 larvae of the 557 registered during the monitoring of the population fluctuation, which is equivalent to 30.3% of natural control. The parasitised larvae showed a yellow colouration (Figure 5), in contrast to the brown healthy larvae.

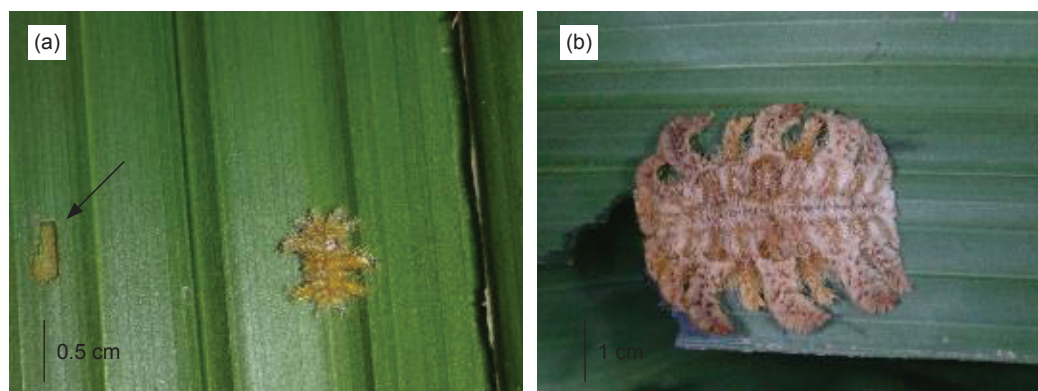


Figure 3. Larvae of *P. hipparchia*: (a) Third instar feeding on the superficial part of the leaf blade (indicated with the arrow), and (b) eighth instar larva consuming the entire leaf blade.

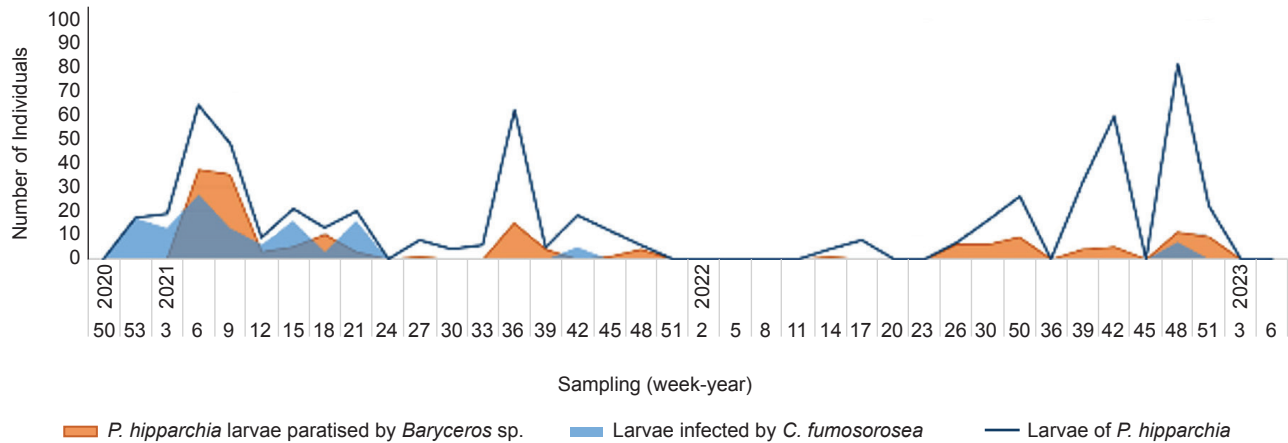


Figure 4. The number of healthy larvae of *P. hipparchia*, infected larvae with *C. fumosorosea* and larvae parasitised by *Baryceros* sp. registered on each sampling date of oil palms in the municipality of Agustín Codazzi, Cesar, Colombia.



Figure 5. *Phobetrion hipparchia* larva parasitised by *Baryceros* sp., where it is possible to observe the larva of the parasitoid developing inside the host.

The second most important mortality factor of larvae is the infections caused by fungus *Cordyceps fumosorosea* (Hypocreales: Cordycipitaceae), which was observed infecting 95 larvae of the 557 registered during the monitoring of the population fluctuation, which is equivalent to 17.0% of the larvae collected. This strain of *C. fumosorosea* was conserved in the collection Entomopathogenic Microorganisms Associated with the Oil Palm (MEAPA for its acronym in Spanish) and was encoded as MEAPA0295, for future use in biological control studies.

No association was registered between the population fluctuation of *P. hipparchia* larvae and the environmental factors studied (Table 3), but a strong association was registered between the population fluctuation of *P. hipparchia* larvae and the biological control caused by *Baryceros* sp. ($\rho = 0.685$; $p < 0.0001$) and by *C. fumosorosea* ($\rho = 0.505$; $p < 0.001$).

The duration of the life cycle of *P. hipparchia* was similar to that registered for other species of the Limacodidae family that are also pests of oil palm

TABLE 3. SPEARMAN'S CORRELATION COEFFICIENT RELATING THE FLUCTUATION OF THE *P. hipparchia* POPULATION WITH CLIMATIC FACTORS AND THE NATURAL CONTROL IN AN OIL PALM LOT

	Precipitation	Temperature	Relative humidity	<i>Baryceros</i> sp.	<i>C. fumosorosea</i>
<i>P. hipparchia</i>	0.052	-0.041	0.295	0.685	0.502
Sig.	0.756	0.805	0.072	0.0001**	0.001**
<i>Baryceros</i> sp.	0.044	0.111	0.082		
Sig.	0.794	0.505	0.626		
<i>C. fumosorosea</i>	-0.276	0.379	-0.279		
Sig.	0.093	0.019*	0.090		

Note: * - Significant at $\alpha = 0.05$; ** - Significant at $\alpha = 0.01$.

crops, such as *E. elaeasa* Dyar, 1906, *S. megasomoides* Walker, 1866 and *A. fusca* Stoll, 1781 under similar conditions (Alvarado et al., 2014; Martínez et al., 2014; Mexzón et al., 1996). Morphologically, the different stages of development are typical of specimens of the Limacodidae family (Epstein, 1996); however, unlike other species such as *A. fusca*, *E. elaeasa*, *S. megasomoides* and *A. extensa* Schaus, 1896 (Alvarado et al., 2014; Martínez & Plata-Rueda, 2013; Pacheco-Flores et al., 2006), the setae that present the larvae and those that cover the cocoons of *P. hipparchia* are not urticating.

The foliar consumption of the larvae of *P. hipparchia* (682.9 ± 103.6 cm² of leaves) is much higher than that recorded for other species of defoliating insects of the oil palm that are considered a pest of economic importance to the oil palm plantations in Colombia such as *E. elaeasa* (66.0 cm² of leaves), *A. fusca* (402.3 cm² of leaves) *S. impressella* Busck, 1914 (36.7 cm² of leaves), and *Opsiphanes cassina* Felder, 1862 (294.4 cm² of leaf) (Alvarado et al., 2014; Barrios et al., 2014; Martínez et al., 2014; Salles, 2016). This high rate of consumption implies that *P. hipparchia* has the potential to generate heavy defoliation in oil palms and could easily and quickly reach the status of an economically important pest. For this reason, their populations must be kept under permanent monitoring, especially during the second period of the year, when the highest population densities occur. Estimating the foliar consumption of defoliating insects is necessary information for determining economic damage thresholds (Salles, 2016), so this information can be used in the future for this purpose.

Similarly, the study of population fluctuation shows the existence of a strong correlation between the presence of *P. hipparchia* larvae and the presence of the natural enemies *Baryceros* sp. and *C. fumosorosea*; these organisms had already been recorded as important natural enemies of larvae of other species of the Limacodidae family (Gates et al., 2012; Vera, 2020). Other investigations have recorded that viruses of the Polihedrovirus type are one of the main regulators of the populations of larvae of the Limacodidae family (Sambiran et al., 2017; Zeddarn et al., 2003); however, in the present investigation, no *P. hipparchia* larvae with symptoms of viral infections were recorded. In turn, other studies carried out on oil palm show the importance of natural control in the regulation of pest insect populations such as *S. cecropia* and *E. elaeasa* (Alvarado et al., 2014; Sendoya & Bustillo, 2016). Monitoring the population dynamics of *P. hipparchia* shows that this insect was not permanently recorded in the oil palm plot, but rather that its presence is associated with the decrease of its natural enemies, due to this it should be considered as an occasional pest of the

oil palm in the Colombian Caribbean, concluding that maintaining adequate natural control, it is possible to keep the populations of these insect pests at levels that do not cause economic damage to the crop and reducing dependence on chemical insecticides.

CONCLUSION

Phobetron hipparchia has a short life cycle and its larvae are voracious and consume a high amount of oil palm leaf blade, which could cause severe defoliation that could affect the productivity of the oil palm, due to this, its populations should be monitored especially during the second semester, when the highest population peaks occur. On the other hand, the registered natural enemies seem promising to be used in biological control plans for *P. hipparchia*.

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GENOME SURVEY AND PRELIMINARY GENOMIC CHARACTERISATION OF A KEY OIL PALM POLLINATOR, *Elaeidobius kamerunicus*

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ABSTRACT

Elaeidobius kamerunicus is essentially the sole insect pollinator that efficiently pollinates oil palm, a major commodity crop accounting for 31.4% of the world's oils and fats production. Despite its importance, in-depth molecular studies of this pollinator are severely lacking. The scarcity of molecular information to complement the current biological knowledge of *E. kamerunicus* warrants an investigation into the genome features of the pollinator. This aids in understanding the molecular mechanisms of plant-insect interaction during pollination events. Genome size estimation was conducted on Malaysian-bred males and females *E. kamerunicus* using flow cytometry and k-mer analysis on genome survey sequencing data. The cytometric analysis underestimated the genome size compared to the computational sequence-based method with differences of 157-179 megabase (Mb) for the female and male *E. kamerunicus*, respectively. Genome sizes estimated from k-mer analysis were 365.93 Mb (female) and 380.73 Mb (male). Low sequence repeats ratios of 43.49% (female) and 43.70% (male) and high heterozygosity ratios of 1.92% (female) and 1.62% (male) were obtained. The average guanine-cytosine (GC) content of the female genome was 33.48%, and 31.71% for the male. These results lay the groundwork for *E. kamerunicus* genomic studies.

Keywords: flow cytometry, genome size, genome survey, k-mer analysis, pollinating agent, sex difference.

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INTRODUCTION

Replacing rubber as the primary economic crop, the oil palm industry is now a major contributor to Malaysia's economy, making oil palm one of the world's leading oil crops (Parveez et al., 2021). In 2021, Malaysian palm oil and its products generated RM108.52 billion in export revenue (Parveez et al., 2022). The global demand for vegetable oils may reach 240 million tonnes by

2050, as estimated by Barcelos et al. (2015). The growing demand for palm oil products, both edible and non-edible, necessitates an increase in yield productivity which has remained at around 3 t ha⁻¹ yr⁻¹ for decades (Woittiez et al., 2017). To support sustainability of the oil palm industry, increasing production without expanding into new land for oil palm cultivation is much needed. Enhancing in-field palm fruit formation can potentially improve oil yield to meet the growing demand for palm oil.

Proper fruit development therefore requires efficient pollinators, usually insects, for the fertilisation of flowers. The oil palm (*Elaeis guineensis*) is a monoecious plant, where independent male and female flowers developed separately on the same plant. *Elaeidobius kamerunicus*, an entomophilous pollinator, is specific to the oil palm, with male flowers serving

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as their preferred site for feeding, laying eggs and reproducing (Meléndez & Ponce, 2016). Both male and female weevils are vital for effective pollination, despite slight body size differences. Their pollen-carrying abilities are comparable (Dzulhelmi et al., 2022). Both are attracted by the anise-like scent of estragole released by male and female inflorescences during anthesis, enabling pollen transfer from the weevil to the stigma of female inflorescences. Its introduction into Malaysia in the early 1980s has had a positive impact on the oil palm industry, significantly enhancing palm oil production (Mohamad et al., 2023). Malaysia's success paved the way for the global introduction of *E. kamerunicus* in other oil palm-growing nations like Indonesia, Papua New Guinea, Ecuador, Colombia, Costa Rica and Central and South America. This has notably boosted oil yields in these countries (Appiah & Agyei Dwarko, 2013). This underscores the economic significance of the weevil to the palm oil industry, highlighting the importance of safeguarding their well-being.

Despite being a key oil palm pollinator, little is known about the molecular biology of *E. kamerunicus*. Apriyanto and Tambunan (2021) briefly studied its genome, based on a single male individual. A total of 26,566 predicted genes and 281,668 simple sequence repeat (SSR) markers were annotated on their 269.79 Mb genome assembly. Genome size, or deoxyribonucleic acid (DNA) C-value, is the total DNA in a haploid chromosome set, crucial for evolution, species differentiation, and hybrid identification (Bureš et al., 2004; Morgan-Richards et al., 2004; Zonneveld, 2001). Closely related species can have varying genome sizes due to repetitive sequence changes such as segmental duplications or deletions driven by environmental adaptation (Boulesteix et al., 2006; Biémont, 2008). As of 2021, genome size data exists for only 0.135% of the nearly one million known insect species, with just nine reports on curculionids (Gregory, 2023).

To estimate the genome size of a species, flow cytometric analysis was traditionally seen as the "gold standard" (Mounsey et al., 2012). However, the rapid progress of next-generation sequencing has led to the use of computational sequence-based methods, now common in many insect genome projects (Pflug et al., 2020). As molecular information on *E. kamerunicus* is rather limited, we first set out to determine the genome size of both sexes of *E. kamerunicus* as different sexes of an organism may have different genome sizes. In our study, we employed flow cytometry to estimate the genome sizes of both male and female *E. kamerunicus* weevils. We then expanded our research by using k-mer analysis with Illumina genome skim sequencing data following the

procedure of Sarmashghi et al. (2019). We compared the genome size estimates from these two methods. Our results enhanced the comprehension of genome size in the insect taxa, especially within the curculionidae family. This sequencing data also offers a valuable genomic foundation for future functional studies. The data on both sexes, together with the male genome sequence by Apriyanto and Tambunan (2021), can be used to support future efforts to generate a good *E. kamerunicus* reference genome.

MATERIALS AND METHODS

Sampling of Insects

Male and female *E. kamerunicus* weevils were captured at the MPOB Research Station in Jerantut, Pahang, Malaysia (GPS coordinate: 4°17'14"N 102°24'44"E). The weevils were randomly sampled from male inflorescences. Briefly, the fully anthesised spikelets from male inflorescence were excised and pulled into two whereby the weevils were then easily accessible. The male and female *E. kamerunicus* were manually separated (Figure 1a), flash-frozen in liquid nitrogen, and stored at -80°C for DNA extraction and flow cytometric analysis. *Drosophila melanogaster* was used as the external reference standard. *Drosophila* cultures were obtained from the Universiti Kebangsaan Malaysia (UKM) and maintained under a 12 hr light: 12 hr dark photoperiod at 25 ± 2°C in the laboratory.

DNA Isolation, Library Construction and SkimSeq Sequencing

DNA was extracted with the DNeasy Blood and Tissue kit (Qiagen) following the manufacturer's instructions. Genomic DNA quantity and quality were assessed with the Biotek Instruments Synergy HTX multi-mode reader, followed by visualisation on a 1% agarose gel. Seven male and seven female *E. kamerunicus* DNA samples were sent to Novogene, USA, for library preparation and whole-genome sequencing using the Illumina HiSeq platform. DNA from seven individuals was adequate for library preparation with the NEBNext® DNA Library Prep Kit (NEB). Briefly, 1,500 nanograms (ng) of genomic DNA was randomly sheared into approximately 350 base pairs (bp) fragments. The sheared DNA was subjected to library construction followed by end repair, dA-tailing, and further ligation with NEBNext adapters. The required fragment sizes (300-500 bp) were PCR-enriched with P5 and indexed P7 oligos. Paired-end sequencing (PE150) was conducted on the libraries. The quality control

steps for the raw sequences included removal of the adapter sequences, discarding paired reads with 10% or more ambiguous nucleotides on either read, and eliminating paired reads if over 50% of either read contained low-quality nucleotides (base quality ≤ 5) (Cock et al., 2009). The *E. kamerunicus* SkimSeq sequencing data is available at <http://genomsawit.mpob.gov.my/index.php?track=30&nu=1&info=3>.

K-mer Estimation of Genome Size, Heterozygosity Ratio and Repeated Sequences

K-mer analysis was performed on clean reads from both males and females to predict genome size, assess heterozygosity ratio, and identify repeated sequences before assembly analysis. Typically, k-mer values between 17 and 27 (Pflug et al., 2020), are used for prediction and analysis. The genome size was calculated by dividing the total k-mer count with the highest value in the k-mer frequency distribution, according to the following formula: Genome size = k-mer number/k-mer depth (Marçais & Kingsford, 2011). The revised genome size was then calculated by excluding the k-mer error. The Genomeye program (Novogene) was used to determine the genome size, heterozygosity rate and repeat content.

Flow Cytometry Analysis

Samples were prepared according to Galbraith et al. (1983), with minor modifications. Approximately seven adult weevils and seven *Drosophila* heads were dissected on a petri dish. The samples were fully homogenised in 700 μL ice-cold Galbraith's buffer (pH 7.0) containing 45 mM MgCl_2 , 20 mM MOPS [3-(N-morpholino) propanesulfonic acid], 30 mM sodium citrate and 0.1% Triton X-100. The homogenate was filtered into a 1.5 mL tube through a 40 μm cell strainer and incubated with RNase at a final concentration of 20 $\mu\text{g mL}^{-1}$ for 10 minutes at 25°C. The solution was then centrifuged at 8,000 rpm for 5 min. The pellet was resuspended in 700 μL phosphate buffer (pH 7.0), stained with 50 μL propidium iodide (PI) (1 mg mL^{-1}) and incubated in darkness at 4°C for 10 min. The suspension obtained in the final step was analysed using the BD FACSCalibur™ flowcytometry system (BD Biosciences). Cellular DNA content was measured using the fluorescence intensity of each sample exposed to 488 nm wavelength. The same parameter settings were applied to *D. melanogaster* head samples. Estimation of the nuclear DNA content was based on *D. melanogaster*, 1C = 175 Mb, as an external reference standard. Fluorescence intensity from all samples were compared with the fluorescence intensity of *D. melanogaster* to obtain the ratio, then

multiplied by the genome size of *D. melanogaster* (1C = 175 Mb). The fluorescence intensity peaks and genome size of samples were analysed using CellQuest3^R software (BD Biosciences).

The total quantity of DNA in each sample was calculated as Equation (1):

$$\text{Sample IC value} = \frac{\text{Sample 2C mean peak position}}{\text{Reference 2C mean peak position} \times \text{IC reference}} \quad (1)$$

The genome size (Mb) is reported as 1C, i.e., the mean amount of DNA in one copy of a single complete genome. One megabase is equal to one million base pairs (1 Mb = 1,000,000 bp). The entire flow cytometry experiment was repeated for a total of five replications, amounting to the use of a total of 35 males and 35 females *E. kamerunicus* individuals, and 70 *D. melanogaster* individuals.

De novo Genome Assembly

The SOAPdenovo assembler (Li et al., 2010) was used with a k-mer of 41 and default parameters to create an initial genome assembly based on a de Bruijn graph (Luo et al., 2012). After that, GC content and average sequencing depth were calculated to assess sequencing bias. To assess data contamination, 10,000 pairs of processed reads were randomly selected for a BLASTN search against the National Center for Biotechnology Information (NCBI) nonredundant (NR) nucleotide database with defined options for processors, program name, and expectation value (-a 6 -p blastn -e 1e-05). Only sequences above 100 bp were selected for assembly statistics.

RESULTS AND DISCUSSION

Genome Size Prediction of *E. kamerunicus*

This study aimed to estimate the genome sizes of both the male and female *E. kamerunicus* (Coleoptera; Curculionidae) and to also supplement the recently reported male genome size (Apriyanto & Tambunan, 2021). Genome size estimation was carried out with two approaches, a k-mer sequence-based approach and flow cytometric analysis. A single major peak in the k-mer plot provides a good approximation of the actual genome size (Figure 1). Thus, the estimated genome sizes through k-mer analysis for *E. kamerunicus* were 365.93 Mb for the female and 380.73 Mb for the male (Table 1), suggesting that the male weevil genome is bigger than the female.

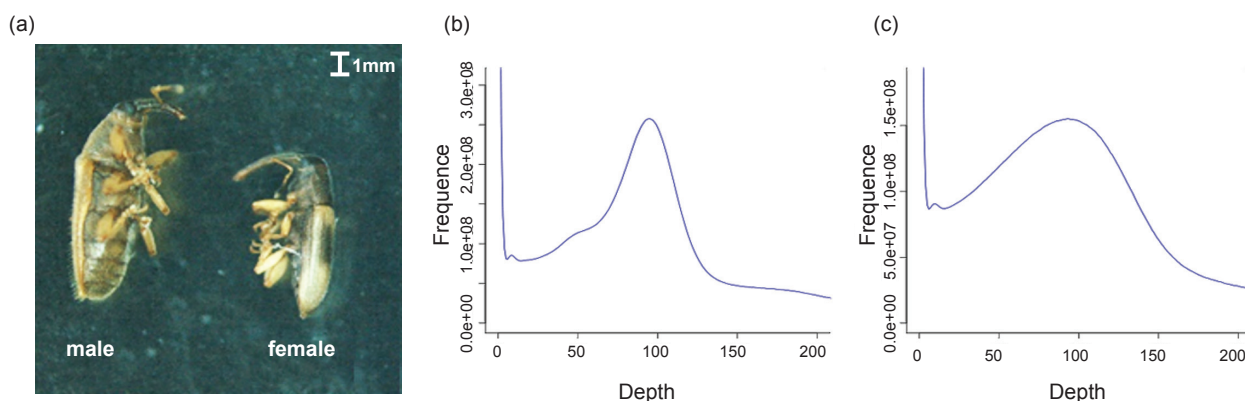


Figure 1. *E. kamerunicus* morphology and k-mer analysis plots. (a) Male and female *E. kamerunicus* adult weevils. Distribution of 17-mer depth and frequency for (b) female, and (c) male *E. kamerunicus*. The x-axis shows k-mer depth, and the y-axis represents the proportion relative to the total frequency.

TABLE 1. K-MER ANALYSIS AND *E. kamerunicus* GENOME SIZE EVALUATION, COMPARED TO GENOME SIZE ESTIMATED THROUGH FLOW CYTOMETRY

Sex	k-mer depth	Total number of 17-mer	Estimated genome size from k-mer analysis (Mb)	Heterozygosity rate (%)	Repeat rate (%)	Revised estimated genome size from k-mer analysis (Mb)	Estimated genome size (haploid) from flow cytometry (Mb)
Female	92	3.43×10^{10}	372.53	1.92	43.49	365.93	208.74
Male	94	3.64×10^{10}	386.75	1.62	43.70	380.73	201.88

Using flow cytometry analysis, the *E. kamerunicus* genome was estimated to be slightly larger than *D. melanogaster* based on the differences in their fluorescence intensities (Figure 2). The DNA content was converted into genome size using the relationship: 1 picogram (pg) of DNA equals 980 Mb (Bennett et al., 2000). The mean nuclear DNA content of *E. kamerunicus* was 0.213 ± 0.014 pg for female ($n = 5$) and 0.206 ± 0.002 pg for male ($n = 5$)

(Table 2). Using the known fruit fly genome size of 175 Mb, we estimated the haploid *E. kamerunicus* genome sizes at approximately 208.74 Mb (female) and 201.88 Mb (male) (Table 1). Flow cytometry analysis suggested that the male genome was smaller than the female genome, contrary to the k-mer analysis results. Furthermore, the genome size estimates from k-mer analysis were approximately twice the size from flow cytometry estimates.

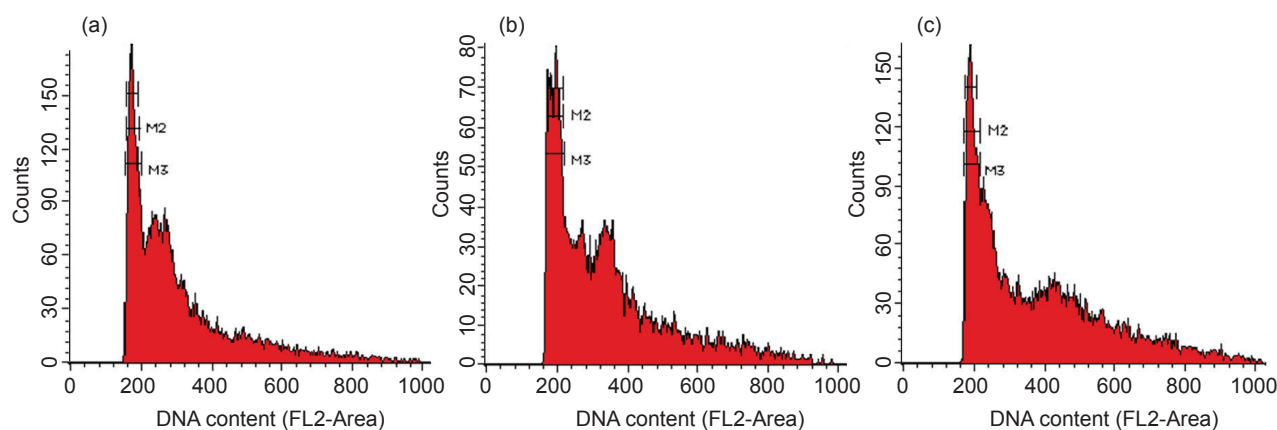


Figure 2. Flow cytometry profiles of (a) *D. melanogaster* reference standard, (b) female, and (c) male *E. kamerunicus*.

TABLE 2. FLOW CYTOMETRIC ESTIMATION OF GENOME SIZE USING THE MEAN NUCLEAR DNA CONTENT

Male/replicate	DNA content/1C value (pg)
1	0.206
2	0.207
3	0.205
4	0.204
5	0.206
Mean ± SD	0.206 ± 0.002
Femalereplicate	DNA content/1C value (pg)
1	0.214
2	0.204
3	0.221
4	0.221
5	0.206
Mean ± SD	0.213 ± 0.014

Note: pg - picogram; SD - standard deviation.

Differences of 6.86 and 14.80 Mb between the fluorescence and sequence-based analyses were obtained for the male and female genome sizes, respectively. The 13.72 Mb difference between male and female diploid genome sizes estimated through fluorescence-based analysis is relatively small, roughly 3.4% of the estimated male genome. The 14.8 Mb difference between the sexes, estimated through sequence-based analysis, also accounts for only approximately 3.9% of the estimated male genome size. Both methods yielded conflicting estimates: Cytometric analysis suggested a larger female genome, while k-mer analysis indicated a larger male genome. Therefore, the k-mer approach overestimated the male genome by about 178.85 Mb and the female genome by about 157.19 Mb compared to the cytometric technique. Apriyanto and Tambunan (2021) suggested that the *E. kamerunicus* has a genome size of 269.79 Mb (based on a single male), which was larger than our cytometry estimate by 69.90 Mb but smaller by 110.94 Mb when compared to k-mer estimates. The use of pooled weevil individuals, rather than the single isolated male in Apriyanto and Tambunan (2021), may be a reason for the genome size differences. However, pooling of several individuals was necessary to provide sufficient DNA for the SkimSeq technology. Genome size estimates using flow cytometry and sequence-based analyses for other insects also showed similar discrepancies. The genome size of *Bemisia tabaci* was ~682.30 Mb based on k-mer analysis and ~690.00 Mb according to flow cytometric analysis, using chicken red blood cell as the standard reference. This represents just a 10.00 Mb difference (Chen et al., 2015). Conversely,

Guo et al. (2015) reported a larger genome size for *B. tabaci* using the k-mer approach (720 Mb), which is ~60 Mb larger than the ~653 Mb estimate from flow cytometry, with *D. melanogaster* as the reference. These studies highlight that, apart from the choice of reference standards, cytometric analysis often provides variable estimates compared to k-mer methods. Other comparative studies, such as He et al. (2016), found differences in genome size estimates using k-mer and cytometric analyses. For *Laodelphax striatellus*, estimates were 657 and ~555 Mb, a 102 Mb difference. In the case of *Micropentila cingulum*, k-mer analysis underestimated the genome size at 136 Mb, 25 Mb less than the ~157 Mb from cytometric analysis. Pflug et al. (2020) concluded that although certain sequence-based methods, like k-mer distribution or read mapping, aligned with flow cytometry estimates for some species, no single technique consistently agreed with flow cytometry. While these two methods are expected to yield similar results, there are often some inconsistencies. These variations may be due to the use of different external standards, tissue samples at various growth stages in flow cytometry and potential cell endoreplication in insects (Yu et al., 2021).

The average coleopteran species (Order) genome size is 760 Mb (oscillating from 160- 5,020 Mb (Gregory, 2023). On the other hand, the average genome size for the curculionids species (Family) is 568 Mb (i.e., 205-842 Mb) (Gregory, 2023), though only 10 species were recorded, with the biggest being the rice water weevil, *Lissorhoptrus oryzophilus* (981 Mb). Holometabolous insects, like *E. kamerunicus*, which undergo complete metamorphosis with egg, larvae, pupae and adult stages, have been hypothesised to be constrained to a genome size limit of 2 pg or 956 Mb (Gregory, 2002; Hanrahan & Johnston, 2011). Significant gaps in taxon sampling for holometabolous insects persist, with entire orders still unrepresented. It is unclear whether this perceived restriction signifies an actual genome size threshold for holometabolous insects, or if expanded sampling might reveal larger genomes among them. Still, the estimated *E. kamerunicus* genome size from this study remains below the size limit for curculionids, which supports the hypothesis mentioned earlier. Our findings, using k-mer and flow cytometric analyses, showed that *E. kamerunicus* has a haploid genome size (C-value) of between 201-380 Mb (0.20-0.38 pg). Specifically, the genome size of the male *E. kamerunicus* is 202-381 Mb and 209-366 Mb for the female *E. kamerunicus*. The male genome size of 269.79 Mb reported by Apriyanto and Tambunan (2021) is within the range tabulated from this study.

Regarding the genome sizes estimated through genome sequencing assembly, beetle genomes have generally been on the smaller side, with an average assembly size of 286 Mb, ranging from 160 to

782 Mb (Cunningham et al., 2015; Hazzouri et al., 2020; Keeling et al., 2013; Meyer et al., 2016; Parisot et al., 2021; Powell et al., 2021; Richards et al., 2008; Vega et al., 2015). The model species for beetles, *Tribolium castaneum*, has a relatively small genome size of 160 Mb only (Richards et al., 2008) with a later improvement to 165 Mb (Herndon et al., 2020). The first assembled curculionidae genome, *Dendroctonus ponderosae*, is 205.8 Mb (<http://www.genomesize.com>), which is relatively similar to the 204 Mb genome reported by Keeling et al. (2013). The red palm weevil, *Rhynchophorus ferrugineus*, another pest from the curculionidae family, has a genome size of 782 Mb while the rice weevil, *Sitophilus oryzae*'s genome is 770 Mb (Hazzouri et al., 2020; Parisot et al., 2021). Assembled genome sizes of ground beetles are typically smaller compared to k-mer sequence-based methods, possibly because some repeated genomic regions remain unassembled (Pflug et al., 2020). In contrast, k-mer-based methods analyse the entire genome. A smaller assembly than expected may be due to incomplete or repeat collapses, while a larger assembly can be caused by independent haplotype assembly redundancies (Li et al., 2019). In brief, our *E. kamerunicus* genome size estimates using k-mer and flow cytometry align with coleopteran species' assembly sizes.

Obtaining precise estimations for complex genomes from actual sequencing data remains a challenging task to date. In future, efforts should be directed towards creating advanced algorithms that can effectively utilise genome sequences from other known insect species to enhance the accuracy of estimating genomic features. Fortunately, the *E. kamerunicus* genome is relatively small or falls within the intermediate size range among published insect genomes. This implies that future genome assembly and annotations for *E. kamerunicus* may be relatively simpler.

Genomic Features of Male and Female *E. kamerunicus* Genomes

K-mer analysis predicts genome size and reveals genomic features, like heterozygosity ratio and repeat rates. The GC content can be derived from the subsequent assembly analysis. Heterozygosity ratio, repeat rate and GC content are fundamental features for evaluating genome assembly quality and estimating genome size, particularly when using sequence-based approaches (Liu et al., 2013). Heterozygosity, which represents the proportion of differing nucleotides between an individual's inherited chromosomes from their parents, is a crucial measure for understanding genetic diversity (Bryc et al., 2013). Since the weevil samples used in this study are not inbred, the high heterozygosity level in the *E. kamerunicus* species (high heterozygosity ratio $\geq 0.8\%$, $0.5\% \leq$ low heterozygosity $< 0.8\%$)

is expected. High heterozygosity is common in various insect genomes, including Lepidoptera (Li et al., 2019). For example, the *Papilio glaucus* (Eastern tiger swallowtail) exhibits genome heterozygosity as high as 2% (Cong et al., 2015). The *Plutella xylostella* (diamond back moth) maintains a high level of heterozygosity even after ten generations of laboratory inbreeding. Remarkably, it was the first successfully sequenced insect genome that possess a significant degree of heterozygosity (You et al., 2013). However, our findings reveal that the male weevil has a 1.62% heterozygosity rate, while the female has a 1.92% rate (Table 1). Genome assembly may become challenging when genomic heterozygosity exceeds 1.00% (Zhou et al., 2022). Hence, the *E. kamerunicus* genome may be relatively difficult to assemble, as suggested by the short N50 scaffold lengths obtained (Table 3).

The genomes of both male and female *E. kamerunicus* belong to the category of low repetitive genomes, as their repeat ratios were below 50%. Specifically, the female *E. kamerunicus* has a repeat ratio of 43.49%, and the male has a repeat ratio of 43.70% (Table 1). The *E. kamerunicus* genome is considered a relatively simple repetitive genome, which may be linked to the species' small to medium genome size. High levels of heterozygosity, repeat sequences, and sequencing errors can reduce the precision of genome size estimation when utilising k-mer frequency (Liu et al., 2013). The high heterozygosity in the *E. kamerunicus* genome may explain the differences between our k-mer estimation and the cytometric approach. Genomic features statistics, such as repeat ratio, heterozygosity ratio, and GC content, were generally similar for both male and female *E. kamerunicus*.

High or low GC content exceeding the normal range ($>65\%$ or $<25\%$) on the Illumina sequencing platform can introduce sequencing bias, potentially leading to reduced coverage of sequencing regions and affecting genome assembly (Zhou et al., 2022). Yet, GC content within the range of 30%-50% does not notably impact genome sequence quality (Shangguan et al., 2013). Both female and male *E. kamerunicus* genomes had similar GC content (Figure 3). The GC content of 31.71% in the male *E. kamerunicus* genome here was consistent with the male *E. kamerunicus* genome draft assembly (Apriyanto & Tambunan, 2021). Genomes of other curculionidae species also exhibit similar levels of GC content. *Dendroctonus ponderosae* and the model beetle *T. castaneum* had similar GC content (36% and 33%, respectively) (Keeling et al., 2013; Richards et al., 2008). The coffee borer beetle, *Hypothenemus hampei*, had a GC content of 32.46% (Vega et al., 2015). The red palm weevil, *R. ferrugineus*, had a GC content of about 32%, while the rice weevil *S. oryzae* had a GC content of 32.9% (Hazzouri et al., 2020; Parisot et al., 2021).

TABLE 3. GENOME ASSEMBLY STATISTICS FOR *E. kamerunicus*

Item	Sex	Total number of contigs and scaffolds	Maximum length (bp)	N50 length (bp)	N90 length (bp)
Contig	Female	2,837,454	79,424	310	117
	Male	2,631,773	85,498	280	110
Scaffold	Female	2,729,248	79,424	334	120
	Male	2,467,789	193,303	318	113

Note: bp - base pairs.

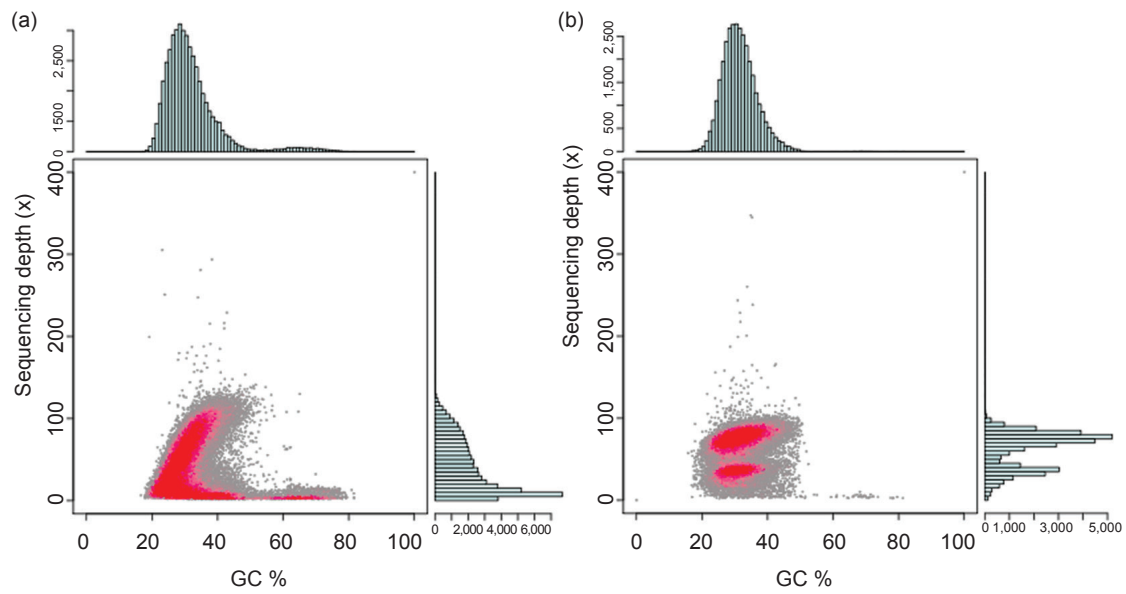


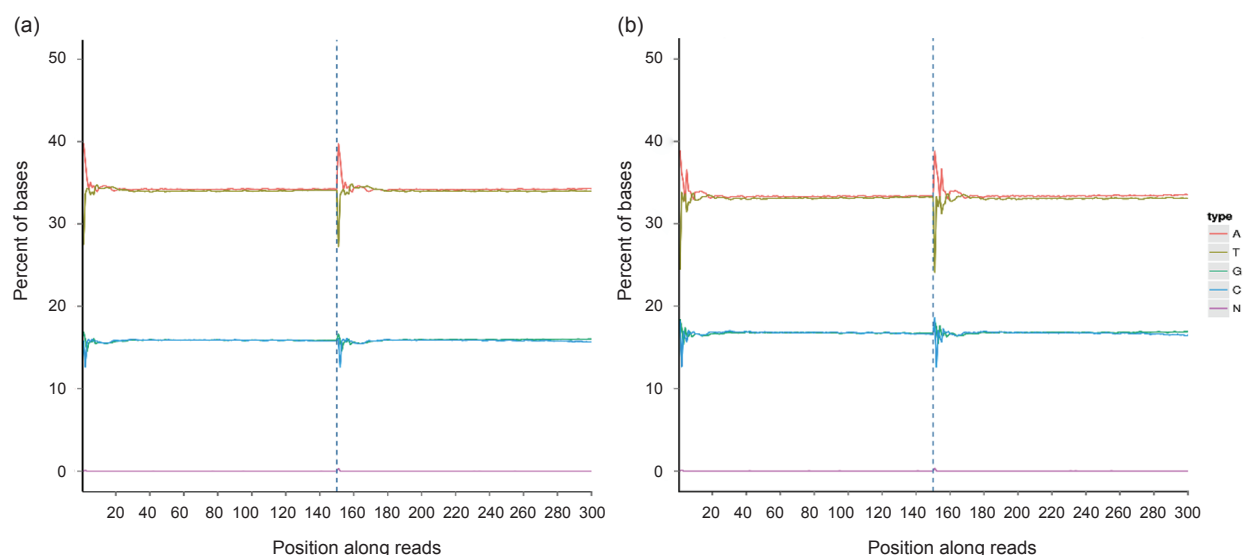
Figure 3. GC content and depth correlative analysis of (a) female, and (b) male *E. kamerunicus*. Red regions represent relatively dense portions of the scatter plot. The right bar chart shows sequencing depth distribution, while the upper-left one represents GC content distribution.

De novo Short-read Genome Assembly

A total of 213.84 and 215.16 Gb of raw sequence data were generated from female and male *E. kamerunicus*, respectively. Quality trimming and filtering, including a step to remove contaminating sequences, provided 63.90 Gb (female) and 64.50 Gb (male) of clean bases with Q20 scores >97%. The overall base error rate was a mere 0.03%. Figure 4 shows the proportion of single bases, which is used to distinguish whether AT and GC separation is present. The results indicated good sequencing quality, with balanced A/G and C/T base content.

For assembly analysis, a k-mer of 41 was used due to the low repeat ratio of around 43% for both male and female *E. kamerunicus* (Table 1). From the female and male *E. kamerunicus*, 2.8 million and 2.6 million contigs were assembled, with N50 values of 310 and 280 bp, respectively. Subsequently, the draft female and male genome assemblies consist of 2.7 and 2.5 million scaffolds, with slightly higher N50 values of 334 and 318 bp, respectively (Table 3). The slight N50 value increase in the scaffold assemblies suggests no significant improvement over the contig assemblies.

The high heterozygosity rate (1.62%-1.92%) in the *E. kamerunicus* genome may affect genome size estimation and assembly quality. According to Li et al. (2020), the shorter N50 lengths of contigs and scaffolds (183 and 186 bp) in the Sichuan pepper genome may be due to its high heterozygosity rate of 1.73%. A BLASTN search was performed, examining 10,000 pairs of randomly processed clean reads from both male and female datasets for contamination assessment. BLAST results showed that *E. kamerunicus* sequences had their closest match in the mountain pine weevil *D. ponderosae*, with low homology (Table 4). These beetles are from different subfamilies. *Dendroctonus ponderosae* is in the Scolytinae subfamily, which comprises phloem-feeding beetles, while *E. kamerunicus* is in the curculioninae subfamily. Similar to *E. kamerunicus*, *Acinetobacter* bacterial sequences were found in the *D. ponderosae* assembly, indicating the presence of possible gut symbiont bacteria in coleopteran species (Keeling et al., 2013). However, *Acinetobacter* sequences in *E. kamerunicus* were relatively low, about 4%, and were only detected in the female *E. kamerunicus* sequencing data. The low percentages (3.2% to 4.5%) of *Wolbachia*, *Streptomyces*

Figure 4. GC content distribution of (a) female, and (b) male *E. kamerunicus*.TABLE 4. BLASTN HITS* STATISTICS FOR THE *E. kamerunicus* GENOME

Sex of <i>E. kamerunicus</i>	Hit 1	Hit 2	Hit 3	Hit 4	Hit 5
Female	<i>Dendroctonus ponderosae</i> , 20.79%	<i>Wolbachia</i> endosymbiont of <i>Culex quinquefasciatus</i> Pel, 4.49%	<i>Acinetobacter baumannii</i> , 3.93%	<i>Bombyx mori</i> , 3.37%	<i>Plautia stali</i> symbiont, 2.81%
Male	<i>Dendroctonus ponderosae</i> , 24.19%	<i>Homo sapiens</i> , 8.06%	<i>Bombyx mori</i> , 4.84%	<i>Streptomyces lividans</i> TK24, 4.03%	<i>Wolbachia</i> endosymbiont of <i>Culex quinquefasciatus</i> Pel, 3.23%

Note: *Hit: Species name and percentage of reads with hits annotated to it.

bacteria, *Bombyx mori* moth, and even *Homo sapiens* sequences in the *E. kamerunicus* data could be due to environmental contamination or minor sequence similarity among these species. These low contamination levels were deemed acceptable since they are consistent with findings in other insects, such as the coffee berry borer (Vega et al., 2015). Hence, *E. kamerunicus* genome data likely contains a small portion of cobiont genome sequences, possibly due to natural gut bacteria or cobionts within these weevils. These cobionts' genomes need to be considered when conducting genomic analysis such as de novo assembly. These factors, such as high heterozygosity, cobiont genomes, and pooled samples, coupled especially with the low coverage of the SkimSeq data, likely negatively affected the assembly results. While our assembly appears fragmented, future studies could benefit from combining long and short read data from various sequencing platforms, potentially improving and increasing the average sequence size used for assemblies (Amarasinghe et al., 2020).

CONCLUSION

Insects often show differences in genome size estimates when using sequence-based and flow

cytometric methods. Not surprisingly therefore, genome size estimations for the male and female *E. kamerunicus* measured using these two approaches were different. The genome size estimate for the male *E. kamerunicus* is 202-381 Mb, with an almost similar range of 209-366 Mb for the female *E. kamerunicus*. The SkimSeq data also suggests *E. kamerunicus* has a simple repetitive genome with high heterozygosity rates. The current genome database for insects consists of only nine curculionids, thus our study contributes sequence data for the *E. kamerunicus* genome, encompassing both sexes.

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MULTIDIMENSIONAL ANALYSIS SUSTAINABILITY OF CORN-SOYBEAN INTERCROPPING MODEL IN SMALLHOLDER OIL PALM REPLANTING

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ABSTRACT

In the global agricultural sector, corn-soybean intercropping offers significant long-term benefits for increased productivity, improved agroecology and land management. While the impact of intercropping on corn and soybean yields has been studied extensively, information on their sustainability is limited. This study aims to analyse the status and sustainability determinants of the intercropping model on smallholder oil palm land through multidimensional analysis which includes economic, social, cultural, environmental, technological and institutional dimensions. Utilising a multi-dimensional scaling method with RAPFISH's modified software, called Rapid Appraisal for Palm Oil. The analysis results show that the sustainability index for oil palm plantations using the corn-soybean intercropping model has a good status in the economic dimension, with price-sensitive production attributes for smallholders. The value of the sustainability index in the socio-cultural dimension of good status with sensitive attributes of community access to replanting requirements. The continuous index value in the environmental dimension of the status is very good with the attribute of sensitive pest disturbance. The value of the sustainable index in the technology dimension is in good status with sensitive attributes of post-harvest handling. The value of the sustainable index in the institutional dimension is in good status with sensitive attributes of smallholder's group access to banking.

Keywords: corn, intercropping, palm oil, replanting, soybean, sustainability.

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INTRODUCTION

A model in which two or more crops are grown simultaneously or nearly simultaneously on the same farm in a regular cropping pattern is called intercropping. Intercropping is the new green

revolution because it can improve land efficiency by using information on how species complement each other and provides a way to achieve sustainable intensification of agriculture (Xu et al., 2020). It has become the best cropping system for corn-soybean and plays an important role in contributing to

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modern and sustainable agriculture. Chang et al. (2020) stated that intercropping can increase crop competitiveness and improve resource utilisation on a given farm. Intercropping corn-soybean land is an effective and sustainable model. It is more effective than monocropping (Ren et al., 2017). This model can utilise soil nitrogen and other minerals and convert them from inorganic to organic forms that can be used for soil uptake. This increase in size can improve nitrogen production and resource availability (Li et al., 2013). With low-input and corn-soybean intercropping model provides significant benefits to smallholders (Iqbal et al., 2019).

Oil palm plantations in Indonesia have a strategic role in the economy as a source of foreign exchange (Purnomo et al., 2020), employment (Puder, 2019) and improving community welfare (Ayompe et al., 2021). Oil palm plantation land in Indonesia in 2022 has an area of 16.83 million hectares and 36.58% of this area is oil palm smallholder plantation. Riau Province has the largest area of oil palm plantations at 3.49 million hectares, of which 1.8 million hectares are smallholder plantations (Directorate General of Plantations, 2022).

Oil palms exceed economic value at 25 years of age (Afifah et al., 2021) and after 25-30 years, the trees are felled due to reduced oil content (Nuryawan et al., 2022). Oil palm productivity can be increased through replanting (Syarfi et al., 2019). The problem for smallholders to finance oil palm replanting because it requires large capital. Smallholders lose their income during the immature period of oil palm (0-3 years) (Triastutik & Hartati, 2020). Corn-soybean intercropping model programmes can address the loss of income during replanting (Rachman et al., 2022).

Nasution et al. (2022) showed that the application of the corn-soybean intercropping model on oil palm land is quite successful. The intercropping model can ameliorate the negative impact of oil palm on soil physico-chemical properties (Dhandapani et al., 2020). Although this model is considered to replace lost income, corn-soybean intercropping still needs to be analysed for sustainability in oil palm replanting programmes. Overall, conducting a sustainability analysis of corn-soybean intercropping in an oil palm replanting programme within an agricultural enterprise system is economically viable, environmentally friendly, and socially beneficial. This holistic approach can contribute to the long-term sustainability and resilience of agricultural communities.

Corn-soybean intercropping is expected to be a sustainable solution for smallholder oil palm replanting models. To date, no information or research shows the sustainability status of the corn-soybean intercropping model on oil palm replanting land specifically. The application of the concept of

sustainability of the corn-soybean intercropping model has not been formulated with appropriate and measurable indicators, both in the context of policy, and its overall impact comprehensively. This study aims to analyse the index, status and determinants of the sustainability of the corn-soybean intercropping model in smallholder oil palm plantations, as well as policy strategies to address its sustainability.

MATERIALS AND METHODS

Description of Study Area

The research was conducted at a pilot location of corn-soybean intercropping in smallholder oil palm replanting land from January to December 2019. It is in Bukit Jaya Village, Ukui District, Pelalawan Regency, Riau Province with coordinates of 00°09'35.2"S and 102°06'23.5"E at an altitude of 44 m above sea level. The research site was on dryland that had been planted with oil palm for one cycle (25 years). It has Ultisol soil type with soil characteristics in certain parts such as acidic and organic soil, brackish groundwater, rather high humidity with an air temperature of 21.6°C-34.8°C, average rainfall of 151.33 mm, and rainy days of 9.5 mm/day (Badan Pusat Statistik Kabupaten Pelalawan, 2023).

Sampling and Data Collection

This research was conducted using a survey method, namely research to obtain facts, identify problems, and obtain justification for ongoing practices by certain units at a place and time with a sample. The selection of respondents is adjusted to the surrounding environmental conditions and understanding of the problem being researched. In this study, the selection of respondents using the purposive sampling method, also known as judgmental sampling, is a non-probability sampling technique used in research to select participants based on certain characteristics or qualities relevant to the study. A total of 50 respondents were selected with the criteria in which 35 respondents were smallholders as programme participants, administrators and Village Unit Heads and 15 respondents from the expert team with the criteria of having expertise in accordance with the field under study. Some considerations in determining the experts used as respondents are:

- a) Have competent experience in the field studied.
- b) Have a reputation or position that is competent in the field being researched.
- c) Have high credibility and willingness and are in the location under study.

The data collected in this study was through (1) a literature review on corn-soybean intercropping and oil palm replanting, identifying key sustainability factors, (2) a field survey on economic, socio-cultural, technological, environmental and institutional components, and (3) development of a sustainable intercropping management model based on the analysis results.

Data Analysis

Analysis of the index and sustainability status of intercropping models in smallholder oil palm plantations using multi-dimensional scaling method (MDS) analysis techniques, assisted by the Rapid Appraisal for Fisheries (RAPFISH) add Ins Microsoft Office Excel application called RAP-PALM OIL (Rapid Appraisal for Palm Oil) approach. RAP-PALM OIL was modified to evaluate the sustainability of overlapping models on smallholder oil palm plantations from the RAPFISH approach developed by the University of British Colombia, Canada to assess the sustainability of fisheries (Kavanagh & Picher, 2004). RAPFISH software was used to define each measurable indicator. A multidimensional scale is a statistical technique that attempts to reduce the dimensions in a dataset by dividing it into simpler groups. Each dimension has properties that indicate sustainability (Akhmad & Suzy, 2005).

Multidimensional scale analysis (MDS) is carried out in several stages, namely, (1) determination of parameters covering five aspects (economic, social cultural, environmental, technological and institutional), (2) evaluation of each attribute based on sustainability criteria in each measurement, (3) ordination of analysis of sustainability index values, (4) to identify sensitive variables that affect sustainability, a sensitivity analysis was conducted and (5) to consider aspects of uncertainty is down with Monte Carlo analysis (Kavanagh & Picher, 2004).

The results of the MDS analysis will obtain (1) the status or index of each dimension and (2) sensitive attributes or influential attributes based on the root mean square (RMS). The normalisation test for model feasibility uses the determination coefficient (R^2), if the R^2 value is close to 1 the model is said to be good, or it means that the data is normally distributed. The feasibility test of the model is carried out to determine whether it is necessary to add attributes to the model and test the accuracy of the model compared to the actual situation. Meanwhile, leverage analysis is to find sensitive attributes or influential attributes that affect the sustainability index value in each dimension referring to the highest RMS value.

The ordination analysis process in the form of index values, errors may occur so it is necessary to evaluate the influence of errors on the process so that

it is necessary to carry out Monte Carlo simulations as a test of validity and accuracy. If the Monte Carlo simulation results do not change significantly or have a small difference in ordination values, it can be concluded that the ordination results have been able to overcome the random error (Pitcher et al., 2001). The simulation also serves to see the level of stability of the ordinance results. Monte Carlo simulation is a method to analyse the propagation of uncertainty where the goal is to determine how random variations or errors affect the sensitivity, performance, or reliability (consistency) of the system being modelled (Rubinstein, 1981).

The sustainability index value from the data processing ranges from 0 to 100 and is divided into four sustainability categories. The sustainability assessment index used in this study is shown in *Table 1*. A visualisation of the research flow chart describing the stages and steps can be seen in *Figure 1*.

TABLE 1. SUSTAINABILITY INDEX VALUE

Index category	Value
0.00-25.00	Unsustainable
25.01-50.00	Less sustainable
50.01-75.00	Sustainable enough
75.01-100.00	Sustainable

Source: Kavanagh and Pitcher (2004).

RESULTS AND DISCUSSION

The results of the MDS analysis presented in *Table 1*, indicate that the index values and sustainability statuses of the economic, social cultural, technological and institutional dimensions fall within the moderately sustainable category, with an average value ranging from 56.23-65.22. Conversely, the environmental dimension is classified as highly sustainable, with an index of 85.31. A visualisation depicting the sustainability statuses across dimensions is provided in a kite diagram (*Figure 2*). Furthermore, *Table 2*, presents the results of the comparison between the MDS analysis and Monte Carlo values.

The difference between the MDS sustainability index and the Monte Carlo analysis shown in *Table 2* is deemed acceptable as a predictor of the sustainability index value if it falls below 5% (Pitcher, 2013). Specifically, the disparity between the sustainability index value and the Monte Carlo value ranges from 0.96-4.13, which suggests that the RAP-PALM OIL model, employed for evaluating the sustainability of the corn-soybean intercropping models on oil palm replanting land, is highly accurate and justifiable. Mahida et al. (2019) stated that the difference between MDS and

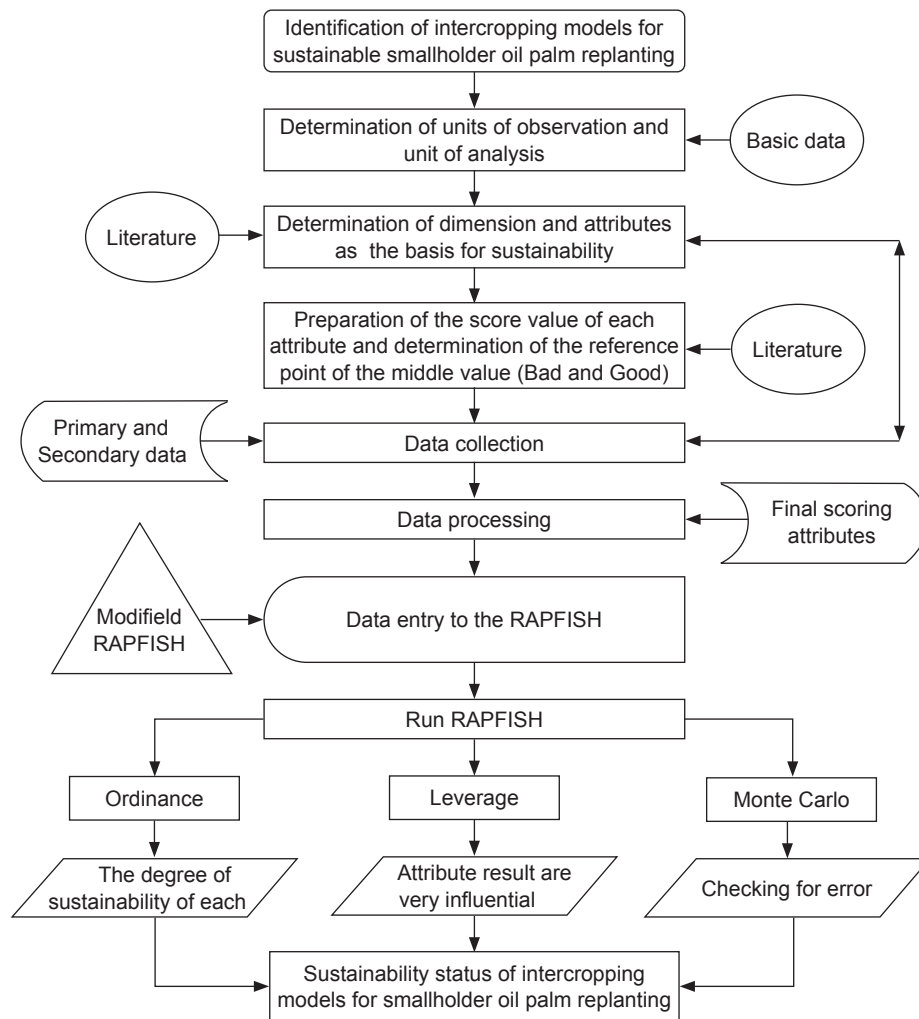


Figure 1. Stages of sustainability analysis of the intercropping model in the community oil palm replanting.

Monte Carlo results shows the confidence interval of the RAP-PALM OIL results. If the difference between MDS and Monte Carlo is smaller than the value of 1, then the resulting index is more than 90% accurate. If the results of the Monte Carlo analysis do not change significantly or have a small difference in ordination values, it can be concluded that the results of MDS coordination have been able to overcome the existence of random errors (Pitcher, 1999).

The percentage difference between the MDS and Monte Carlo is greatest in the environmental dimension 4.13, while the smallest difference occurs in the technological dimension 0.96. The high value of the environmental dimension is due to overlapping improving soil physical, chemical and biological properties, reducing surface erosion and increasing soil pH.

The results of the sensitivity analysis on each attribute are the determinants of the sustainability of the multi-state model of intercropping corn-soybean on oil palm replanting land. The results showed that of the 63 attributes analysed, 12 attributes influenced the corn-soybean

intercropping model on oil palm replanting land. Key attributes were farm-gate prices and family labour (economic); access to replanting, empowerment and infrastructure (socio-cultural); pest infestation and soil type (environmental); post-harvest handling (technological); and bank access, land use consistency and ownership (institutional).

Economic Dimension

The results of corn-soybean overlap in oil palm replanting areas based on the economic dimension fall into the moderately sustainable category with an index of 60.02 (Figure 3) at a coefficient of determination (R^2) of 95% (Table 2). These results indicate that the economic dimension is a good model and can capture the problems that occur. Therefore, there is no need to add attributes to address the actual situation. The results of the Monte Carlo simulation of economic status show a value of 58.83 (Table 2), which indicates that there are no significant errors associated with the economic dimension.

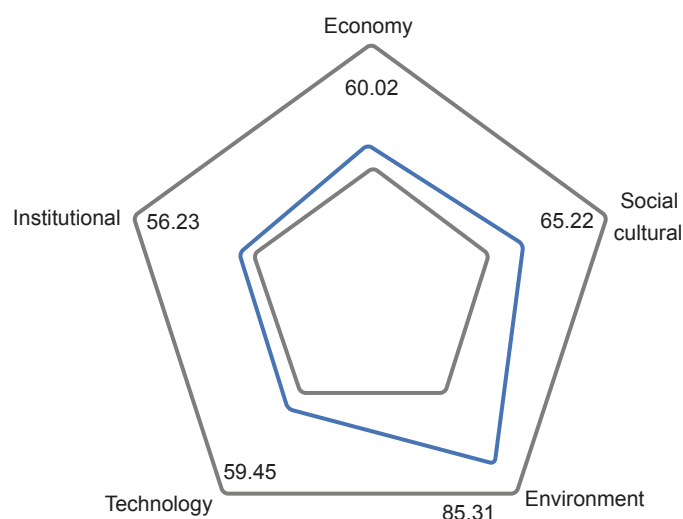


Figure 2. Inter-dimensional sustainability analysis kite diagram.

TABLE 2. INDEX AND SUSTAINABILITY STATUS OF INTERCROPPING MODEL IN REPLANTING SMALLHOLDERS' OIL PALM PLANTATIONS

Dimensions	R ²	Index ordination	Categories	Simulation Monte Carlo	Difference between indices and Monte Carlo
Economy	0.95	60.02	Sustainable enough	58.83	1.98
Social culture	0.94	65.22	Sustainable enough	64.08	1.75
Environment	0.95	85.31	Sustainable	81.79	4.13
Technology	0.94	59.45	Sustainable enough	58.88	0.96
Institutional	0.94	56.23	Sustainable enough	54.77	2.60

The results (Figure 3) of the leverage analysis show that there are two sensitive attributes out of 15 attributes forming the economic dimension that affect the sustainability of corn and soybean overlap on smallholder oil palm replanting land, namely the price of production at the farm level Index 3.82 and the adequacy of family labour for farming Index 3.44.

This study found that the selling prices of corn and soybean are lower than the government price. The selling price of corn at the farm level ranges from USD0.24-USD0.29/kg, which is lower than the government price of USD0.31. Likewise, the selling price of soybeans at the farm level is USD0.43/kg, lower than the standard price set by the government at USD0.53/kg. This is due to the lack of access to price information by the smallholders, cooperation not being well established, middlemen playing a dominant role, and markets being located far from smallholders. If this situation is not addressed immediately, it is feared that it will greatly affect the sustainability of the agricultural system.

Strategies to increase selling prices at the farm level can be done by ensuring that information on market prices of food crops and horticultural

commodities reaches smallholders by providing information services, strengthening the function of village unit cooperatives as price stabilisers. According to Villanoa et al. (2019), the creation of good and easy market access and support for pricing policies that do not harm smallholders will be a factor in the successful development of agricultural enterprises.

The adequacy of family labour for agriculture is very limited with a RMS value of 3.44. This is due to the absence of family members at the farm location on account of continuing their education or working outside the area. The use of simple technology on intercropped land can overcome labour limitations. Effendi et al. (2014) stated that to overcome labour limitations and efforts to increase agricultural productivity, post-harvest technology and the development of agricultural mechanisation can be carried out.

Social Cultural Dimensions

The results of the social-cultural ordination analysis in the sustainability of corn-soybean intercropping for oil palm replanting land showed an index dimension of 65.22 with

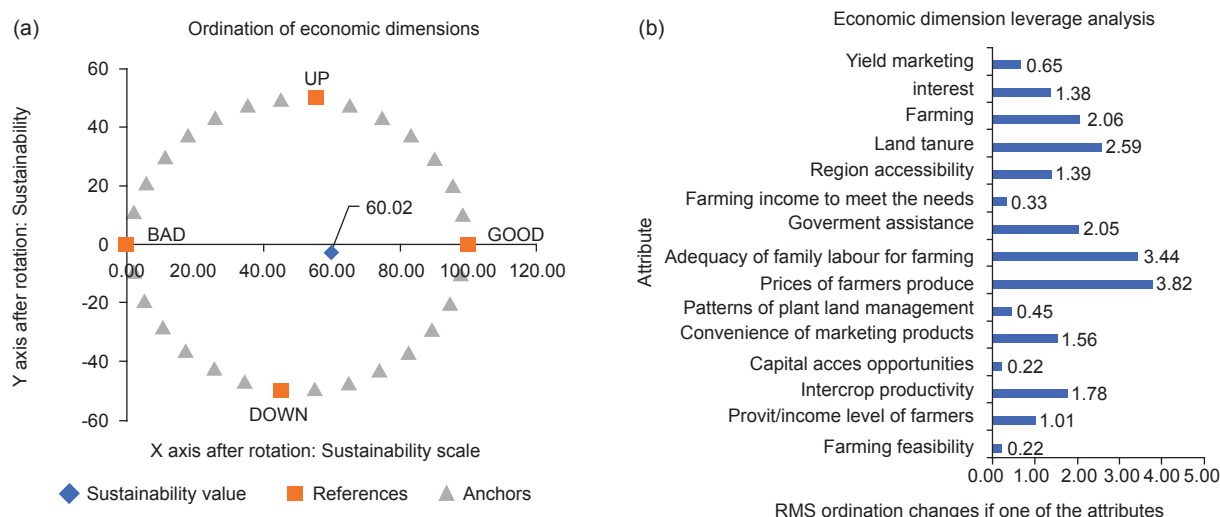


Figure 3. (a) Ordination index and (b) sensitive attributes of the sustainability status of corn-soybean intercropping in the economic dimension.

the category of sustainable enough (Figure 4). Similarly, the coefficient of determination was 94% and the Monte Carlo analysis status value was 64.08 (Table 2), which indicates that there is no significant disturbance in the absence of disturbance associated with social and cultural dimensions.

The results of the leverage analysis (Figure 4) identified three sensitive attributes out of 13 attributes of the socio-cultural dimension affecting community access to replanting requirements (RMS 5.17), community empowerment (RMS 4.71) and community access to replanting infrastructure (RMS 4.18). Based on the 2016 Minister of Agriculture Regulation on general guidelines for oil palm replanting (Directorate General of Plantations, 2022), 14 requirements must be met to participate in replanting and the process takes a long time. In addition to the requirements, there are still many procedures that must be followed before verification at the stage of land replanting, which must pass verification at the district and provincial levels, therefore, it is necessary to convey the regulation of replanting to smallholders more broadly.

A sensitive attribute in the empowerment of farming communities is the suboptimal role of extension workers as a companion to smallholders in groups to improve skills, applying technological innovations and decision-making. This is related to limited intellectual abilities that are not in accordance with field conditions. To reduce the impact of sensitivity on the strategies carried out, it is necessary to conduct technical skills training for smallholders to produce products when utilising productive land, improve the performance of assistants, improve the performance of smallholder groups and encourage cooperation.

Regarding the issue of community accessibility to replanting infrastructure, in this case especially

inputs in the form of certified superior seeds and subsidised fertilisers, the strategy is to convey to the community the availability of certified superior oil palm seeds, the characteristics of superior seeds and how to obtain quality seeds and fertilisers. Good cooperation within smallholder groups and supported by adequate infrastructure will create a resilient and sustainable local food system (Lutz et al., 2017).

Environmental Dimensions

Intensive agriculture can have negative impacts on the environment such as the greenhouse effect, toxic gas release, soil erosion and environmental degradation (Yang et al., 2021). One of the efforts to reduce these negative environmental impacts is intercropping (Dhandapani et al., 2020). This result is with the regulation of the sustainability of corn-soybean intercropping on oil palm replanting land based on the environmental dimension which has an index of 85.31 with a very sustainable category (Figure 5). The coefficient of determination was 95% and the Monte Carlo simulation analysis value obtained was 34.20 (Table 2).

In Figure 5, the leverage analysis results showed two sensitive attributes that are influential factors, namely pest nuisance (RMS 6.83) and soil type (RMS 6.66). Pest infestations that occur in the research location are domesticated animals such as pigs and cows. An effective way to deal with pig pests has not yet been invented, and cow owners are advised not to freely release their livestock. Other pests were not found, so the use of pesticides in this area is very low. This is certainly very good for the safety of the food produced. According to Ademola et al. (2019), if such excessive inputs endanger food production,

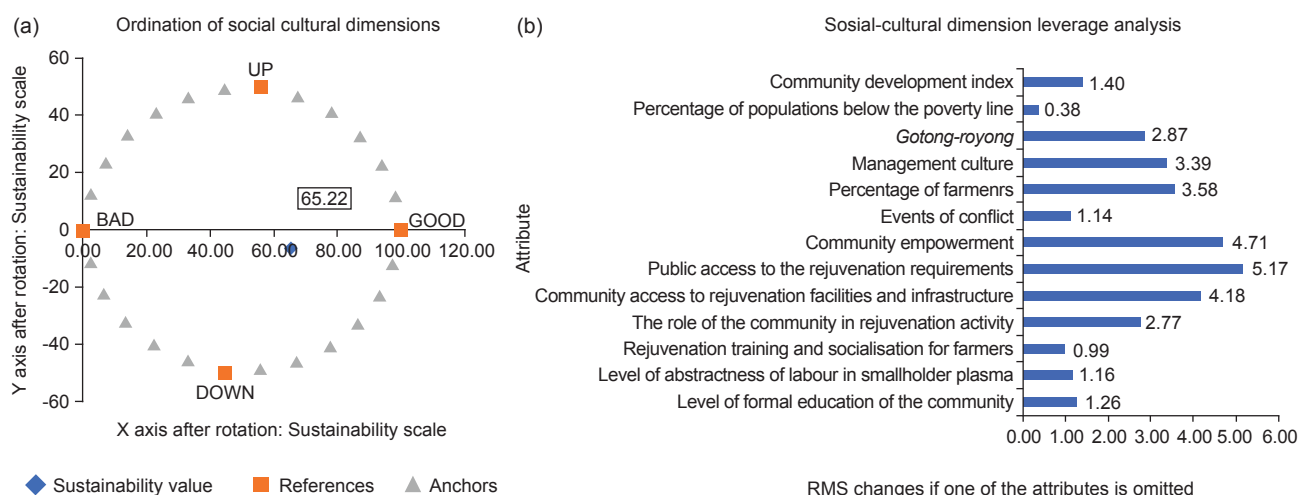


Figure 4. (a) Ordination index and (b) sensitive attributes of the sustainability status of corn-soybean intercropping in the socio-cultural dimension.

food security is the basis of sustainable agriculture. Archer et al. (2018), argued that to realise the sustainability of agriculture in the long term, it is necessary to reduce the use of chemical pesticides and the implementation of an integrated pest control system.

The second influential factor is soil type due to low nutrient content and pH. The pH value at the replanting site was low because the soil was overturned during tree felling and land clearing. To raise the pH of the soil, lime was used before planting. The application of organic and inorganic fertilisers is beneficial in improving the physicochemical properties of the soil at the research site. To increase soil fertility and pH, lime was applied before planting (Bossolani et al., 2021), organic and inorganic fertilisers, and legume intercropping (Duchene et al., 2017).

Dimensions of Technology

The results of the ordination of the sustainable status of corn-soybean intercropping agriculture in oil palm replanting land in the technological dimension with an index value of 59.45 (Figure 6), the sustainable category with a determination coefficient value of 94% and a Monte Carlo simulation has a value of 58.88 (Table 2). In Figure 6, the results of the leverage analysis show that post-harvest handling (RMS 2.71) and processing opportunities (RMS 2.64) are sensitive attributes that affect the sustainability of corn-soybean intercropping on oil palm replanting land. Improving smallholders' knowledge of agricultural technology innovations is an important part of the innovation adoption process and smallholders' empowerment. Gershom et al. (2019) stated that in accelerating agricultural development,

individual knowledge has an important meaning because knowledge can increase the ability to adopt new technologies in agriculture. Jayne et al. (2019) stated that the determinants of agricultural sustainability are the availability of capital, technology, and smallholders' knowledge. This is in accordance with research by Syahza et al. (2021), which stated that good skills and mastery of technology by smallholders will improve their agricultural performance while increasing their income.

The quality of corn and soybean yields is currently not optimal due to poor post-harvest handling. To overcome this, a drying warehouse that can handle large-scale harvesting is needed. In terms of marketing, the harvested corn production is processed in the form of peeled corn and sold directly to traders, while soybeans are sold to tofu and tempeh processors. To increase the selling price of these products, the government needs to aid in the form of simple corn processing facilities that can convert dried corn into granular corn ready for livestock consumption. This is expected to increase market uptake and increase the selling value of the product. This is in accordance with Villanoa et al. (2019), that the sustainability of agricultural businesses is not only the responsibility of smallholders but also government policy.

Institutional Dimension

Ordination result of the sustainability status of intercropping agriculture in oil palm community replanting areas show that the institutional dimension ordinance is quite sustainable with an index of 56.23 (Figure 7) with a determination coefficient of 94% and a Monte Carlo simulation status value of 54.77 (Table 2). In Figure 7 the

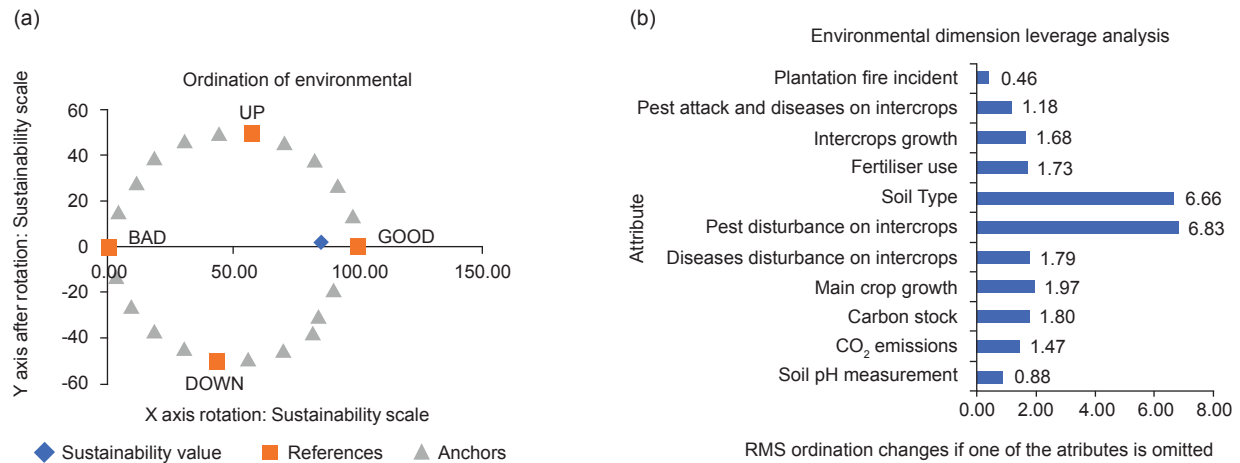


Figure 5. (a) Ordination index and (b) sensitive attributes of the sustainability status of corn-soybean intercropping in the environmental dimension.

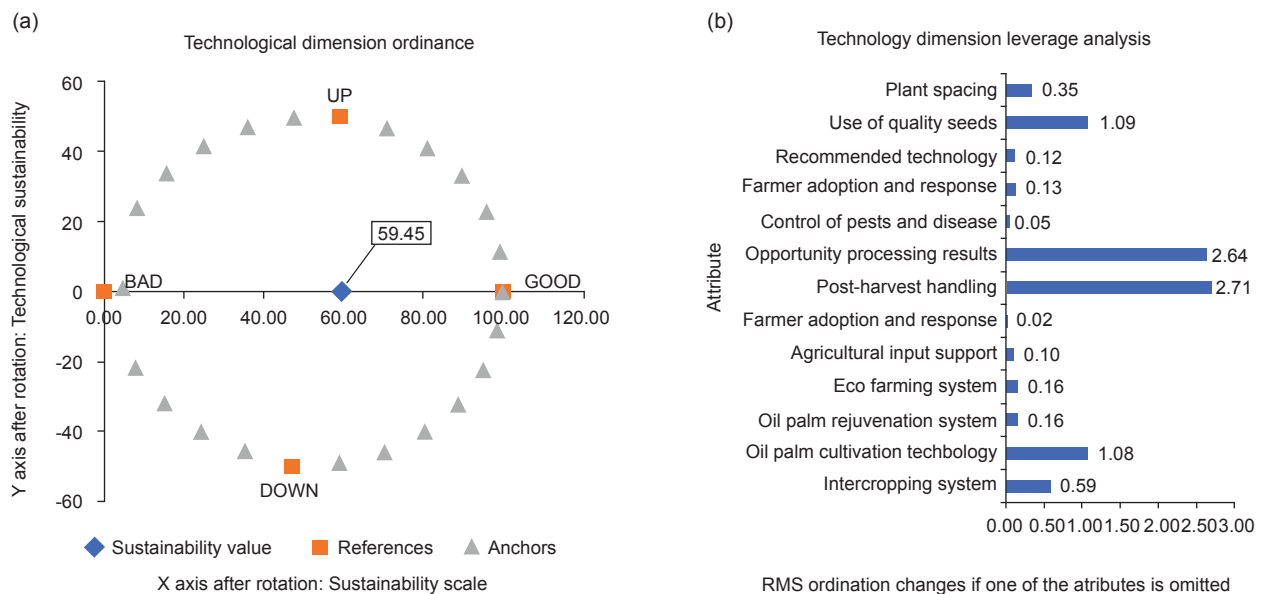


Figure 6. (a) Ordination index and (b) sensitive attributes of the sustainability status of corn-soybean intercropping in the technological dimension.

results of the leverage analysis show that in the institutional dimension, three sensitive attributes affect the 11 attributes analysed, namely, smallholders group access to banking (RMS 5.48), consistency of land use for plantation development (RMS 5.26) and land ownership status (RMS 4.72).

Policy Strategy for Sustainability of Intercropping Model

Sensitive attributes identified as key issues that can affect the sustainability of land regeneration in oil palm smallholdings need to be the focus of planning and problem-solving. The solution is the need for policy intervention against the influence of sensitive

attributes that can improve the sustainability of oil palm plantation regeneration which is inseparable from various other related elements and resources, so a systematic and integrated approach is needed. This will prevent the phenomenon where one event or action triggers a series of similar events or chain reactions that will impact other aspects / dimensions and worsen land sustainability status regeneration in the oil palm plantation.

Based on the sensitive attributes that affect the viability of the corn-soybean intercropping model in oil palm replanting, a policy strategy is needed to address these sensitive attributes. There are 12 policy strategies to address the influence of sensitive attributes on each dimension as shown in Table 3.

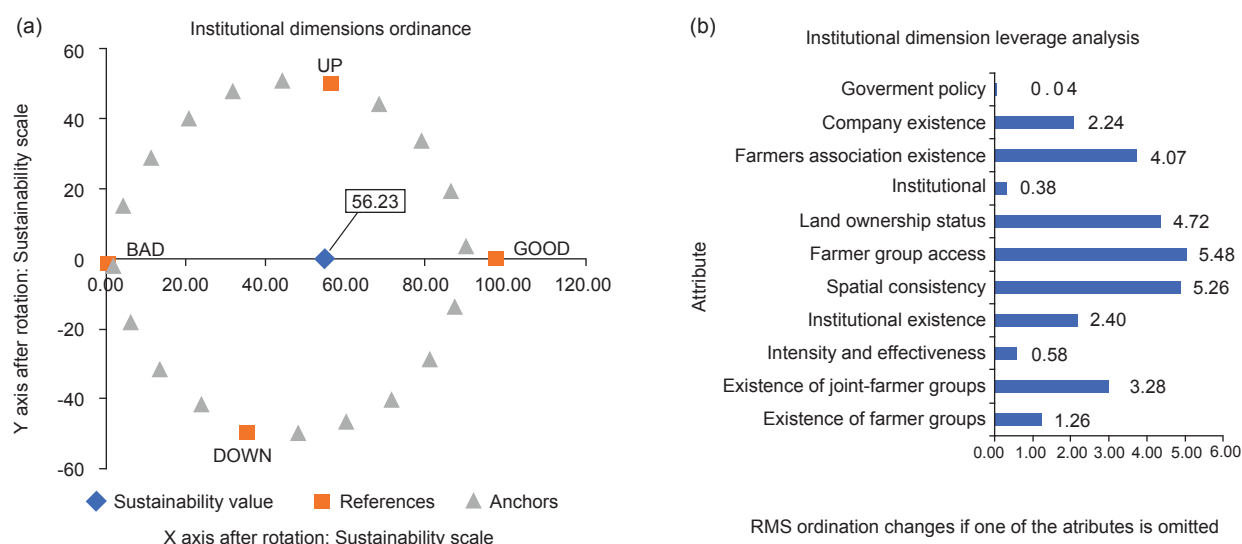


Figure 7. (a) Ordination index and (b) sensitive attributes of the sustainability status of corn-soybean intercropping in the institutional dimension.

TABLE 3. POLICY STRATEGY FOR INTERCROPPING MODEL IN OIL PALM REPLANTING AREAS

Dimensions	Sensitive attributes	Policy strategy
Economy	1. Yield at farm level	1. Improving access to market information on crop commodity benchmark prices.
	2. Sufficiency of family labour for farming business	2. Using simple farming tools for planting.
Social culture	1. Community access to replanting requirements	1. More widespread socialisation of the requirements and procedures for replanting oil palm smallholdings.
	2. Community empowerment	2. Training on intercropping cultivation techniques and other productive business.
	3. Community access to replanting infrastructure	3. Socialisation and information about the availability of superior seeds, characteristics of superior seeds, and fertilisers.
Environment	1. Intercrop pest infestation	1. Integrated pest control in collaboration with local village stakeholders.
	2. Soil type	2. The use of lime and organic fertilisers.
Technology	1. Post-harvest handling	1. Post-harvest handling /tools/facilities assistance.
	2. Result processing opportunities	2. Assistance with corn chopper.
	3. Smallholder groups' access to bank	3. Facilitate the transfer of smallholders' land certificates.
Institutional	1. Consistency of land use for plantation development	1. Coordinate with relevant ministries on the handling of forest plantations.
	2. Land ownership status	2. Coordinate with land agencies.

CONCLUSION

The sustainability of oil palm plantations with corn-soybean intercropping models is good in all dimensions. Economically, farm-level production prices are a key factor, requiring improved market access, smallholder cooperation and stronger village cooperatives. Socio-culturally, access to replanting programmes requires simplification

and better outreach. Environmentally, pest control, especially for pigs, remains a challenge. In the technological dimension, post-harvest handling is critical and improving smallholders' knowledge on innovations can improve crop quality. Institutionally, better access to banking and financial literacy for smallholder groups is needed to improve loan management and the use of financial technology.

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QUANTIFYING THE EFFECT OF FERTILISER APPLICATION ON NITROUS OXIDE PULSES IN OIL PALM PLANTATION

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ABSTRACT

The environmental impacts of oil palm production systems have raised global concerns due to the large quantities of greenhouse gases released (including nitrous oxide, N₂O) into the atmosphere. To understand the effects and the response to the nitrogen fertiliser application on N₂O flux for mitigation planning, emissions were monitored intensively over the course of two field campaigns from 2015-2016. The study was conducted in an oil palm plantation cultivated on peat in Sarawak, Malaysia. N₂O flux was highly temporally variable, with large increases in N₂O flux following fertiliser application. The mean (± standard error) of N₂O flux from the first and second sampling campaigns were 129.000 ± 9 mg N m⁻² d⁻¹ and 1.021 ± 150 mg N m⁻² d⁻¹, respectively. The magnitude of the N₂O flux and the timing of the emissions peak varied for different sampling campaigns, reaching a maximum of 384.000 ± 77 mg N m⁻² d⁻¹ 30-days after the first fertiliser application event and 3.777 ± 777 mg N m⁻² d⁻¹ 7-days after the second application. During fertilisation events, environmental variables played only a weak role in modulating N₂O fluxes in this ecosystem, suggesting that N₂O flux was principally determined by fertiliser input rates.

Keywords: fertiliser, nitrous oxide, oil palm, peatland.

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INTRODUCTION

Oil palm ecosystems are globally significant environments as they take up approximately 19 million hectares of agricultural land worldwide (Murphy et al., 2021). The palm oil industry has emerged as a significant player in the global food industry, driven by the remarkable versatility and high productivity of oil palm, it accounts for a substantial portion, approximately one-third, of the world's total production of vegetable oils and fats (Kushairi et al., 2018). This prominence underscores

the pivotal role that palm oil plays in meeting the escalating demand for edible oils worldwide. As the availability of suitable mineral soil sites has declined in recent years, the oil palm industry has expanded cultivation into peatlands, especially in Southeast Asia, specifically Malaysia and Indonesia.

Tropical peatlands extend over an area of 25 million hectares in Southeast Asia and are estimated to contain 11%-14% (68.5 Gt) of the global peat carbon (C) reservoir (Page et al., 2011). Population growth, industrialisation and economic expansion have accelerated land use change across the tropics, with heightened demand for food and natural resources encouraging small and large farmers in Southeast Asia to expand into tropical peatlands over the past two decades. For example, Miettinen et al. (2016) reported that both commercial and smallholder oil palm plantations cover almost 50% (7.8 million hectares) of peatlands area in insular Southeast Asia. However, cultivation of peatlands has led to negative ecological, hydrological and atmospheric impacts, which national governments, industry bodies and civil society groups now aim

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to curb or reverse through sustainability measures such as national “No Peat” conversion policies, national and industry certification schemes that prohibit growers from developing new plantations on peatland (e.g., Malaysian Sustainable Palm Oil scheme, Indonesian Sustainable Palm Oil certification scheme, Roundtable for Sustainable Palm Oil) (Carlson et al., 2018; Sumarga & Hein, 2016). In addition, research has been intensified to find more climate and environmental-friendly approaches to manage existing peatland plantations, in order to reduce or mitigate the effects of past peatland conversion on greenhouse gas emissions and regional flood risk (Manning et al., 2019; McCalmont et al., 2021).

In order to achieve optimum plant growth and high yields, managed tropical peatlands need to be drained, which leads to enhanced decomposition of peat, compaction of soil and subsequent land subsidence (McCalmont et al., 2021). Drainage often leads to enhanced carbon dioxide (CO₂) emissions to the atmosphere due to aerobic conditions in the upper peat profile and the use of fertilisers that can accelerate peat oxidation via microbial priming effects (Hirano et al., 2014; Hooijer et al., 2015; Jauhiainen et al., 2014; McCalmont et al., 2021; Sakata et al., 2015). What is less well established are the impacts of drainage and fertilisation on nitrogen (N) cycling and nitrous oxide (N₂O) flux. Theoretically, these two management interventions could greatly enhance the potential of managed tropical peatlands to emit N₂O by alleviating constraints on N₂O flux imposed by redox and N substrate availability, respectively (Davidson et al., 2000). These predictions are supported by evidence from managed temperate or boreal peatlands, where N₂O emissions range from 0.1 to 37.0 kg N ha⁻¹ yr⁻¹, and can exceed emissions from agricultural systems on mineral soils (Klemetsson et al., 2005; Maljanen et al., 2003; Regina et al., 2004). In these higher latitude systems, this increase in N₂O emissions is attributable to the mineralisation of fertiliser N combined with accelerated rates of soil organic N mineralisation (Klemetsson et al., 2005; Maljanen et al., 2003; Regina et al., 2004; Schrier -Uijl et al., 2013; Stichnothe & Schuchardt, 2011). However, measurements of N₂O emissions from managed tropical peatlands are fewer than those for the temperate or boreal zone (Skiba et al., 2020) and show a higher degree of variability; literature estimates a range from -1.1-259.0 kg N ha⁻¹ yr⁻¹ (Hadi et al., 2005; Inubushi et al., 2003; Melling et al., 2007; Takakai et al., 2006; Terry et al., 1981). Critically, we have relatively little data on N₂O emissions from tropical peatlands converted to oil palm (Skiba et al., 2020), making it difficult to evaluate the wider regional and global consequences of oil palm production on the global N₂O budget. There is a critical shortage of

information on the temporal variability of N₂O fluxes within oil palm plantations, making it difficult to accurately predict annual emission rates (Skiba et al., 2020). For example, Melling et al. (2007) estimated N₂O emission in the Malaysian oil palm plantation was at 566.0 kg CO₂-eq ha⁻¹ yr⁻¹; however, uncertainties were large and data were too limited either to distinguish background emissions from event-based emissions due to fertiliser application.

This study addresses these knowledge gaps by focusing on the temporal variability in N₂O fluxes from oil palm planted on peat soils in Sarawak, Malaysia. A critical component of this study is a detailed examination of the temporal dynamics of N₂O flux after fertiliser application. Considering the substantial increases in N₂O fluxes typically observed after fertilisation, neglecting these events may result in significant underestimations of the emission potential, impacting the accurate estimation of annual N₂O budgets in these systems, as highlighted by previous studies (Groffman et al., 2009; Skiba et al., 2020). Our hypothesis anticipates that N₂O emissions are strongly influenced by temporal heterogeneity and would increase significantly after N fertiliser application.

MATERIALS AND METHODS

Site Description

The study was conducted in an oil palm plantation located in tropical peatland near Bintulu, Sarawak, Malaysia (3° 12.691'N, 113° 30.008'E) planted with *Elaeis guineensis* Jacq. (oil palm) in 2007. Soils are classified as histosols on deep peat, reaching a depth of 3 to 4 m (Manning et al., 2019; McCalmont et al., 2021). Mean annual precipitation is approximately 3,200 mm yr⁻¹. The northeast monsoon from October to January has the highest rainfall, with a slightly drier southwest monsoon occurring between May and August while the two principal dry seasons fall between February to April and in September. The mean annual temperature is approximately 26.5°C (Manning et al., 2019; McCalmont et al., 2021).

Sampling Design and Fertiliser Application Method

This experiment was designed to quantify the temporal response of soil N₂O flux to fertiliser application. A spatially stratified sampling design was used to account for spatial heterogeneity in the landscape, with flux data collected from landscape management units (hereafter referred to as “microforms”). Sampled microforms included: Field drains (FD), frond piles (FP), harvest paths (HP),

inter-row/avenues (IR) and vegetation paths (VP). These microforms are regular repeating landscape components managed for a specific purpose and experience different levels of disturbance (Figure 1). FD are small drains in each planting block, used to drain water from the soil profile to a collecting drain; FP are the locations where the pruned fronds are stacked on the ground for nutrient recycling; HP are 50-75 cm wide area, present on every other palm row and completely free of weeds to allow access for workers; IR are lines of palms parallel to harvest paths and VP are pathways leading to the bushy vegetation.

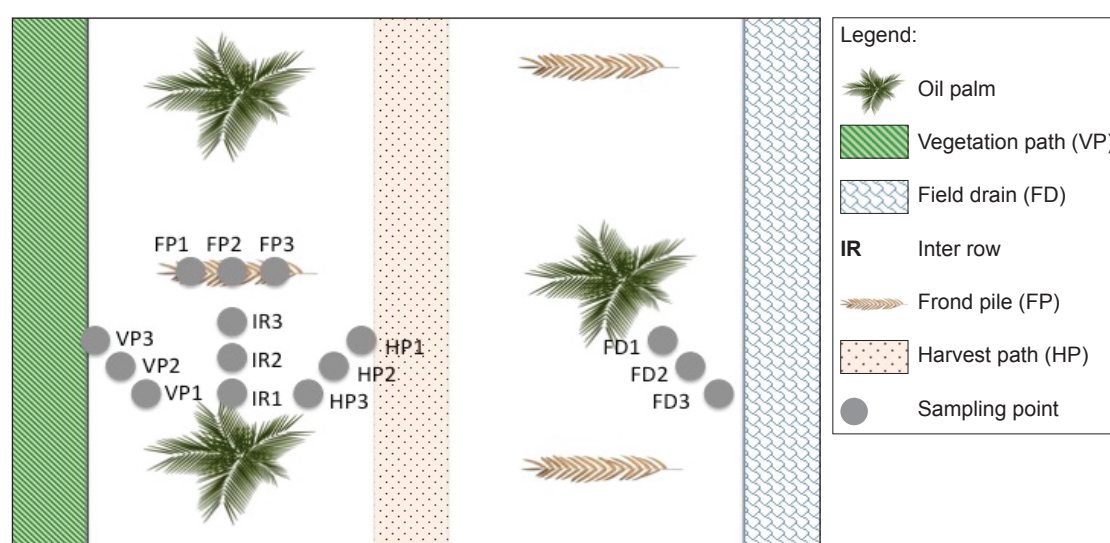
Urea was used as the N input and was applied by the broadcasting method, ensuring homogenous distribution within the circular footprint ($r = 0.0505$ m) of each flux chamber, split into two even applications over the course of the year, mimicking the estate practice. The first application of $0.925 \text{ kg urea m}^{-2}$ was made on 8 August 2015 (first campaign), with gas fluxes quantified over 212 days until 15 March 2016. The second application of $0.925 \text{ kg urea m}^{-2}$ was made on 26 April 2016 and gas fluxes were quantified for 121 days until 24 August 2016 (second campaign), thus completing a whole annual cycle of measurements.

Soil Flux Measurements

Nitrous oxide (N_2O) flux was measured using a chamber-based methodology with permanently installed static flux chambers (Parkin & Venterea, 2010). High-density polyvinyl chloride (PVC) soil collars (10.1 cm diameter and 10.0 cm high) were installed at least 5.0 cm into the ground and extended no more than 5.0 cm above the surface at 15 different sampling points surrounding the palm.

Any herbaceous vegetation was cut and removed before the collars were pushed into the soil, although root in-growth inside the collars from the surrounding palms was permitted. Gas chambers with dimensions of 10.1 cm in diameter and 16.0 cm high were placed directly on the soil collars to measure gas fluxes.

For the first field campaign, flux chambers were sampled in all five microforms and at three different distances from palms (0.5, 1.0 and 1.5 m) over a period of 212 days (i.e., $n = 3$ distances $\times$ 5 microforms per distance $\times$ 3 replicates per distance = 45 replicates). N_2O flux was quantified from August 2015 to March 2016, at 12 time points; one day prior to fertiliser application (representing the pre-fertilised condition), 1, 3, 7, 14, 30, 60, 91, 121, 152, 182 and 212 after fertiliser application. For the second field campaign, the experimental design was simplified by sampling from flux chambers only 1.5 m from each palm, rather than for all three distances from the palm over a period of 121 days (i.e., $n = 1$ distance $\times$ 5 microforms per distance $\times$ 3 replicates per distance = 15 replicates). N_2O flux was quantified from April 2016 to August 2016, at ten time points; one day prior to fertiliser application (representing the pre-fertilised condition), 1, 3, 5, 7, 14, 30, 60, 91 and 121 after fertiliser application. The reduced duration of the second field campaign was informed by the findings from the first field campaign, which indicated that a shorter sampling period was sufficient to capture the spike in N_2O flux linked to fertiliser application. We were also forced to use different flux sampling techniques because of logistical reasons and the breakdown of our instantaneous gas analyser (INNOVA 1412; INNOVA Air Tech Instruments, Denmark) that



Note: Distance between palm/drain and each sampling points are 0.5 m.

Figure 1. The layout of each microform where N_2O fluxes were measured.

was used during the first field campaign, which meant that we were forced to adopt a more labour-intensive flux sampling technique using Exetainers (Labco Ltd., UK) and gas chromatography (GC) to collect and analyse gas samples for the second field campaign. Gas concentrations were quantified using either a portable photo-acoustic spectrophotometer (INNOVA 1412, INNOVA Air Tech Instruments, Denmark) for the first campaign or by gas chromatography (Agilent 6890, Santa Clara, USA) for the second campaign. For the photo-acoustic method, concentration measurements were collected for each chamber over a period of 3 min, with at least five concentration measurements collected over that enclosure period. A Genie Membrane Separator (Lumasense Technologies, Denmark) were used, connected externally in-line, to remove water droplets and water vapour from the sample gas, so as to prevent known interferences for water vapour in our measurements (Iqbal et al., 2013; Rosenstock et al., 2013). For the GC method, gas samples were collected using a polypropylene syringe at 0, 10, 20 and 30 min intervals and stored in 20 mL evacuated Exetainers (Laboco Ltd., UK), sealed with 20 mm chlorobutyl rubber/PTFE septa. Samples were analysed at the University of Aberdeen using an Agilent 6890 GC equipped with an electron capture detector (ECD) for N_2O determination and a methaniser with a flame ionisation detector (FID) for CO_2 determination. Analytes were separated on a Haysep Q column at 45°C , with the sample volume injected by an autosampler (HT29748, HTA, Italy). The GC was calibrated for CO_2 and N_2O using chemical standard of known concentrations.

Measurement of Environmental Parameters

The volumetric soil water content (θ_v) was measured by a portable moisture meter (HH2 Moisture Meter, Delta T Devices, Cambridge) and sampled close to the chamber (within 1.0–5.0 cm). Concomitant with flux measurement, soil temperature, for the top 12.5 cm soil depth, and air temperature were measured using a probe T-bar thermometer (ATP Instrumentation Ltd, Leicestershire). The depth of the water table was collected from 2.0 m piezometers that were installed in each plot.

For climate monitoring of the area, a rain gauge was installed and daily rainfall and air temperature were measured. The water-filled pore space (WFPS) was calculated from the soil volumetric moisture measurements and using bulk density values obtained by other members of our research group (Manning et al., 2019; McCalmont et al., 2021).

Soil samples were taken at 10 cm depth twice, at the beginning and the end of the research study. The soil was homogenised, air-dried, ground and passed

through a 2 mm sieve prior to laboratory analysis. The soil was analysed for soil pH, and total C and N. Several attempts were made to sample soil water for inorganic N analysis, but these were unsuccessful because termites destroyed our soil sampling apparatus (i.e., mini-rhizons).

Statistical Analysis

Linear mixed-effects models were utilised to analyse the relationship between key independent variables (e.g., campaign, time since fertilisation, microform) and N_2O flux. Multiple regression was used to explore the relationship between environmental variables (e.g., soil temperature ($^\circ\text{C}$), air temperature ($^\circ\text{C}$), water table depth (m), WFPS, soil N, soil C, pH and N_2O flux). Data were tested for heteroscedasticity and homogeneity of variances and transformed where necessary to meet the assumptions of the parametric tests. Wherever treatments were found to be significant, pairwise testing with a t-test or means comparison test (e.g., Tukey-Kramer HSD) was performed among the main and subplot treatments due to its effectiveness in comparing multiple treatment means while controlling the familywise error rate, providing a robust statistical approach to identify significant differences between groups following an analysis of variance (ANOVA) test. All statistical analysis was performed using JMP version 15 (SAS Institute Inc., Cary, North Carolina, USA).

RESULTS AND DISCUSSION

The response of N_2O flux to fertilisation events was analysed using a linear mixed effects model that used campaign, time since fertilisation, and microform as fixed effects, while the plot was treated as a random effect. The instantaneous or cumulative N_2O flux was the dependent variable.

Effects of Fertiliser Application on Nitrous Oxide Flux Over Time

Campaign time since fertilisation and microform significantly influenced instantaneous and cumulative N_2O flux ($p < 0.0001$). Instantaneous and cumulative N_2O flux was both significantly lower for the first campaign as compared to the second one ($p < 0.0001$). The mean instantaneous flux was 129.0 ± 9 and $1021.0 \pm 150 \text{ mg N m}^{-2} \text{ d}^{-1}$ for the first and second campaigns, respectively. This represents an increase of approximately 1.9 and 15 times, respectively, relative to the pre-fertilisation flux rate of $68.0 \pm 71 \text{ mg N m}^{-2} \text{ d}^{-1}$. The mean cumulative flux, calculated over 121 days, was 14.9 ± 0.4 and $76.4 \pm 0.7 \text{ g N m}^{-2}$ for the first and second campaigns, respectively.

Instantaneous and cumulative N_2O flux increased significantly over time after fertiliser application for both campaigns ($p < 0.001$; Figure 2 and 3). However, the temporal patterns for instantaneous and cumulative fluxes differed for the two campaigns. For the instantaneous flux, peaks in emissions differed in time for the two campaigns. In the first campaign, instantaneous flux increased gradually, reaching a maximum 30 days after fertiliser application that was almost six times higher than the pre-fertilisation flux rate (i.e., $384 \pm 77 \text{ mg N m}^{-2} \text{ d}^{-1}$ vs. pre-fertilisation flux of $68 \pm 71 \text{ mg N m}^{-2} \text{ d}^{-1}$; Tukey-Kramer HSD, $p < 0.05$; Figure 2). Instantaneous flux declined gradually after this period only to rise again to reach a second maximum 91 days after fertiliser application ($285 \pm 52 \text{ mg N m}^{-2} \text{ d}^{-1}$; Tukey-Kramer HSD, $p < 0.05$; Figure 2). In contrast, instantaneous flux showed only one maximum in the second campaign, rising rapidly after fertiliser application to reach a peak 7 days after fertiliser application that was 56 times higher than pre-fertilisation flux rates (i.e., $3,777 \pm 777 \text{ mg N m}^{-2} \text{ d}^{-1}$ vs. pre-fertilisation flux of $68 \pm 71 \text{ mg N m}^{-2} \text{ d}^{-1}$; Tukey-Kramer HSD, $p < 0.05$; Figure 2). Instantaneous flux oscillated after this point but did not show another statistically significant increase (Tukey-Kramer HSD, $P > 0.05$). This temporal response is generally consistent with other studies of oil palm plantations on peat, where enhanced emissions are typically observed within the first month after fertiliser application (Chaddy et al., 2019; Oktarita et al., 2017; Sakata et al., 2015).

N_2O flux was at the higher end as compared to other managed tropical peatlands in Southeast Asia, where post-fertilisation flux rates typically range from $0.02\text{--}235.00 \text{ mg N m}^{-2} \text{ d}^{-1}$ (Groffman et al., 2009; Hadi et al., 2005; Inubushi et al., 2003; Melling et al., 2007; Oktarita et al., 2017; Sakata et al., 2015; Takakai et al., 2006; Toma et al., 2011). This difference could have several possible explanations. One possibility is that fertiliser application rates for other studies were lower than in this experiment, leading to a proportionately lower N_2O flux (Groffman et al., 2009). For example, Chaddy et al. (2019) applied fertiliser at a rate of $0\text{--}124.3 \text{ kg N ha}^{-1}$, Melling et al. (2007) at a rate of $103.0 \text{ kg N ha}^{-1}$ and Oktarita et al. (2017) at a rate of $21.0\text{--}103.0 \text{ kg N ha}^{-1}$. Another potential reason is that fertiliser applied was substantially higher amount within the experimental plots as compared to other investigators due to differences in the underlying assumptions used to calculate experimental fertiliser application rates. To briefly explain, even though 158.36 kg N is applied for every 1 ha of land in the plantation, this amount of fertiliser is only applied to areas that contain palms. This means that the soil containing palms receives a substantially higher amount of fertiliser

(i.e., $8,510.00 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) as compared to palm-free soil, which does not receive fertiliser. Here we mimicked the industry practice, applying fertiliser to the footprint of our flux chambers at the higher input rate. If other investigators used plantation-scale application rates (e.g., $158.36 \text{ kg N ha}^{-1}$) rather than the application rate for individual palms (e.g., $8,510.00 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), then differences in flux rates between our study and theirs could be explained by differences in N input (Groffman et al., 2009). It is difficult, however, to ascertain from prior publications precisely how much fertiliser was added to experimental treatments because these details are often absent from the text (Chaddy et al., 2019; Oktarita et al., 2017; Sakata et al., 2015). A third possibility is that the majority of the published studies, with the exception of Oktarita et al. (2017), did not explicitly aim to track pulses in N_2O flux linked to N fertiliser application, and consequently sampled at a lower frequency than was necessary to identify emission peaks (Chaddy et al., 2019; Melling et al., 2007; Oktarita et al., 2017; Sakata et al., 2015; Skiba et al., 2020). As a consequence, these other studies may probably have underestimated the emissions from their study sites (Groffman et al., 2009; Skiba et al., 2020).

Given the significant effects of microforms, separate temporal analyses were conducted with disaggregated data to determine if individual microforms diverged from these general patterns. However, it was found that temporal trends for individual microforms from the palm were broadly consistent with the patterns found for the pooled data.

For the cumulative flux data, the two campaigns also showed different patterns of change over time (Figure 3). The first campaign showed a gradual and relatively linear increase over 121 days of observation (Figure 3) with 50% of total emissions occurring after ~ 51 days after fertiliser application. In contrast, the second campaign showed an asymptotic increase, rising rapidly over the first 20 days after fertilisation, before shifting towards a slower rate of increase for the remainder of the observation period (Figure 3). Half of (50%) to total emissions occurred in ~ 24 days after fertiliser application; i.e., twice as quickly compared to the first campaign.

Another interesting feature of the data is that the timing and magnitude of N_2O flux differed for the two field campaigns, suggesting that patterns of soil microbial activity varied for each campaign. The slower, more gradual increase in N_2O flux (i.e., over 30 days) for the first field campaign, combined with a lower mean and peak flux, suggests that microorganisms took longer to access fertiliser N pools and that N_2O production was more substrate-limited. In contrast, the more rapid rise in N_2O flux (i.e., 7 days) for the second field campaign, combined with a significantly higher mean and peak flux,

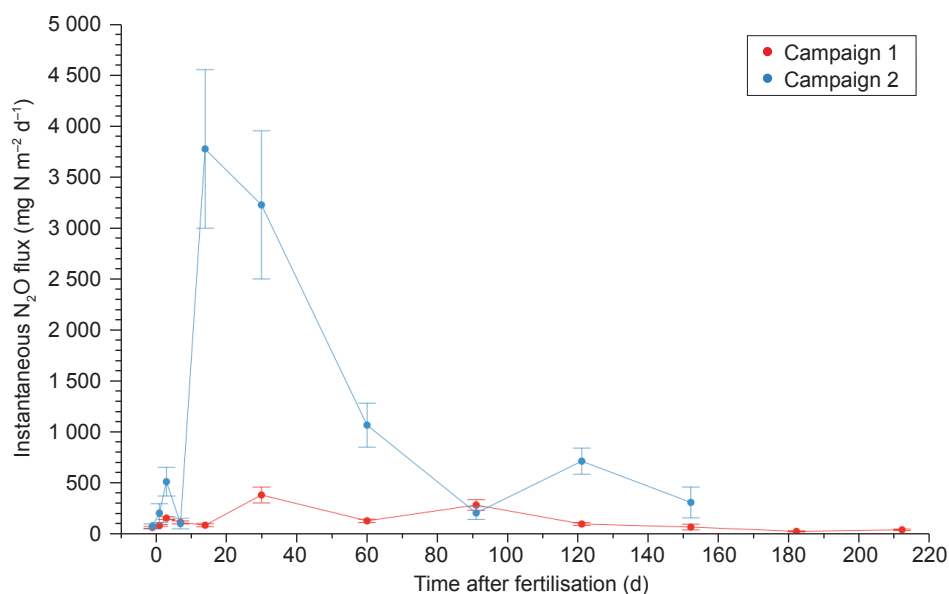


Figure 2. Instantaneous N₂O flux for the first (red) and second (blue) field campaigns. Bars indicate standard errors.

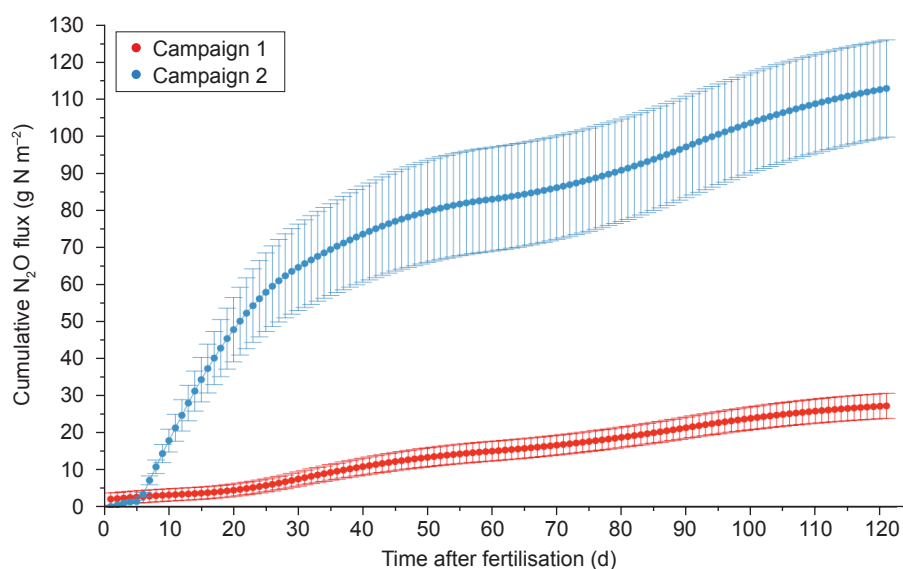


Figure 3. Cumulative N₂O flux for the first (red) and second (blue) field campaigns. Bars indicate standard errors.

suggests that microorganisms were able to access fertiliser N more rapidly and were less substrate-limited. This high variability among individual fertilisation events appears consistent with other studies (Chaddy et al., 2019; Oktarita et al., 2017; Sakata et al., 2015), although it is unclear what causes variability among individual fertilisation events. One explanation is that differences in plant N demand could have influenced substrate availability for N₂O production between campaigns. For example, greater competition by palms for N during the first field campaign could have limited microbial access to N and suppressed N₂O production (Groffman et al., 2009; Pardon et al., 2016). If plant N demand was weaker during the second campaign, then more fertiliser N could

have been accessed by soil microorganisms. Plant N demand is also varied at different stages of oil palm growth, target yield, soil type, fertility and climate that might affect the variability. The greater competition by palms for N during the dry season compared to the wet season may be attributed to reduced water availability, diminished microbial activity, decreased decomposition rates, altered physiological responses, and intensified root exploration in response to limited nutrient accessibility.

A second explanation is that there were significant differences in other competing sinks for N between the two field campaigns, such as ammonia volatilisation, leaching or run-off (Pardon et al., 2016). For example, volatilisation alone can

lead to >40% loss of the urea applied to oil palm soils; hence, changes in these processes, driven by environmental variables, could have significant consequences for plant uptake or downstream microbial processes such as denitrification (Pardon et al., 2016). Alternatively, ammonium sulphate is a source of N and sulphur (S), however, it tends to decrease soil pH from oxidation of ammonium ion to nitrate and leaching of the nitrate ion (Lau et al., 2023).

In our study site, differences in rainfall between the two campaigns may have affected the relative partitioning of N between different pathways. The first field campaign took place during the dry season, whereas the second occurred in the wet season. It is possible that more N was lost via ammonia volatilisation during the first (dry season) campaign, whereas more N was processed via microbial pathways in the second (wet season) campaign, given that rainfall is known to significantly reduce rates of ammonia volatilisation (Black et al., 1987; Bouwmeester et al., 1985). However, rainwater can lead to the runoff of fertilisers, affecting fertiliser effectiveness through nutrient losses (Guo & Chen, 2022). Thus, optimising water and N management might be one of the most effective ways to reduce N₂O emissions from agricultural soils. Most studies have reported increased N₂O emissions after the application of N fertiliser, especially with high soil moisture. N₂O is generally emitted most rapidly when the soil has >60% water-filled-pore when the soil pore water displaces the amount of available oxygen in the soil pores and therefore leads to anaerobic soil moisture conditions, which are conducive to the production of N₂O. Under such conditions, the soil NO₃⁻ is reduced by facultative anaerobic bacteria (e.g., *Pseudomonas citronellolis*) to NO₂⁻, N₂O and then N₂ (Wang et al., 2021).

A third possibility is that ongoing peat decay and N mineralisation could strongly influence temporal patterns in N₂O flux by supplementing any fertiliser-derived N₂O (Toma et al., 2011). While many investigators have assumed that fertiliser N inputs drive N₂O flux in managed tropical peatlands (Chaddy et al., 2019; Melling et al., 2007; Oktarita et al., 2017; Sakata et al., 2015; Skiba et al., 2020). Toma et al. (2011) challenged this assumption when they found that there was no significant correlation between annual N₂O emission and fertiliser application rates. Instead, they found that long-term management plays a greater role in modulating N₂O flux, where management practices or the history of cultivation have altered the quality of the peat, characteristics of the soil microorganisms and subsequent rates of N₂O flux. This implies that N₂O derived from peat organic matter could play a significant role in trace gas exchange.

Total Cumulative Flux over 121 Days

Total cumulative flux, an estimate of the total amount of N₂O released over 121 days, was analysed using a linear mixed effects model using campaign, microform from palm as fixed effects, with plot treated as a random effect. Campaign and microform were significantly related to total cumulative flux ($p < 0.0001$). Total cumulative flux was significantly higher in the second campaign as compared to the first campaign, differing by a factor of four ($p < 0.001$; 112.7 ± 13.1 vs. 27.3 ± 3.4 g N m⁻², respectively). For the microforms, IR and HP showed the highest total cumulative flux, VP and FD showed intermediate total cumulative flux, and the FP showed the lowest total cumulative flux (Tukey-Kramer HSD, $p < 0.05$; Figure 4). The large N₂O emissions observed in this experiment suggest that a substantial proportion of the applied fertiliser could have been lost from the soil as N₂O. Total cumulative flux, calculated over 121 days, suggests that up to 27.3 ± 3.4 g N m⁻² of fertiliser could have been lost as N₂O during the first campaign, while up to 112.7 ± 13.1 g N m⁻² could have been lost during the second campaign. This equates to 6.4% and 26.5% of the applied fertiliser in the first and second field campaigns, respectively. These values are in the same order of magnitude as those found in comparable studies in oil palm plantations on peat soil (Chaddy et al., 2019; Oktarita et al., 2017; Sakata et al., 2015). For example, Chaddy et al. (2019) found that total cumulative N₂O flux could account for as much as 33.4%-80.7% of the applied fertiliser, while Sakata et al. (2015) found as much as 52.6%-92.8% of the applied fertiliser could have been lost as N₂O. This suggests that these soils are behaving similarly to those in other studies, despite very high instantaneous flux rates.

Spatial Patterns in N₂O Flux

Linear mixed effects models indicated that microform significantly influenced instantaneous and cumulative fluxes ($p < 0.0001$). For the microform data, HP and IR showed the highest instantaneous and cumulative fluxes. VP and FD showed intermediate fluxes, and FP showed the lowest fluxes (Tukey-Kramer HSD, $p < 0.05$; Figure 5, data only shown for instantaneous flux measurements).

Significant differences in N₂O flux were observed among microforms consistent with studies of trace gas exchange in other spatially stratified peatland ecosystems (Jones et al., 2018; Manning et al., 2019; Teh et al., 2011, 2014, 2017). With respect to the role of microforms, N₂O flux was generally higher in HP and IR microforms, and significantly lower in other microforms. It is possible that differences in WFPS among the microforms influenced how much fertiliser was converted either

to N_2O or N_2 by denitrification (Wang et al., 2021). HP and IR microforms were generally drier than the others, with WFPS averaging $41 \pm 1\%$ and $53 \pm 2\%$ respectively. In contrast, WFPS in FD, FP and VP microforms were significantly higher, averaging

$65 \pm 2\%$, $78 \pm 1\%$ and $56 \pm 2\%$ WFPS, respectively. Higher N_2O flux rates from HP and IR microforms may thus reflect more incomplete denitrification, while wetter microforms may experience more complete denitrification (Wang et al., 2021).

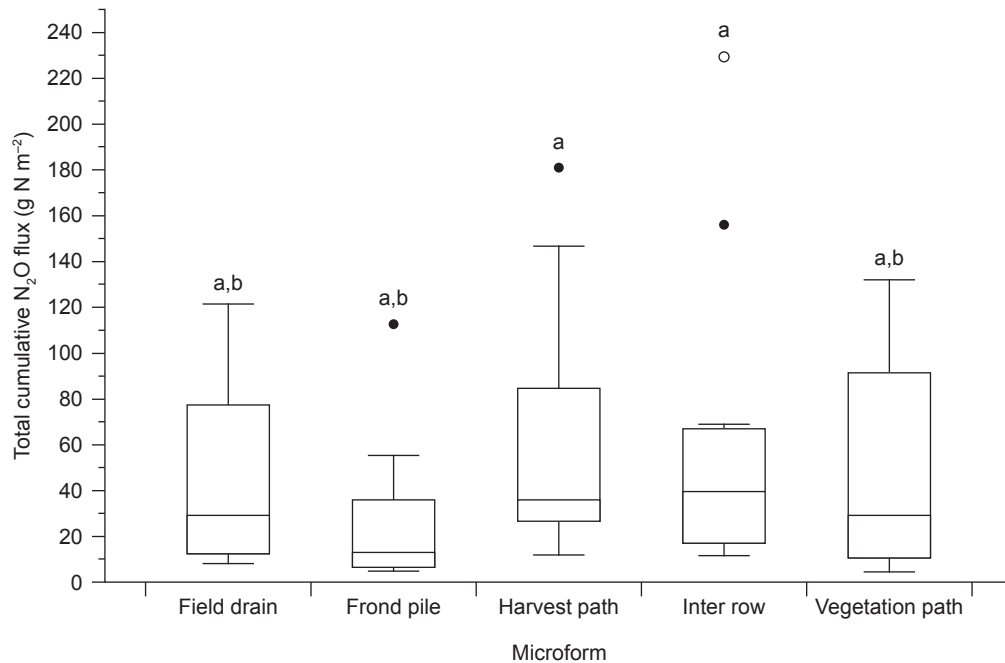


Figure 4. Total cumulative flux, calculated at 121 days, for different microforms. Data was pooled from both field campaigns. Boxes enclose the interquartile range, whiskers indicate the 90th and 10th percentiles and the solid line within each box indicates the median. Lowercase letters indicate significantly different means (Tukey-Kramer HSD, $p < 0.05$).

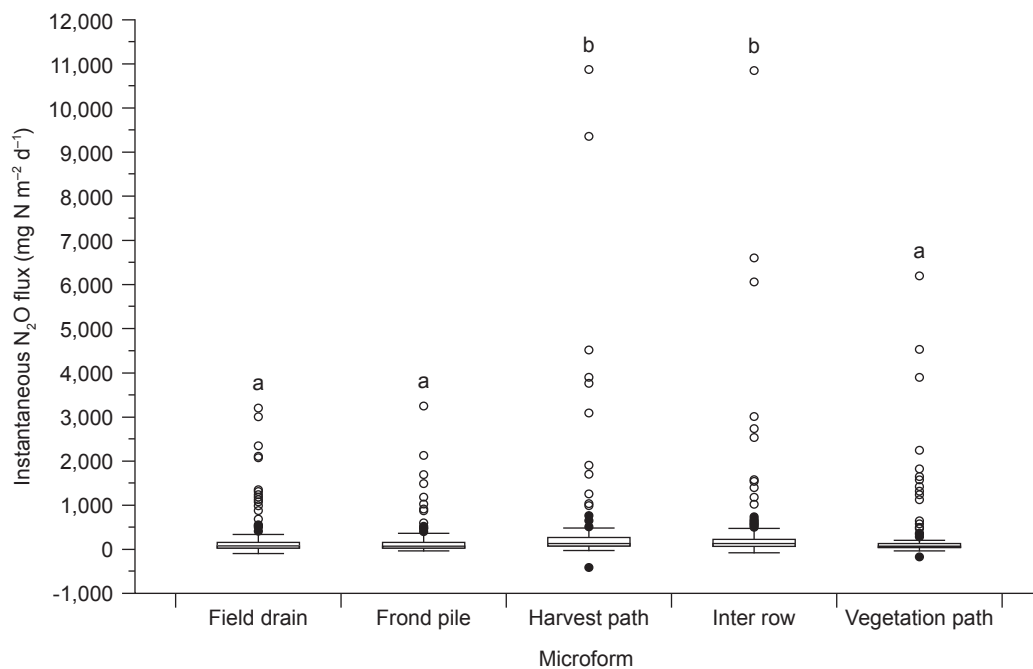


Figure 5. Instantaneous N_2O flux for different microforms. Data was pooled for both field campaigns. Boxes enclose the interquartile range, whiskers indicate the 90th and 10th percentiles and the solid line within each box indicates the median. Lowercase letters indicate significantly different means (Tukey-Kramer HSD, $p < 0.05$).

Role of Environmental Variables in Regulating N₂O Fluxes

The relationship between environmental variables (i.e., water table depth, WFPS, soil temperature, air temperature, soil N, soil C and pH) and instantaneous N₂O flux was examined using multiple regression models. Two separate multiple regression analyses were conducted, one containing all the environmental variables and a second focusing only on water table depth, WFPS, soil temperature and air temperature. The second analysis was performed because soil chemical variables (i.e., soil N, soil C, pH) were only measured for a subset of samples ($n = 90$), whereas soil moisture and temperature data for all flux measurements ($n = 690$). The second analysis of moisture and temperature variables would provide a more representative picture of the relationship between these drivers and N₂O flux, given the much larger sample size.

Analysis of all the environmental variables suggests that N₂O flux was negatively correlated with WFPS ($r^2 = 0.28$, $p < 0.0003$, $n = 90$), whereas the analysis of only moisture and temperature data suggests that N₂O flux was weakly predicted by water table depth (negative correlation), soil temperature (positive correlation) and air temperature (positive correlation) ($r^2 = 0.12$, $p < 0.0001$, $n = 690$).

When the data were disaggregated and data were analysed separately for each campaign, slightly different patterns emerged. For the first campaign, N₂O flux was weakly correlated with water table depth (negative correlation), WFPS (negative correlation) and air temperature (positive correlation) ($r^2 = 0.07$, $p < 0.0001$, $n = 540$). For the second campaign, analysis of the moisture and temperature data suggest that only water table depth (negative correlation) and soil temperature (positive correlation) were correlated with N₂O flux ($r^2 = 0.12$, $p < 0.0009$, $n = 150$).

In our study, patterns of N₂O emissions were affected by soil WFPS, perhaps because greater soil moisture favoured denitrification. The negative relationship between WFPS and N₂O fluxes observed in this study could be due to increasing N₂O reductase activity at higher soil moisture content, as the soils become more anaerobic. In anaerobic soil, near saturated WFPS (>60% WFPS) conditions, denitrification and nitrifier denitrification are recognised as the potential processes of N₂O consumption in soils (Wang et al., 2021). Although it was negative correlation, the relationship between WFPS and N₂O fluxes in the first campaign was weak ($R = -0.2$), therefore, it is not surprising that the second fertiliser addition campaign showed no evident response. Even the occurrence of rainfall events stimulates soil N

mineralisation (Xu et al., 2023; Zhang et al., 2020) and wetted soils often have high carbon and N availability that is linked to high denitrification rates (Sakata et al., 2015). However, a lower C:N ratio promotes increased N mineralisation and N₂O emissions (Liimatainen et al., 2018) and soil pH has a marked effect on the products of denitrification. Denitrification rates would be slower under the strong acid conditions in the peat soils. This is commonly attributed to the sensitivity of N₂O reductase to proton activity, and it is also likely that all denitrifying enzymes are susceptible at low soil pH and produce N₂O from other intermediate products (Nägele & Conrad, 1990). Finally, numerous studies have demonstrated that the N₂O:N₂ ratio decreases with increasing pH (Žurovec et al., 2021).

CONCLUSION

The high N₂O flux and N losses observed in this study site suggest that current fertiliser application rates may be higher than is necessary to sustain palm growth and yields, given that such a large proportion of fertiliser N is lost as N₂O. Further site-specific trials may be required to fine-tune fertiliser application rates for this plantation to minimise N loss and mitigate greenhouse gas emissions. Generally, adapting to better fertiliser management such as tailoring the nutrient application to crop requirements, implementing precision agriculture techniques, the use of controlled-release fertilisers, and improving soil management practices can help reduce the high N₂O flux and losses.

Large differences in N₂O flux between the first and second fertilisation events suggest major, intra-annual shifts in N dynamics that warrant further investigation, as it is unclear what proportion of the applied N is being taken up by plants or lost by other pathways such as ammonia volatilisation, nitric oxide (NO) flux, dinitrogen (N₂) flux, leaching and run-off. Future studies could exploit stable isotope tracers to more accurately track the fate of fertiliser N in these important production systems. Lastly, significant spatial heterogeneity in N₂O flux emphasises the need for more detailed, spatially explicit sampling of oil palm systems in order to accurately estimate ecosystem-scale patterns in N₂O emission and to understand the role of local management effects on N₂O flux.

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SEPARATION AND THERMAL MODIFICATION OF MONTMORILLONITE FROM TANAH DATAR CLAY AND ITS CATALYTIC APPLICATION IN BIODIESEL PRODUCTION FROM WASTE FRYING OILS

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ABSTRACT

Natural clay comprises a blend of diverse minerals exhibiting distinct structures and characteristics. Montmorillonite exhibits strong acidity and stabilising effects on active species due to its unique layered structure, thus making it a suitable material for use as a heterogeneous catalyst. Therefore, this research aims to examine the effect of separation and thermal modification process of Tanah Datar clay minerals, as well as their catalytic activity in the conversion of waste frying oil (WFO) into biodiesel. The catalysts were evaluated using X-ray diffraction (XRD), X-ray fluorescence (XRF) and Laser Particle Size Analyser (LPSA). The uncalcined clay primarily consists of montmorillonite, along with kaolinite, hematite and quartz. Upon calcination, montmorillonite transforms into meta montmorillonite. The particle size of montmorillonite increases after separation and calcination. The catalysts were employed in a lab-scale biodiesel production via transesterification reaction, employing a mole ratio of oil and methanol of 1:6, catalyst amount of 3.00% (w/w), stirring speed of 500 rpm, and a temperature of 70°C for 3 hr. Both uncalcined and calcined montmorillonite catalysts can enhance biodiesel yield by up to 80.11%. This observation demonstrates the considerable potential of the implemented treatment as a catalyst in biodiesel production.

Keywords: biodiesel, catalyst, clay, montmorillonite, transesterification.

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INTRODUCTION

Energy is one of the factors in sustaining human life activities. Energy consumption always increases every year. Continuous energy use results in a shortage of energy originating from fossil fuels. The use of fossil fuels is also a global problem due to excessive levels of CO₂ emissions, environmental

impacts and threats to the economy, environment, and human welfare (Azni et al., 2023; Rehman et al., 2021). One renewable energy source that can lessen the scarcity of fossil fuels and lessen environmental pollution is biodiesel (Pacheco-López et al., 2021; Pikula et al., 2020). Biodiesel fuel can replace fossil fuels used for diesel engines. It is more attractive than diesel fuel because it is non-toxic, biodegradable, and can be produced from renewable sources, making it a sustainable alternative fuel (Nguyen et al., 2023; Siddeg et al., 2022). Additionally, it can also help reduce greenhouse gas emissions by up to 86% compared to petroleum diesel (Suwarno et al., 2021; Xu et al.,

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2022). It can be made through several methods, such as dilution, pyrolysis, microemulsion, and transesterification (Irawan et al., 2021; Mishra & Goswami, 2018; Supriyanto et al., 2021). The transesterification method is preferred for biodiesel due to its low cost compared to other methods. In addition, the transesterification process is also relatively simple (Gouran et al., 2021; Salaheldeen et al., 2021).

Making biodiesel by transesterification method uses raw materials containing triglycerides, such as vegetable oils. Some types of vegetable oils that have been used are sunflower (Jabbari, 2018; Khan et al., 2023; Vital-López et al., 2023), castor oil (Bibin et al., 2020; Mustafa et al., 2023; Ubaid et al., 2022), coconut oil (Bambase et al., 2021; Lugo-Méndez et al., 2021), and palm oil (Nang et al., 2009; Palaniselvam et al., 2023; Syukri et al., 2022). However, the use of waste frying oil (WFO) is of great interest. This is because biodiesel from WFO is an environmentally friendly process. After all, WFO can be utilised, making it more economical and effective in waste management (Gouran et al., 2021; Sukkathanyawat & Wichianwat, 2023). The transesterification reaction also requires a catalyst. The catalysts used can be homogeneous catalysts and heterogeneous catalysts. Homogeneous catalysts have disadvantages such as difficulty in catalyst separation, high effluent generation (Pasae et al., 2021; Silva et al., 2015), and equipment corrosion, while heterogeneous catalysts offer advantages like reusability, selectivity, and low cost (Faruque et al., 2020; Gaide et al., 2023; Kibar et al., 2023). Therefore, many heterogeneous catalysts are now developed in the transesterification reaction, one of which is montmorillonite.

Montmorillonite belongs to the smectite group and has a 2:1 layer structure (Belghazdis & Hachem, 2022; Uddin, 2018; Yaghmaeiyan et al., 2022). Alumina and silica make up the majority of montmorillonite, a typical cationic clay; other metal oxides make up a small portion (Tripol'Skaya et al., 2009). Montmorillonite exhibits strong acidity and a stabilising effect on active species, such as metal nanoparticles, due to its unique layered structure. This makes it a suitable material for use as a heterogeneous catalyst (Takabatake & Motokura, 2022). Several studies have used montmorillonite as a catalyst for transesterification reactions, such as montmorillonite KST in producing biodiesel from palm oil (Kansedo et al., 2009), the novel modified montmorillonite to produce biodiesel from castor and *Jatropha* oil (Negm et al., 2017), and barium-loaded montmorillonite for the synthesis of biodiesel from waste cooking oil (WCO) (Sharma & Bhavani, 2021). Each of these reactions produces high yields of biodiesel. However, the chemical modification given to the montmorillonite catalyst requires

complicated steps and is expensive (Shapkin et al., 2017). Meanwhile, physical modifications can also be given to montmorillonite, such as thermal modifications (Abdrakhimova & Abdrakhimov, 2007; Wu et al., 2022). Previous researchers reported that clay calcined at 850°C could increase biodiesel yield (Admi et al., 2022).

The montmorillonite that is widely used in catalyst application is synthetic montmorillonite, which consequently increases production costs. However, montmorillonite is one of the minerals contained in natural clay. Separating montmorillonite from natural clay can be a solution to reduce catalyst production costs. Montmorillonite was separated from Boyolali natural clay by previous researchers and applied as an oil-bleaching agent (Taslimah et al., 2008). However, montmorillonite, separated from natural clay has never been used as a catalyst. Therefore, this research applies montmorillonite separated from natural clay as a catalyst in the transesterification reaction. Based on data in 2021, the availability of clay in Indonesia is very abundant. There are about 10,387.209 million tonnes of natural clay available in West Sumatera Province. Therefore, the catalyst raw material used in this study was taken from one of the areas in West Sumatera Province, namely Tanah Datar Regency because there have been no reports regarding the separation of montmorillonite minerals from clay in that area. The Tanah Datar area has abundant clay, which is characterised by the presence of brick production sites.

This study aims to determine the effect of separation process and thermal modification on mineral composition, physical and chemical properties of minerals, and catalytic activity of uncalcined and calcined montmorillonite from Tanah Datar clay in the process of converting WFO into biodiesel.

MATERIALS AND METHODS

Materials Preparation

The clays used in this study were taken from Andaleh, Batipuh District, Tanah Datar Regency, West Sumatra Province, Indonesia. The area is located at latitude 0°27'12.4"S and longitude 100°26'09.7"E in the form of a plateau area at an altitude of 946 m above sea level. The map of natural clay (*n-clay*) sampling locations can be seen in Figure 1. The *n-clay* was soaked in distilled water for 24 hr, filtered, and dried at 105°C for 3 hr. The clay was crushed and sieved with a sieve of 90 µm (Syukri et al., 2020). The heated clay (*h-clay*) catalyst was obtained ready to be separated from the montmorillonite mineral. The transesterification reaction was carried out following the methods

reported by previous researchers (Febiola et al., 2023) with some modifications using three times WFO and methanol (Merck) as raw materials. Ammonia hydroxide (NH_4OH) was used as a dispersing agent for montmorillonite from Tanah Datar clay following the method reported by previous researchers (Taslimah et al., 2008).

Separation and Thermal Modification of Montmorillonite

Montmorillonite was separated by dispersing it in an NH_4OH solution. In brief, 25 g of *h-clay* was stirred in 400 mL NH_4OH 2% (v/v) with a magnetic stirrer for 15 min and left for two days (Taslimah et al., 2008). The suspension was separated by centrifugation at 1,000 rpm for 5 min. The precipitate was separated and dried in an oven at 105°C to obtain the montmorillonite clay (*Mt-clay*) catalyst. Thermal modification is carried out by calcining the *Mt-clay* catalyst at a temperature of 850°C in a furnace for 4 hr to obtain the calcined montmorillonite clay (*c-Mt-clay*) catalyst.

Catalyst Characterisation

The catalyst's crystallinity was assessed through the utilisation of X-ray diffraction (XRD) equipment by X'Pert MPD (PANalytical) which employed Cu $\text{K}\alpha 1$ radiation ($\lambda = 1.54059 \text{ \AA}$). The equipment operated at 40 kV and 30 mA, covering an angular range of 2θ 10° to 90° . The oxide composition was determined using X-ray fluorescence (XRF) equipment by NEXCG Rigaku. Particle size was assessed using the Laser Particle Size Analyser (LPSA) equipment by Labtron, LLPA-C10, which has a measuring range of 0.01-2,000.00 μm .

Preparation and Determination of Free Fatty Acids in WFO

The preparation of WFO is done by heating it at 105°C until the water bubbles disappear. Free fatty acid (FFA) content was determined following the acid-base titration method reported by previous researchers (Febrianto et al., 2020) with the Equation (1):

$$\% \text{ FFA} = \frac{V_{\text{NaOH}} \times N_{\text{NaOH}} \times \text{Bm}_{\text{fatty acid}}}{M \times 1000} \quad (1)$$

where, V_{NaOH} is the volume of 0.1 N NaOH titration (in mL), N_{NaOH} is normality of NaOH, $\text{Bm}_{\text{fatty acid}}$ is molecular weight of fatty acid (in g) and M is mass of oil sample.

Biodiesel Production Through Transesterification Reaction and Its Characterisation

The WFO used in this study has a density of 0.90 g cm^{-3} and FFA of 1.65%. The FFA value is lower than 3% so WFO can be used directly in the transesterification reaction. Each catalyst was used in the transesterification reaction of WFO by refluxing method. The molar ratio of WFO and methanol used was 1:6 with 3.00% (w/w) catalyst. The transesterification reaction was carried out for 3 hr at 70°C with a stirring speed of 500 rpm. The catalyst and glycerol were separated from the product mixture by centrifugation. The biodiesel layer was washed with 50°C aquadest at a volume of 1:1 and homogenised for 5 min. The mixture was allowed to stand so that the dense water would separate as the bottom layer and drained out. The remaining water in the biodiesel was removed



$0^\circ 27' 12.4'' \text{S } 100^\circ 26' 09.7'' \text{E}$

Figure 1. Map of natural clay sampling locations.

using anhydrous Na_2SO_4 . The yield of biodiesel was calculated using the Equation (2) (Munir et al., 2019):

$$\% \text{ Yield} = \frac{\text{Mass of biodiesel produced (g)}}{\text{Mass of oil used (g)}} \times 100 \quad (2)$$

Product Analysis

The type and content of methyl esters produced in biodiesel products are identified using Gas Chromatography-Mass Spectrometry/GC-MS (Shimadzu QP 2010 Ultra). The GC-MS instrument is equipped with a DB5 MS column with Helium as a carrier gas and an injection temperature of 250°C. The biodiesel product was determined for density at 15°C (ASTM D6751) and moisture content (ASTM D2709).

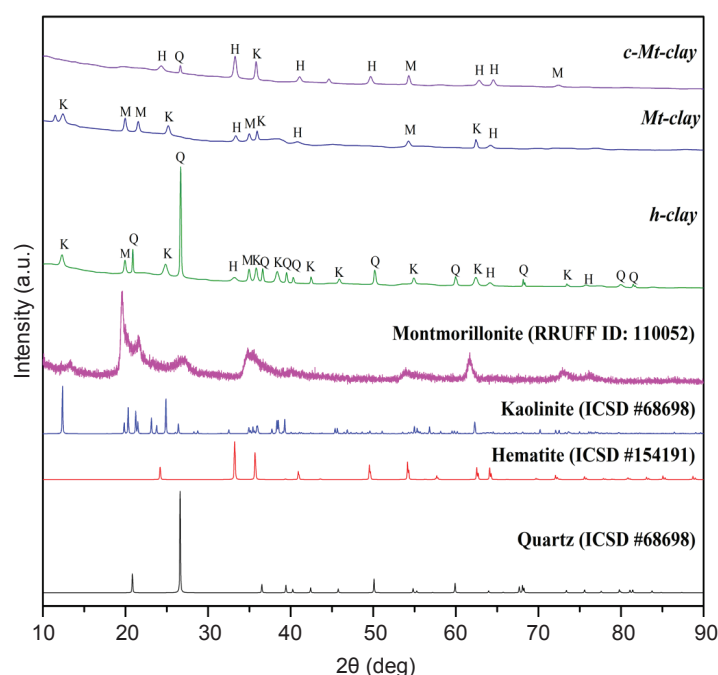
RESULTS AND DISCUSSION

X-ray Diffraction Characterisation

Figure 2 shows the X-ray diffraction pattern of the *h-clay*, *Mt-clay* and *c-Mt-clay* catalysts scanned at 2θ from 20°-90° that the Tanah Datar clay contains the minerals montmorillonite and kaolinite. The presence of montmorillonite (RRUFF ID: 110052) was found to have diffraction intensities at 2θ : 19.9° and 34.9°. Kaolinite in the *h-clay* sample was confirmed based on Inorganic

Crystal Structure Database (ICSD) No. 68698 with the appearance of diffraction intensity at 2θ : 12.3°; 24.8°; 36.5°; 38.3°; 42.4°; 45.8°; 54.8° and 62.3°. The presence of quartz (ICSD No. 68698) was found with high intensity at 2θ : 26.6°. Quartz is a stable phase of silica in the form of an inert compound. When at normal temperature, quartz has a trigonal quartz (α -quartz) structure (Götze et al., 2021). The diffraction intensity of hematite (ICSD No. 154191) was also found at 2θ : 33.1°; 36.5°; 64.0° and 75.7°.

The separation can cause changes in mineral peaks. Montmorillonite peaks appear at 2θ : 21.5° and 54.2°. Meanwhile, the peak that existed before separation remained after separation and there was an increase in intensity, namely at 19.9° and 34.9°. Kaolinite mineral as an impurity in *Mt-clay* also causes the disappearance of the peak at 2θ : 36.5°; 42.4°; 45.8° and 54.8°. Meanwhile, the kaolinite peak that is still present in the *Mt-clay* sample has a low intensity at 2θ : 12.4°; 25.1°; 35.9°; and 62.4°. Hematite also experiences peak loss at 2θ : 36.5° and 75.7°. The thermal modification given to *c-Mt-clay* caused the disappearance of most of the montmorillonite mineral peaks (Figure 2). Montmorillonite peaks were only found at 2θ : 54.2° and 72.5° with very low intensity. The diffraction peaks of *c-Mt-clay* are dominated by hematite peaks, at 2θ : 24.3°; 33.2°; 41.0°; 49.6°; 62.7° and 64.4°. Another interesting point is the appearance of a quartz peak at 2θ : 26.6° albeit with very low intensity, which was previously missing in the *Mt-clay* sample.



Note: M - montmorillonite, K - kaolinite, Q - quartz, H - hematite.

Figure 2. XRD diffractograms of *h-clay*, *Mt-clay*, and *c-Mt-clay*.

X-ray Fluorescence Characterisation

Thermal separation and modification affect the elemental and oxide composition of each catalyst (Table 1). According to the XRF analysis, it is evident that all catalyst samples contain a significant proportion of silicon (Si) and aluminium (Al) elements in their composition, whose levels reach more than 60% of the total of all elements in the sample. This shows that the catalysts used are indeed classified as clay because they have the main constituent composition of clay (Lomertwala et al., 2019). In addition, it can be seen that the type of montmorillonite contained in Tanah Datar clay is Ca-montmorillonite because there is Ca content in the clay (Park et al., 2016). Iron oxide occupies the third highest position as a constituent composition of clay catalysts whose levels reach 19%-32%. Determination of the Si/Al mole ratio on the clay catalyst also needs to be done to determine the potential of the sample as a catalyst. It is known that the *h-clay* has a Si/Al mole ratio of 1.79; *Mt-clay* has a Si/Al mole ratio of 1.27 and *c-Mt-clay* has a Si/Al mole ratio of 1.26.

Laser Particle Sizer Analyser Characterisation

The particle size distribution of *h-clay* and *Mt-clay* catalysts may change due to the montmorillonite and separation process (Figure 3). The *h-clay* catalyst is significantly distributed at a particle size of 5.21 μm , accounting for 3.5% of the total samples. After separation and calcination, the particle size distribution increased to 9.60 μm for 4.37% of *Mt-clay* and to 37.86 μm for 9.37% of *c-Mt-clay*. This causes the specific surface area of particles (S) to decrease due to the separation and

calcination process, where the known S values of *h-clay*, *Mt-clay*, and *c-Mt-clay* are 5.57 $\text{m}^2 \text{g}^{-1}$, 1.73 $\text{m}^2 \text{g}^{-1}$ and 0.24 $\text{m}^2 \text{g}^{-1}$ (Table 2). *H-clay* has a particle size distribution of <0.1-2,000.0 μm . While the particle size of *Mt-clay* and *c-Mt-clay* spread over >0.1-2000.0 μm .

The separation and calcination can cause the standard percentile value of the sample to rise (Table 2). The D10 of *h-clay* (0.51 μm) increased in *Mt-clay* (1.54 μm) and *c-Mt-clay* (13.01 μm). This shows that 10% of the samples are below 0.51, 1.54 and 13.01 μm in size. The D50 value of *h-clay* (4.74 μm) increased at *Mt-clay* (9.14 μm) and *c-Mt-clay* (33.01 μm). This indicates that 50% of the samples are smaller than this value for each sample. Similarly, the D90 value becomes 44.14, 54.37 and 83.72 μm in *h-clay*, *Mt-clay* and *c-Mt-clay* which means that 90% of the samples have a small size of that value. The Dav (average particle size) value also increased in *h-clay* 20.75-23.94 μm in *Mt-clay* and 43.02 μm in *c-Mt-clay*. The increase in particle size on the catalysts after separation and calcination may be due to Van der Waals forces increasing the ability for particles to gather to form agglomerations (Hamed et al., 2019).

Mechanism for Separating Montmorillonite from Natural Clay

Montmorillonite mineral was separated from *h-clay* by dispersing it in an NH_4OH solution (Taslimah et al., 2008). NH_4^+ ions from NH_4OH enter the interlayer of montmorillonite minerals through interlayer cation exchange events, causing swelling in the montmorillonite structure (Gautier et al., 2010; Tong et al., 2021). Swelling that occurs due to intercalation by NH_4^+ ions can cause the

TABLE 1. CHEMICAL COMPOSITION OF *h-clay*, *Mt-clay*, AND *c-Mt-clay*

Oxide	<i>h-clay</i> (%)	<i>Mt-clay</i> (%)	<i>c-Mt-clay</i> (%)
Al_2O_3	22.854	22.893	25.845
SiO_2	48.356	34.459	38.502
P_2O_5	3.325	3.261	2.912
K_2O	1.108	0.743	0.599
CaO	0.677	0.688	0.597
TiO_2	2.122	1.866	1.777
V_2O_5	0.069	0.096	0.089
Fe_2O_3	20.476	32.657	28.606
ZnO	0.068	0.119	0.099
SrO	0.005	0.003	0.000
ZrO_2	0.076	0.101	0.066
Ag_2O	0.632	0.675	0.0682
PbO	0.014	0.032	0.024
Si/Al ratio	1.790	1.270	1.260

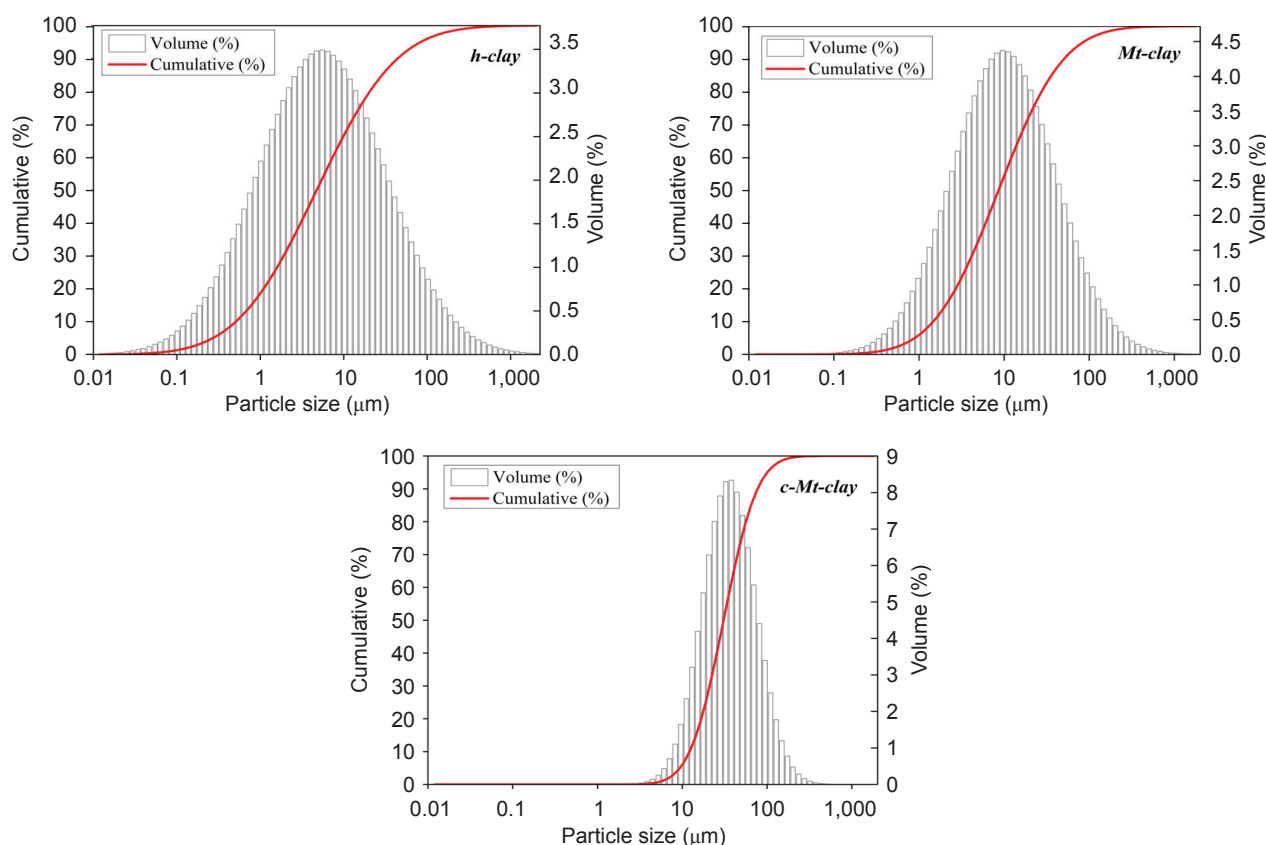


Figure 3. Particle size distribution curves of *h-clay*, *Mt-clay*, and *c-Mt-clay*.

TABLE 2. PARTICLE SIZE DISTRIBUTION CHARACTERISTICS OF *h-clay*, *Mt-clay* AND *c-Mt-clay*

Type of catalyst	D10 (μm)	D50 (μm)	D90 (μm)	Dav (μm)	S (m ² g ⁻¹)
<i>h-clay</i>	0.51	4.74	44.14	20.75	5.57
<i>Mt-clay</i>	1.54	9.14	54.37	23.94	1.73
<i>c-Mt-clay</i>	13.01	33.01	83.72	43.02	0.24

Note: D10, D50, D90 - standard percentiles; Dav - average particle size (agglomeration size); S - specific surface area of particles.

opening of interlayer space in the montmorillonite structure. The distance between the layers of the montmorillonite structure will increase and eventually release so that the montmorillonite can be dispersed in the NH_4OH solution. In addition, the interaction of NH_4^+ ions with montmorillonite can also increase hydration in montmorillonite (Peng et al., 2020). The separation mechanism of montmorillonite using NH_4OH solution can be seen in Figure 4.

Effect of Separation and Thermal Modification on Mineral Type and Composition

Separation of montmorillonite from *h-clay* has a significant effect on increasing the purity of *Mt-clay*. According to the XRD results, new montmorillonite peaks appear at 2θ : 21.5° and 54.2° . Meanwhile, the peak that existed before separation persists after separation and there is an increase in intensity.

The kaolinite mineral as an impurity in *Mt-clay* also decreased substantially and several peaks experienced a decrease in intensity. Hematite also experiences peak loss. The disappearance of the diffraction peaks of montmorillonite mineral in the *c-Mt-clay* sample calcined at 850°C is due to the dehydroxylation of montmorillonite to meta montmorillonite (Werling et al., 2022). During the calcination process, there is a removal of water from the surface and water between the layers of montmorillonite (Taylor-Lange et al., 2015). This means that montmorillonite undergoes thermal decomposition in the form of dehydration and dehydroxylation during the calcination process (Scrivener & Favier, 2015). According to the XRF results, the Si/Al mole ratio decreased due to the separation and thermal modification processes. A small Si/Al mole ratio indicates a high Al content. High Al comes from aluminate which is negatively charged. The large number of negative charges in

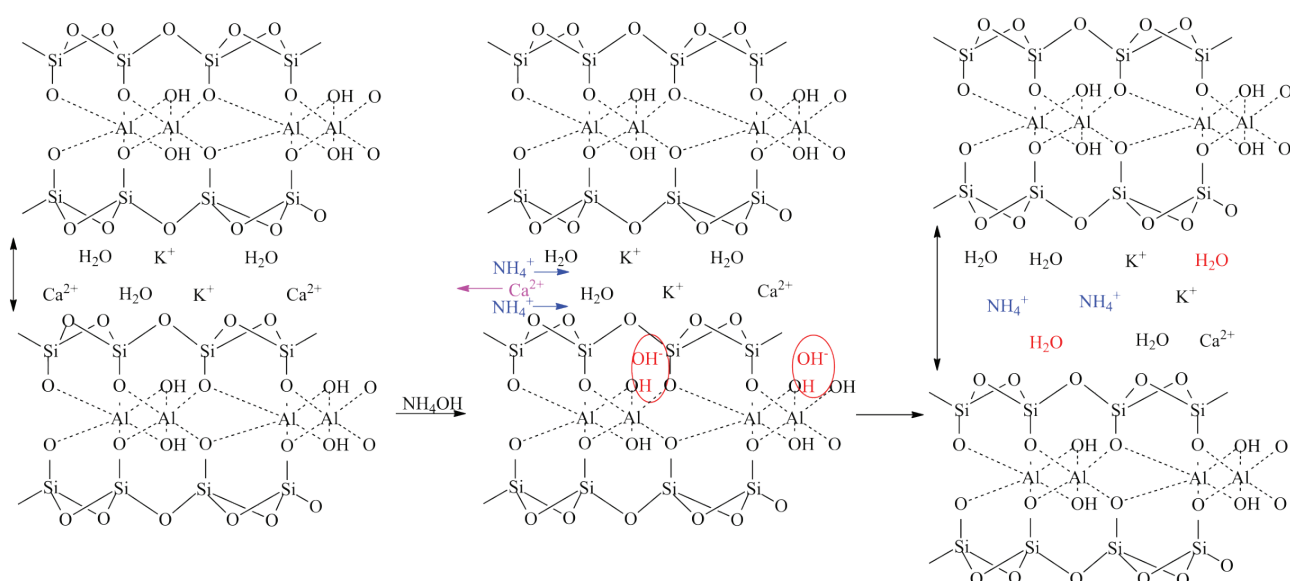


Figure 4. Mechanism of montmorillonite separation using NH_4OH solution.

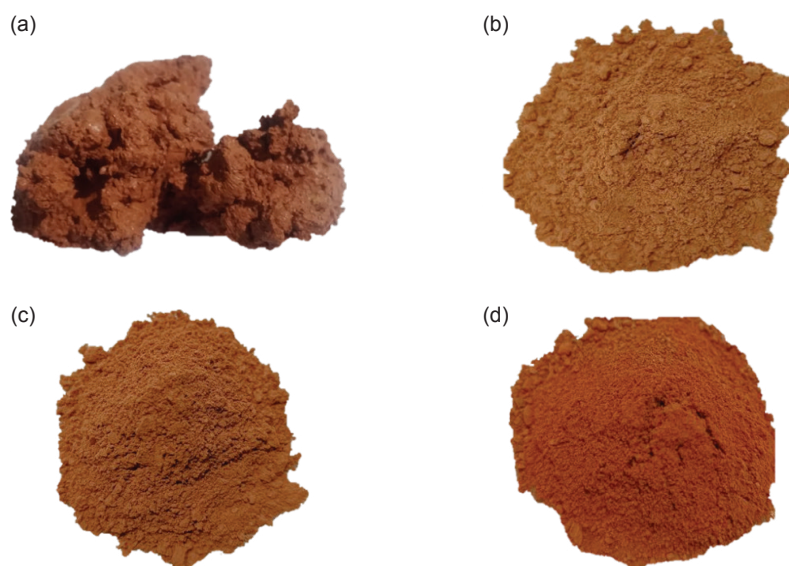


Figure 5. The colour difference of catalyst: (a) n-clay, (b) h-clay, (c) Mt-clay, and (d) c-Mt-clay.

the clay mineral layer will increase the electrostatic interaction with the cations in the clay mineral layer (Strawn, 2021). The change in Si and Al levels in clay is caused by isomorphic substitution in the terrestrial sheet (Kukharensko, 1971). Each catalyst also has a good Si/Al mole ratio (1:2) so that the sample can act as a catalyst. If the Si/Al mole ratio is large (>2), the sample will act as an adsorbent.

Effect of Thermal Separation and Modification on the Physical Properties of the Catalyst

Natural clay which is dark brown (Figure 5a) and becomes light brown (Figure 5b and 5c) in *h-clay* and *Mt-clay*. This is due to the loss of adsorbed

water in the clay due to heating at 105°C . The *h-clay* sample is light brown as shown in the figure. The thermal modification also affects the colour of the catalyst. After coking, *c-Mt-clay* turned a brick red colour (Figure 5d). The colour change in the clay minerals is due to the transformation of iron oxide contained in each sample. When the clays are calcined in the presence of oxygen at 850°C , goethite $[\text{FeO}(\text{OH})]$ transforms into hematite (Fe_2O_3) at 300°C – 650°C . Above 650°C , hematite will be reduced to magnetite (Fe_3O_4). However, the samples that have been calcined are cooled again at room temperature. This cooling process will turn magnetite back into hematite. This is what causes the calcined sample to become reddish (Martirena et al., 2020).

Transesterification Reaction Mechanism with Clay Catalyst

The use of each catalyst in the transesterification reaction involves several stages (Figure 6). In the first stage, the negatively charged siloxane surface of the clay mineral catalyst skeleton structure can remove H^+ from the hydroxyl group of methanol to form alcohol anion (methoxide) and adsorb on the cation interlayer. When the reaction occurs, the methoxide anion will attack the carbonyl C atom on the triglyceride molecule to form an intermediate. Then, the intermediate undergoes rearrangement by taking H^+ from the siloxane surface to form methyl esters and diglycerides (Intarapong et al., 2014). These stages occur repeatedly so that three moles of methyl ester and one mole of glycerol are formed from one mole of triglyceride.

Differences in Catalytic Activity

The separation and thermal modification of *Mt-clay* and *c-Mt-clay* catalysts can improve catalytic activity (Figure 7). The yield of biodiesel in the use of *h-clay* catalyst is 57.67%. The use of *Mt-clay* and *c-Mt-clay* catalysts increased the biodiesel yield to 76.20% and 80.11%, respectively. The increase in catalytic activity is due to the decrease in the Si/Al mole ratio according to the XRF results

shown in Table 1. The decrease in the Si/Al mole ratio value indicates the increase in Al content of aluminate which is negatively charged so that its electrostatic interaction with cations in the clay mineral interlayer also increases (Strawn, 2021). This shows that the active site of the catalyst also increases so that it can increase its catalytic activity. According to LPSA results, it turns out that particle size does not contribute to catalytic activity. Although it is generally assumed that catalysts with higher surface areas have better activity, this is not always the case. In some cases, the shape and geometry of the catalyst also play an important role in determining its activity (Großmann et al., 2023).

The catalyst produced in this research is more selective than the clay catalyst used by previous researchers (Febiola et al., 2023; Syukri et al., 2022). The *h-clay*, *Mt-clay*, and *c-Mt-clay* catalysts have high selectivity in producing methyl palmitate and methyl oleate. Meanwhile, previous researchers produced various methyl ester products in each biodiesel. The yield of biodiesel produced in this research is also almost the same as previous research (which chemically modified the clay (Febiola et al., 2023; Kansedo et al., 2009)). This shows that thermal modification with a simple method and lower cost can also increase the catalytic activity of the montmorillonite mineral.

Description:

X^- = Negatively charged side

Y^+ = Interlayer cations

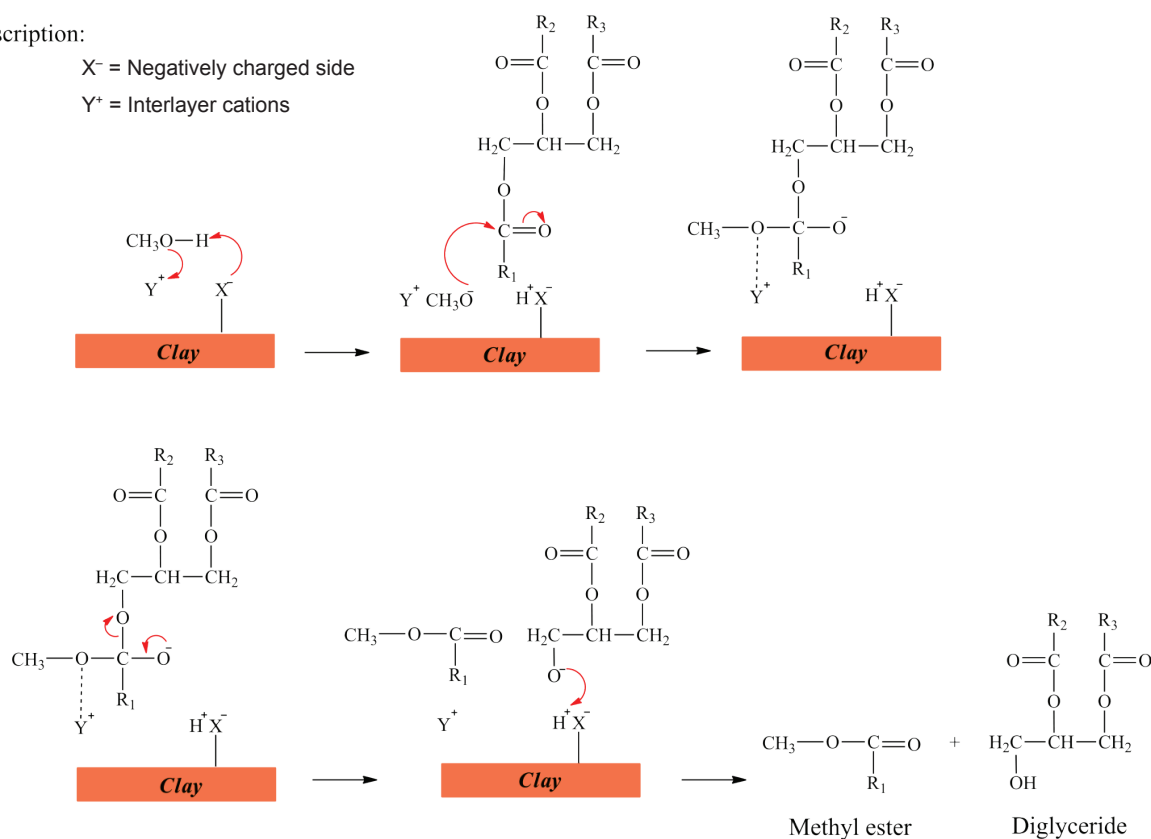


Figure 6. Transesterification reaction mechanism with clay catalysts.

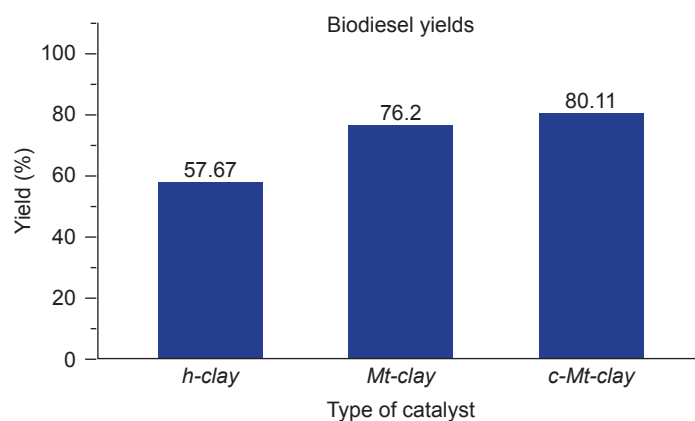
Figure 7. Biodiesel yields catalysed with *h-clay*, *Mt-clay* and *c-Mt-clay*.

TABLE 3. COMPARATIVE STUDY OF MONTMORILLONITE BASED CATALYST ON BIODIESEL YIELD

Catalyst	Feedstock	Temperature (°C)	Time (hr)	Catalyst ratio	Yield (%)	Reference
Cd-Mn-MMT	<i>Prunus cerasoides</i> D. oil	120	5	4.0%	85.00	Munir et al. (2019)
MMT K10	WCO	150	6	4.0%	38.39	Yahya et al. (2020)
Fe-MMT K10	WCO	150	6	4.0%	95.26	Yahya et al. (2020)
Nano-MMT	Crude palm oil	60	3	4.0 g	84.90	Harmawan et al. (2021)
Copper modified MMT	<i>Raphanus raphanistrum</i> L. oil	150	5	3.5%	83.00	Munir et al. (2021)
<i>c-Mt-clay</i>	WFO	70	3	3.0%	80.11	This study

TABLE 4. PHYSICAL PROPERTIES OF BIODIESEL CATALYSED BY *h-clay*, *Mt-clay*, AND *c-Mt-clay*

Properties	Standards	Type of catalyst		
		<i>h-clay</i>	<i>Mt-clay</i>	<i>c-Mt-clay</i>
Density (g cm ⁻³)	0.86-0.90 (ASTM D6751)	0.87	0.86	0.86
Water content (%)	0.05 (ASTM D2709)	0.47	0.03	0.02

Transesterification reactions produce similar methyl esters, namely methyl palmitate and methyl oleate. Methyl palmitate is a saturated methyl ester, while methyl oleate is an unsaturated methyl ester. Saturated methyl esters are the most desirable component in biodiesel. This is related to their stability against oxidation. Unsaturated methyl esters, on the other hand, are more easily oxidised and will form gum in the fuel which can cause fuel corrosion (Lucchini et al., 2011).

Based on the comparison of montmorillonite-based catalyst studies in the Table 3, the yield obtained from this study is higher than the yield obtained in other studies using the MMT K10 synthetic catalyst without treatment and modification. However, when compared with montmorillonite catalysts that were modified by making them nanostructured or by imposition of metal, the yield values obtained in this study were relatively lower.

Physical Properties of Biodiesel Products

The physical properties of the biodiesel product determined are density and water content (Table 4). Based on ASTM D6751, biodiesel has a density of 0.86-0.90 g cm⁻³ at 15°C, while the maximum water content in biodiesel is 0.05% (ASTM D2709). The transesterification reaction products catalysed by *h-clay*, *Mt-clay* and *c-Mt-clay* have densities of 0.87, 0.86 and 0.86 g cm⁻³, respectively. While the water content in *h-clay*, *Mt-clay* and *c-Mt-clay* biodiesel products is 0.47%, 0.03% and 0.02%, respectively. Therefore, biodiesel products catalysed by *Mt-clay* and *c-Mt-clay* are included in the biodiesel group because the values meet with the density and water content under ASTM. While the product catalysed by *h-clay* is not considered biodiesel because the water content exceeds the maximum limit set by ASTM.

CONCLUSION

This research has succeeded in separating the mineral montmorillonite from the natural clay of Tanah Datar. Calcined montmorillonite caused changes in the constituent phases, and the element levels, as well as the oxide levels contained therein. All catalysts contain the main elements of Si, Al, and Fe. Separation and thermal modification caused changes in elemental content and Si/Al mole ratio from 1.79 in *h-clay* down to 1.27 and 1.26 in *Mt-clay* and *c-Mt-clay* samples. Thermal treatment of *c-Mt-clay* caused colour changes from light brown to brick red. Montmorillonite turned into meta montmorillonite after calcined at 850°C. Particle size does not contribute to catalytic activity. Separation and thermal modification of *Mt-clay* and *c-Mt-clay* catalysts can increase biodiesel yield. The *h-clay* catalyst had a biodiesel yield of 57.67%, which increased to 76.20% in the *Mt-clay* catalyst and 80.11% in the *c-Mt-clay* catalyst. Therefore, we suggest that other researchers can use this method to produce a good catalyst for producing biodiesel.

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A BIOMECHANICAL ANALYSIS ON WORK-RELATED MUSCULOSKELETAL DISORDERS USING A MECHANISED CHAINSAW FOR OIL PALM HARVESTING

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ABSTRACT

The demanding nature of harvesting, which requires high cutting force, often leads to extensive physical exertion and fatigue. This study investigates the potential of utilising a pole-mounted mechanised chainsaw to minimise the pulling effort required to overcome friction forces during oil palm harvesting. The powered chainsaw demonstrates a mean peak cutting force of 677.57 ± 337.83 N, with a corresponding Rapid Entire Body Assessment (REBA) score averaging 8.11 ± 1.41 . The mean 5th lumbar vertebra and 1st sacral vertebra (L5-S1) compression and shear forces were 808.63 ± 63.84 and 528.16 ± 190.98 N, respectively, both below the safety threshold. Mechanised tools reduce muscle activation and pain scores across various muscle groups. Peak muscle activation measured less than 65% for the right biceps and mean values were below 30% for all muscle parts. The mean peak pain score was 6 for the right and left biceps and the right anterior deltoid, while the mean peak value was consistently less than 4 for all muscle parts. The tool processes one frond every 25 s, potentially handling 20-30 FFBs/hr. This study suggests that mechanised tools have the potential to enhance worker well-being and efficiency in oil palm harvesting.

Keywords: hand-force, mechanised harvesting tool, oil palm ergonomics, oil palm harvesting, work-related musculoskeletal disorders (WMSDs).

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INTRODUCTION

The oil palm, which originated from West Africa, has been cultivated as a primary food crop for approximately 5,000 years. Today, it is grown in 43 countries worldwide, with Malaysia being one of the prominent producers (Kushairi et al., 2017). The expansion of oil palm plantations has been driven by its versatile applications, including the widespread use of palm oil biodiesel as an alternative energy source. Furthermore, the increasing demand for

vegetable oils, fuelled by dietary and industrial needs, has further propelled the growth of the oil palm industry (Oyedede et al., 2020). In 2016, the oil palm plantation sector employed 429,351 field workers, and this figure continued to rise in subsequent years (Abdullah et al., 2023).

Despite the rapid growth of oil palm production, traditional harvesting methods involving long sickles and chisels are still prevalent, necessitating a considerable workforce (Teo et al., 2023). Harvesters are required to exert significant hand forces for frond cutting, leading to extensive physical exertion and potential fatigue (Abdullah et al., 2023). The manual application of cutting forces and repeated attempts are essential in the actual palm environment, contributing to the physical strain experienced by the harvesters, as highlighted

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in the study by Saibani et al. (2015). Harvesters face challenges associated with uncomfortable working positions and repetitive cutting motions (Tumit et al., 2021), including a 30° neck extension, 90° shoulder flexion, 31° shoulder extension, 45° back flexion, 20° back extension, 120° elbow flexion, 90° hip flexion, 90° knee flexion and 20° ankle flexion. These factors contribute to an increased risk of work-related musculoskeletal disorders (WMSDs), encompassing high physical exertion, awkward postures, and repetitive movements (Tumit et al., 2021).

Previous studies consistently report a high prevalence of lower back and upper back pain among oil palm plantation workers, attributed to spinal loads, inducing vertebral stress (Henry et al., 2015). The lumbar region, particularly L5-S1, experienced the most compression forces. Body pain may result from WMSDs, which are common among palm harvesters (Ahmad & Yahya, 1998b; Bhuanantanondh et al., 2021). Most studies assessing the risk of WMSDs among palm workers employ an indirect measurement and qualitative approach, by utilising ergonomic assessment tools such as rapid upper limb assessment (RULA), rapid entire body assessment (REBA) and OVAKO working posture analysis system (OWAS), based on video recording and interviews (Abdullah et al., 2023; Nawi et al., 2016; Ng et al., 2015).

Mechanisation and automation in oil palm harvesting have been the focus of research and development to address the WMSD issues and improve labour productivity in oil palm harvesting (Aljawadi et al., 2018). Various methods and technologies have been proposed to improve the efficiency and safety of the harvesting process (Hamsi et al., 2020). The advantage of the chainsaw over readily available tools like CANTASTM and CkatTM, lies in its cutting mechanism. While CANTASTM and CkatTM utilise motorised sickles and chisels mounted inside a vibrating mechanism, which can be effective for harvesting fresh fruit bunches (FFBs), the chainsaw, however, introduces a different approach. Another study that will possibly eliminate the risks associated with oil palm harvesting is by developing and evaluating the performance of an oil palm harvesting robot (Oyededeji et al., 2022). By employing a material-removing cutting method, specifically with a chainsaw, this study aims to address a specific challenge and minimise the pulling effort during harvesting. Unlike the vibrating sickles and chisels, the chainsaw's cutting action involves removing material more directly, thus potentially reducing the resistance encountered during the cutting process. This change in approach may offer potential advantages in terms of efficiency, ease of use and safety during oil palm harvesting operations.

A narrow wedge angle in cutting tools such as sickles, increases the friction force, and demands a larger pulling force to overcome resistance to cut the fronds. Chainsaws, however, have wider wedge angles, which reduce the pulling force needed to enhance penetration and reduce binding, thus making them more efficient for cutting thicker fronds with less manual effort. This study represents the first comprehensive investigation into the effectiveness of the chainsaw as a cutting tool in oil palm harvesting. By examining the muscle activity, working postural angles, hand force exertion, lower back forces, ergonomic risks, and body pain, this study aims to provide valuable insights into the potential benefits of this innovative approach for oil palm harvesting.

MATERIALS AND METHODS

Area

The average height of oil palm trees in the study area ranged from 2.5-3.0 m, which shows the suitability of the developed mechanised electric-chain saw in the harvesting process. The study was conducted at Universiti Sains Malaysia's (USM) Engineering Campus in Nibong Tebal, Penang, located at GPS coordinates 5°08'47.7"N; 100°29'27.9"E.

Participants

Ten male students from USM were recruited (age = 23.8 ± 1.17 years old; height = 169.90 ± 6.48 cm; weight = 66.6 ± 9.56 kg). The male gender was chosen because they were predominantly involved in the oil palm harvesting activities (Abdullah et al., 2023; Mehraban et al., 2022). The inclusion criteria of this study included: Individuals who had no health issues and major surgery, as well as consent and compliance with all aspects of the study protocol. The participants were briefed on the experimental protocol, demonstrated the harvesting process, and conducted a trial before data recording. They were equipped with safety helmets and other necessary safety measures. The study was approved by the Human Research Ethics Committee USM (JEPeM) (JEPeM Code: USM/JEPeM/21100665). All participants signed an informed consent document before the study.

Surface Electromyography (EMG)

The muscle activation patterns of the participants were measured using surface electromyography (EMG) from iMotions 9.0 software (iMotions, Copenhagen, Denmark). The

surface electrodes used were 24 mm diameter disposable Ag/AgCl, as used in previous studies by Pelaez et al. (2020) and Tang (2023). Then, the electrodes were placed in bipolar configurations on the right and left biceps brachii (RB, LB), right and left anterior deltoid (RAD, LAD), upper trapezius (UT), middle trapezius (MT), lower trapezius (LT) and erector spinae (ES), as done in the previous study by Abdullah et al. (2023). All participants were coincidentally dominant on the right side. The placement of the electrodes (*Figure 1*) is based on the guidelines provided in the surface EMG for the non-invasive assessment of muscle (SENIAM). The participants carried out maximum voluntary isometric contractions (MVIC) tests, as done by Christova et al. (1999), and determining their maximum muscle activation at a sampling rate of 1024 Hz, as studied by Burns et al. (2010a, 2010b); Chan et al. (2022); Manjarres-Triana et al. (2023) and Reza et al. (2013).

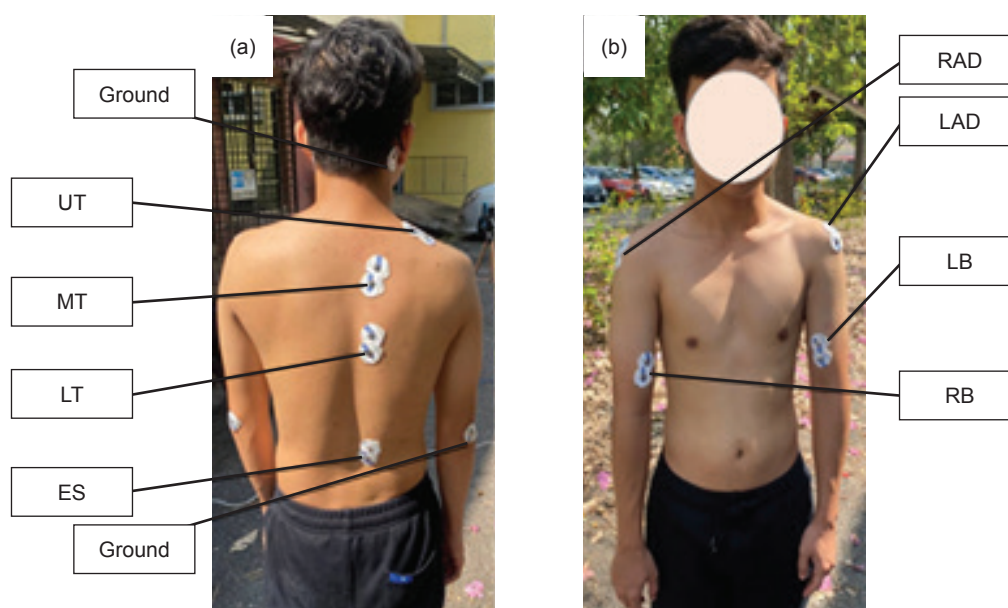
Posture Angle (OpenCap)

OpenCap is a specialised software designed for measuring postural angles. This open-source, markerless, web-based application can estimate 3-dimensional kinematics from videos captured by two separate smartphones (Lima et al., 2023). Posture angles were assessed using two iPhone operating system (iOS) devices (iPhone SE 2020) in conjunction with the OpenCap system. The devices were securely mounted on tripods and strategically placed to obtain a field of view that encompasses

the entire experimental area. Following that, calibration was performed using an appropriately sized checker box. During the calibration, to ease the OpenCap system in detecting and calibrating posture, participants were instructed to stand in a neutral posture, where the OpenCap model scaled the participant size, height and shape. Following a successful calibration, the OpenCap software can be used for the recording process as conducted by Uhlrich et al. (2022).

Mechanised Cordless Chainsaw Cutting Tool and Measurement of Cutting Forces

A mini cordless chainsaw (brand: Santino), was fixed to the top of a pole (*Figure 2*). The chainsaw provides a maximum power of 66.6 W, a chain speed ranging from 3,000-3,700 rpm, and was powered by lithium batteries with a total capacity of 20,000 mAh. Modifications were made to the battery housing and direct current (DC) motor wiring on the cordless chainsaw. A control and monitoring panel (*Figure 2d*) for the DC motor was developed, featuring displays for voltage and current, a DC speed controller, an on/off switch, a battery indicator, and storage of batteries. The total cost for developing the prototype was RM250, which includes items such as the chainsaw machine, pole, lithium battery, DC controller, on/off switch, battery indicator, voltage/current indicator and wires. It is important to note that this cost pertains only to developing the prototype and the actual cost analysis may vary.



Note: RB - right bicep; LB - left bicep; RAD - right anterior deltoid; LAD - left anterior deltoid; UT - upper trapezius; MT - middle trapezius; LT - lower trapezius; ES - erector spinae.

Figure 1. EMG electrode placement to the muscle parts: (a) Back, and (b) front and legend box.

A load cell (S-Type S9M load cell (HBM, Germany)) was attached to the pole, approximately 2.19 m from the top. The length of the pole was approximately 2.50 m and weighed around 7.5 kg. For ease of operation, the control panel was placed at the base of the pole. The load cell was linked to the HX711 load cell receiver, which was then connected to an Arduino Mega, a microcontroller. Arduino was used to record and process the data. For accuracy, the load cell was calibrated by erecting the cutting tool vertically and adjusting the readings to zero to eliminate the reading coming from the weight of the parts, ahead of the load cell (Abdullah et al., 2023; Latha et al., 2017).

Field Setup for Experiment

For the experiment, a suitable oil palm tree measuring between 2.5 and 3.0 m in height was selected. The iMotion software, OpenCap system, and load cell were connected to one computer to synchronise all collected data (Figure 3).

Data Collection, Processing and Statistical Analysis

After participants completed the MVIC test and once calibrated with the OpenCap software, they were instructed to perform the harvesting process

by using the mechanised chainsaw. Participants were instructed to perform five trials to ensure sufficient data points for robust statistical analysis. The data collection begins simultaneously for iMotion, OpenCap and load cell. Participants were instructed to turn on the chainsaw to initiate the frond-cutting task. Each participant completed five repetitions of the frond-cutting task, with recordings paused between cuts. Subsequently, participants completed a pain level form similar to Abdullah et al. (2023) based on the muscles involved.

This study aimed to assess how mechanised tools affect hand force, posture and muscle activation during harvesting. A total of 50 trials were conducted involving 10 participants. In some trials, the posture angles could not be recorded in the OpenCap software, due to the poor connectivity between the smartphone and the server because, there was no real-time indicator that indicated data loss. Eventually, the data from the 19 trials contributed by six participants were included in the analysis.

The collected data, iMotion EMG recordings, OpenCap posture angles and load cell readings, were later exported. For hand force values, the load cell data was tabulated to visualise maximum mean and standard deviations (SD). Muscle activation data, comprising raw MVIC test data and raw

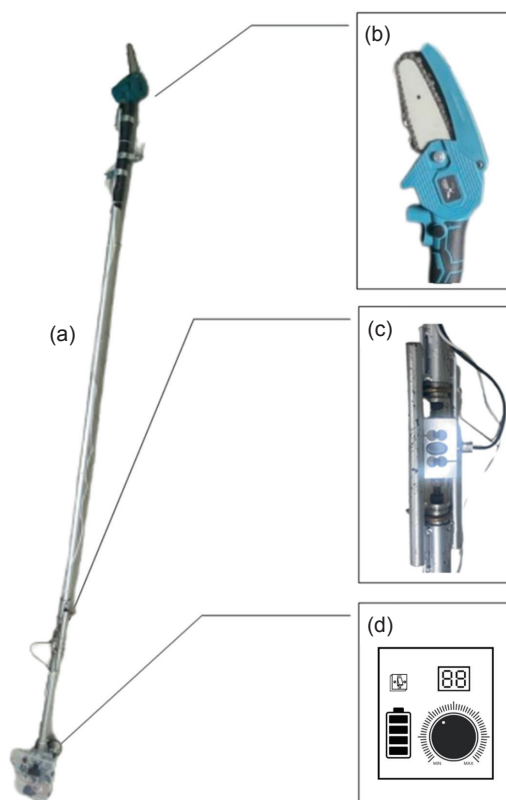


Figure 2. (a) Prototype in detailed view, (b) chainsaw, (c) load cell, and (d) schematic diagram of the control panel.

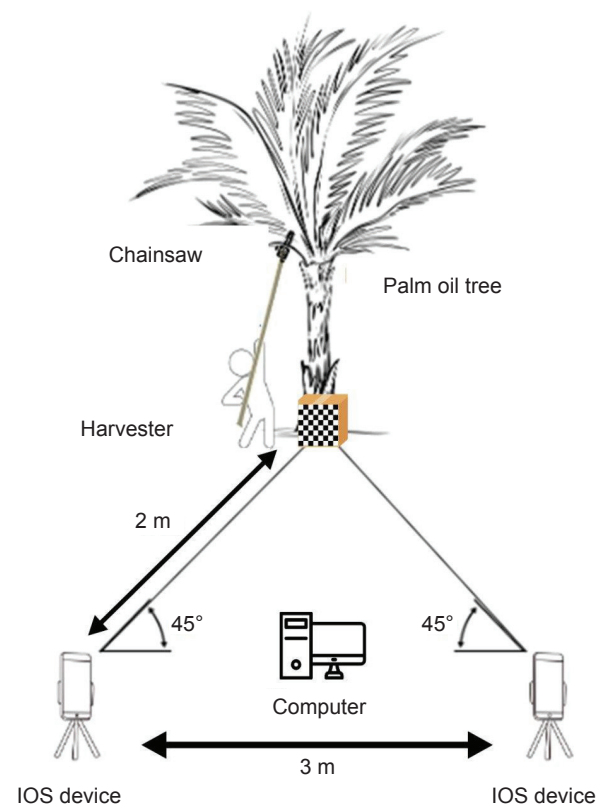


Figure 3. Field experiment setup.

task data during the harvesting process, were exported from the iMotion software in millivolts (mV) into an Excel file for each participant. The normalised muscle activation was then calculated as a percentage and tabulated.

Kinematics data from OpenCap were exported and tabulated for each body part. These kinematics data identified critical movements during the harvesting process. By using hand force values and kinematics data, the spinal loading at L5-S1 (compression and shear forces) was calculated using the 3-Dimension Static Strength Prediction Software (3DSSPS) (Ghezelbash et al., 2020). Additionally, the REBA score was determined for each participant. The REBA score ranged from 1-15 levels, divided into 5 risk levels as recorded in the study by Law et al. (2022). Pain levels during the harvesting process were assessed using a standardised questionnaire administered to participants immediately after task completion as done by Abdullah et al. (2023).

The collected data on hand force, muscle activation, and joint angles were analysed using descriptive statistical methods such as mean and SD. The REBA score and spinal loadings (compression and shear force) at L5-S1 were determined based on the hand force and joint angles of each participant. Descriptive statistical methods were then applied to determine the mean and SD for these variables. To explore the relationship between muscle activation and pain score, a correlation analysis

was conducted using Spearman's correlation. The correlation analysis was performed using the IBM SPSS Statistics software.

RESULTS AND DISCUSSION

Hand Force, Total REBA Score and Spinal Loadings

The mean peak hand forces, total REBA scores, and lower back forces at L5-S1 across different trials (*Table 1*). The mean value of peak hand forces was 677.57 ± 337.83 N, with a range between 190.00 and 1,386.03 N. The REBA score data provides valuable insights into the ergonomic risk associated with tasks performed by the subjects. The mean REBA score from all trials recorded was 8.11 ± 1.41 . The scores ranged from 6-10, indicating varying levels of risk for musculoskeletal disorders. The L5-S1 disc loads were quantified to investigate the forces exerted on the spine of the subjects during the palm harvesting activities. The mean compression and shear forces for all participants were 808.63 ± 63.84 and 528.16 ± 190.98 N, respectively.

A graphical representation illustrates the continuous hand force pattern observed in one of the participants during the execution of the harvesting task, using the mechanised chainsaw (*Figure 4*). The peak value was the maximum pulling force applied by the participant to cut the targeted frond.

TABLE 1. MEAN AND STANDARD DEVIATION (SD) FOR PEAK HAND FORCES, TOTAL REBA SCORE AND LOWER BACK FORCE

Item	Hand force (N)	Total REBA score	Compression force (N)	Shear force (N)
Range	192.29-1386.03	6.00-10.00	700.00-870.00	290.00-750.00
Mean (SD)	677.57 (337.83)	8.11 (1.41)	808.63 (63.84)	528.16 (190.98)

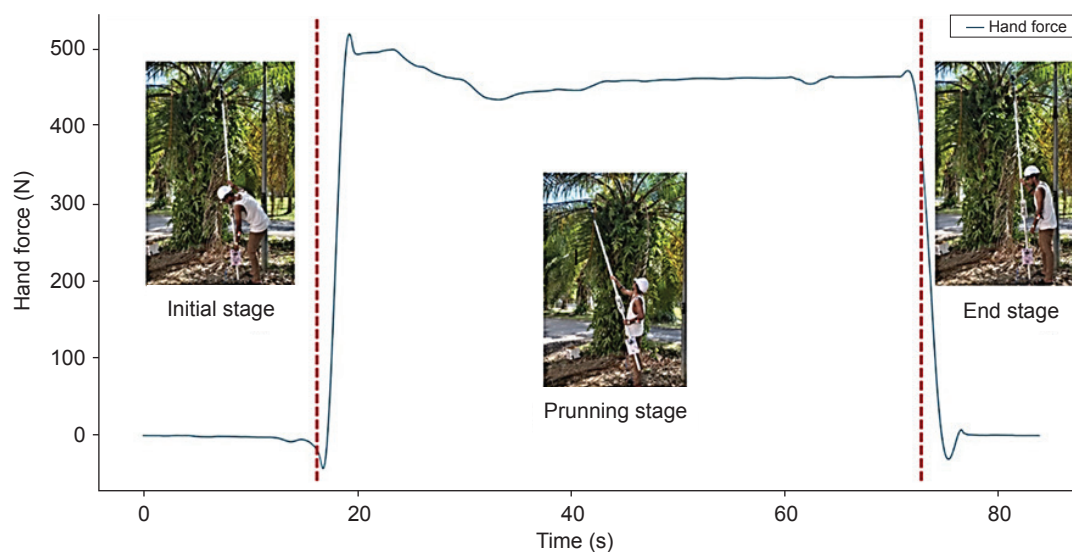


Figure 4. Hand force pattern during the experiment.

Muscle Activation

Various muscle groups were examined to assess the physical effort involved in the harvesting process (Figure 5). The EMG activation was considered moderately activated, with the mean peak value for all muscles being less than 30% MVIC. Notably, the muscles RB, LB, LAD and LT exhibited the highest mean peak activation levels, emphasising their significant role in the harvesting process by generating force and executing repetitive movements during harvesting. The mean peak muscle activation is considered the maximum value in the box plot graph (Figure 5), outliers may negatively bias the entire result of an analysis.

Body Pain Score

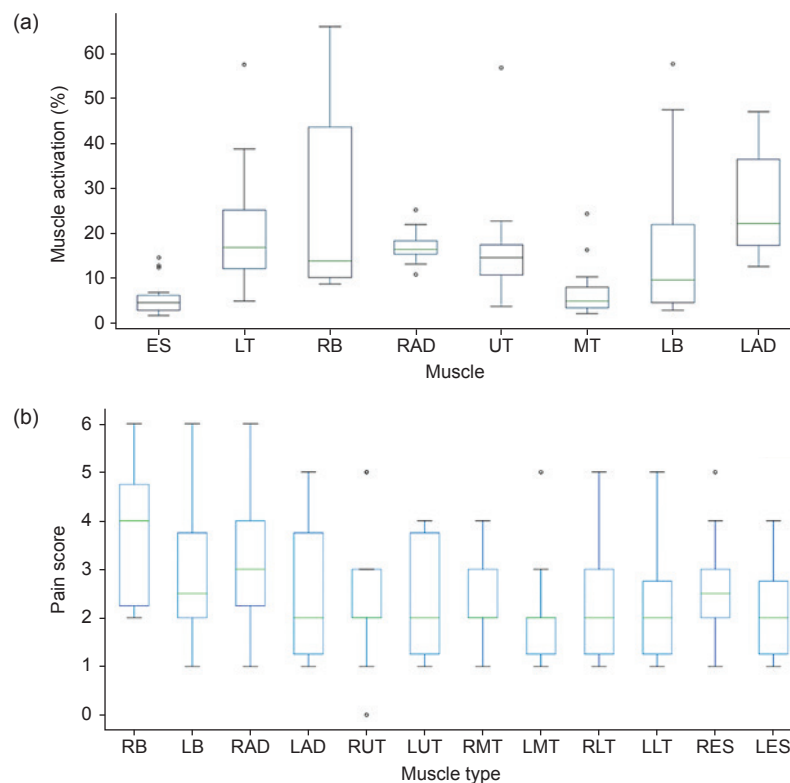
After harvesting, the pain score was recorded by questionnaire form, and the result of questionnaires was plotted in a box-plot graph (Figure 5). The questionnaire form was prepared based on the EMG electrode placement to correlate the muscle activation and pain score. The pain score was measured using a scale of 0 (lowest) to 10 (highest). The peak value obtained was 6 for the right bicep, left bicep and right anterior deltoid.

The mean pain scores for every muscle show less than 4, which was considered a moderated pain rate by the participants.

The Spearman correlation (Table 2) indicates a high degree of significance for all muscles when comparing measured EMG values and self-reported pain scores, except for the upper trapezius, which shows a moderate correlation. It is concluded that there is a linear relationship between muscle activation measured using EMG with the body pain scores. There is mostly a moderate Spearman correlation between the two variables ($p < 0.05$). The grading standards followed a previous study by Yan et al. (2019). The significant correlation suggests a robust relationship between the two variables.

TABLE 2. CORRELATION BETWEEN EMG DATA AND PAIN SCORE

Muscle	Correlation Rs	p-value
Right bicep (RB)	-0.626	0.004
Left bicep (LB)	-0.488	0.034
Right anterior deltoid (RAD)	-0.459	0.048
Left anterior deltoid (LAD)	0.696	0.001
Upper trapezius (UT)	0.450	0.053
Middle trapezius (MT)	-0.606	0.006
Lower trapezius (LT)	0.596	0.007
Erector spinae (ES)	0.520	0.023



Note: RB - right bicep; LB - left bicep; RAD - right anterior deltoid; LAD - left anterior deltoid; RUT - right upper trapezius; LUT - left upper trapezius; RMT - right middle trapezius; LMT - left middle trapezius; RLT - right lower trapezius; LLT - left lower trapezius; RES - right erector spinae; LES - left erector spinae.

Figure 5. (a) Average maximum muscle activation, and (b) reported body pain score in all muscle regions.

Body Posture

The posture angles were captured and obtained during the harvesting process. A comprehensive understanding of these angles was derived from mean and SD values observed across the participants (Table 3).

On the right side, the hip exhibited a slight adduction angle of $4.66^\circ \pm 4.16^\circ$ and a moderate external rotation angle of $16.93^\circ \pm 6.18^\circ$. The knee displayed a slight flexion angle of $7.55^\circ \pm 4.98^\circ$ and a stable standing position angle of $0.13^\circ \pm 0.09^\circ$.

Transitioning to the left side, the hip demonstrated a moderate flexion angle of $12.85^\circ \pm 4.10^\circ$, a slight adduction angle of $8.46^\circ \pm 2.67^\circ$ and a moderate external rotation angle of $8.22^\circ \pm 4.39^\circ$. Similarly, the left knee displayed slight flexion at $5.95^\circ \pm 4.07^\circ$ and a stable position angle of $0.10^\circ \pm 0.07^\circ$. On the right side, the shoulder angles indicated a moderate external rotation of $16.93^\circ \pm 6.18^\circ$ and a flexion angle of $15.57^\circ \pm 4.30^\circ$. The abduction angle was measured at $4.66^\circ \pm 4.16^\circ$. In contrast, the left shoulder exhibited a moderate external rotation of $8.22^\circ \pm 4.39^\circ$, a flexion angle of $12.85^\circ \pm 4.10^\circ$ and an abduction angle of $8.46^\circ \pm 2.67^\circ$.

Analysis of lumbar angles unveiled a moderate extension angle of $9.32^\circ \pm 2.82^\circ$, a slight lateral bending angle of $9.37^\circ \pm 2.14^\circ$ and a moderate rotation angle of $15.12^\circ \pm 3.16^\circ$. These intricate kinematic body posture angles offer comprehensive insights into the joint angles and movements crucial for oil palm harvesting.

The mechanised chainsaw demonstrated a mean peak cutting force of 677.57 ± 337.83 N, representing a reduction of 57.67% compared to the mean cutting force of the traditional sickle (1601.23 ± 424.26 N) (Abdullah et al., 2023). The elimination of the need for additional pulling force, typical of material

removal cutting processes, contributes to the substantial reduction in hand force observed when using a mechanised chainsaw.

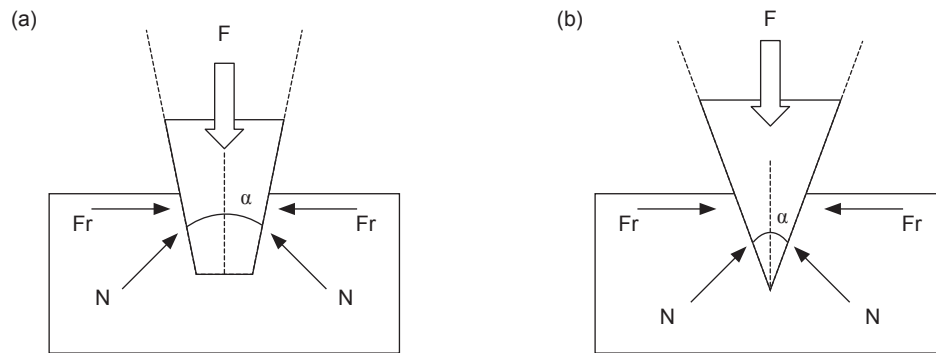
In a previous laboratory study (Ahmad et al., 2020), a sickle cutter with an actuator delivered a force of 12.2 kg/cm^2 (1196411 N/m^2), whereas in the field experiment with a mechanised chainsaw, a mean peak specific cutting force of 9.38 kg/cm^2 was obtained, representing a reduction of about 30% when considering an average frond cross-sectional area of approximately 35.82 cm^2 (Ahmad & Yahya, 1998b). While these results highlight the advantages of the mechanised tool, it is essential to acknowledge other potential influencing factors such as frond moisture, frond maturity and cutting angles, as identified by (Ahmad & Yahya, 1998a; Ahmad et al., 2020), which should be considered to optimise the harvesting methods.

The wedge angle (α), which is crucial in frond cutting with tools like sickles, plays a key role in dictating the force (F) needed for the cutting process to overcome the friction force (F_r) over the normal force (N) (Figure 6). A smaller wedge angle, found in traditional sickles, demands a greater pulling force. In contrast, chainsaws make a wider wedge angle, reducing the pulling force required to cut fronds. This broader angle facilitates penetration and minimises the binding problem, where the wedge gets stuck in the frond. The wedge angle is a critical factor influencing the pulling force: Smaller angles demand more force, while larger angles, as seen in chainsaws, require less force for effective cutting.

The REBA score data reveals varying levels of ergonomic risk associated with tasks performed by participants. The mean REBA score of this study, 8.11 ± 1.41 , was lower than that of a previous study using traditional harvesting tools, which

TABLE 3. JOINT ANGLE DURING FIELD EXPERIMENT FROM 19 TRIALS

Articulation	Posture angle [Mean (SD)] (°)			
	Lateral bend	Axial rotation	Flexion	Extension
Pelvis	12.00 (5.23)	4.99 (2.10)	35.93 (11.45)	35.93 (11.45)
Lumbar	Lumbar bending	Lumbar rotation	Flexion	Extension
	9.37 (2.14)	15.12 (3.16)	-	9.32 (2.82)
Right shoulder	Flexion	Abduction	Adduction	
	16.93 (6.18)	15.57 (4.30)	4.66 (4.16)	
Left shoulder	Flexion	Abduction	Adduction	
	8.22 (4.39)	12.85 (4.10)	8.46 (2.67)	
Right knee	Ulnar deviation	Radial deviation	Flexion	Extension
	-	-	7.55 (4.98)	0.13 (0.09)
Left knee	Ulnar deviation	Radial deviation	Flexion	Extension
	-	-	5.95 (4.07)	0.10 (0.07)
Right wrist	Ulnar deviation	Radial deviation	Flexion	Extension
	8.82 (4.79)	-	-	-
Left wrist	Ulnar deviation	Radial deviation	Flexion	Extension
	-	9.51 (2.96)	-	-



Note: FR - friction force; F - force; N - normal force.

Figure 6. Wedge angle comparison between (a) chainsaw, and (b) sickle.

reported a score of 10.30 ± 1.16 (Abdullah et al., 2023). The use of manual or traditional harvesting tools in harvesting oil palm FFBs exposes workers to musculoskeletal disorders (Shuib et al., 2020). Immediate action, such as providing ergonomics training for workers, should be highly considered to solve this problem (Nawi et al., 2016). The adoption of mechanised harvesting methods can improve workers' productivity and reduce ergonomic risks, leading to a better REBA score (Azaman et al., 2022).

The mean compression and shear forces at the L5-S1 joint across all participants were approximately 808.63 ± 63.84 N, (range from 700 to 870 N) and 528.16 ± 190.98 N, (range from 290 to 750 N), respectively, both of which were lower than the NIOSH safety limits (Afshari et al., 2018). The current study demonstrates a substantial reduction in both compression and shear forces by approximately 67.56% and 63.52% when compared to the previous study by Abdullah et al. (2023) who reported a mean compression and shear forces of $2,493.20 \pm 1,349.48$ and 1446.10 ± 411.00 N, respectively. Study has shown that using ergonomic cutting tools can reduce lower back force during oil palm harvesting activities (Selamat et al., 2023).

The usage of specific harvesting tools, such as the chisel, pole, and loading spike has been found to further contribute to WMSDs (Mohamaddan et al., 2021). In terms of muscle activation, it is evident that the right bicep and lower anterior deltoid muscles display the highest mean peak activation levels. The left trapezius muscle also exhibits significant mean peak activation, indicating its involvement in generating force and facilitating repetitive movements during harvesting.

This study found that muscle activation levels at various muscle locations were reduced for all participants compared to previous studies employing traditional harvesting methods (Abdullah et al., 2023). The right bicep experienced a reduction of 61.4%, the left bicep decreased by 73.4%, the upper trapezius saw a reduction of 69.1%, the middle

trapezius experienced a reduction of 85.9% and the erector spinae showed the highest reduction of 89.0%. In terms of pain scores, reductions of 35.7%, 45.4%, 40.5%, 45.5% and 47.5% were observed in the right bicep, left bicep, upper trapezius, middle trapezius and erector spinae, respectively. These percentage of reductions highlighted the significant ergonomic advantages associated with the use of mechanised chainsaws in oil palm harvesting.

The decrease in muscle activation levels suggests a considerable reduction in the physical strain experienced by workers during the harvesting process (Selamat et al., 2023). The reduction in muscle activation supports the adoption of mechanised tools for oil palm harvesting activities. The evolution of mechanisation in oil palm harvesting has shown a reduction in muscle strain and pain levels among oil palm harvesters (Mohamad, 2022; Selamat et al., 2023). By incorporating these ergonomic devices and mechanised tools, the physical stress and risk factors associated with the manual handling of materials can be minimised, thereby reducing work-related muscle disorders (Rahman et al., 2017).

Previous studies found that workers are exposed to various postural problems, with the latter emphasising the need for immediate action to address these issues (Nawi et al., 2013). The OpenCap software analysed the posture involved in kinematic details, thereby providing a comprehensive understanding of joint angles and movements during the oil palm harvesting activities.

On the right side, the hip exhibited a slight adduction angle and a moderate external rotation angle, suggesting a posture that involves a degree of inward movement and external rotation. The knee displayed a slight flexion angle with a stable position, reflecting a balanced stance, while the ankle angle indicated slight dorsiflexion, contributing to a stable footing. Moving to the left side, the hip demonstrated a moderate flexion angle, coupled with a slight adduction and external rotation angle. These

angles suggest a posture involving a more flexed and rotated position on the left side, potentially influenced by the nature of the harvesting task.

The lumbar angles revealed a moderate extension angle, indicating a slight backward tilt, as a stabilising mechanism during the harvesting process. Additionally, a slight lateral bending angle and a moderate rotation angle suggest a meticulous movement in the lumbar region, contributing to the dynamic nature of the task. This further emphasises the prevalence of unsafe postures, particularly in the upper body, highlighting the imperative for interventions to mitigate these risks. Research by Ng et al. (2015) added to this by identifying specific risk factors for musculoskeletal disorders, including awkward postures, and the need for further investigation into the dynamic of work activities.

The efficiency comparison between the traditional manual sickle, CANTASTM and mechanised electric chainsaw is an essential aspect to be addressed comprehensively. Initial data suggests that the manual sickle can harvest approximately 50 FFBs/hr, while CANTASTM achieves around 75 FFBs/hr (Muhamad & Aziz, 2018). In contrast, the mechanised electric chainsaw prototype currently processes one frond every 25 s, potentially handling approximately 20-30 FFBs/hr. This estimate is based on the average time required for various tasks: Searching for a mature palm oil tree (40.73 s), cutting two fronds (50 s), and cutting one mature FFB (60.26 s) (Saibani et al. 2015). However, further investigation is necessary to determine the actual effectiveness of the electric chainsaw in the oil palm plantation.

This paper attempts to measure the harvesters' hand forces during oil palm harvesting activities under actual field conditions, employing the mechanised chainsaw as the oil palm harvesting tool. Unlike simulated scenarios in a laboratory setup, this approach measures the forces required for cutting palm fronds with a mechanised chainsaw in the field. Furthermore, these findings not only contribute to valuable insights but also serve to complement and address the identified limitations. This unique approach adds depth to the understanding of ergonomic challenges faced by workers in the oil palm industry. Employing students for testing provides insights into muscle activation variations compared to experienced harvesters, enhancing our understanding of biomechanical responses to the new tool. Testing with students allows for the assessment of the new tool's functionality and biomechanical implications before field deployment, informing potential ergonomic refinements for real harvesters.

There are several recommendations aimed at enhancing the effectiveness and applicability of future research activities in the context of oil

palm harvesting. Firstly, it is suggested to increase the sample size and recruit experienced oil palm harvesters to gather feedback on performance, preference, and productivity compared to traditional tools. For the joint angle measurement, the connectivity issues of the smartphone and OpenCap server should be resolved to enable the data capturing successfully. Furthermore, ensuring signal stability for Shimmer and iMotion devices is crucial for improving the reliability of EMG data. The implementation of these recommendations is expected to boost research on motorised saws, leading to enhanced efficiency relevant to the challenges in oil palm harvesting.

CONCLUSION

The mechanised chainsaw cutting tool demonstrates a 57.67% reduction in cutting force compared to traditional sickles. Moreover, the analysis of worker safety metrics reveals a consistent pattern of lower ergonomic risk for participants utilising mechanised chainsaws. The mean REBA score of 8.11 ± 1.41 with reductions in muscle activation levels, ranging from 35.7% to 89.0%, emphasises the considerable decrease in physical strain experienced by workers during the harvesting activity. Pain scores similarly exhibit reductions, ranging from 40.5%-47.5%, further supporting the potential ergonomic benefits of mechanised chainsaws in oil palm harvesting. Additionally, the compression and shear forces at the L5-S1 level demonstrate reductions of approximately 67.56% and 63.52% respectively, indicating a positive impact on lower back health. These findings collectively highlight the comprehensive advantages of adopting mechanised tools in promoting both worker well-being and operational efficiency in oil palm harvesting.

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ENCAPSULATED ACIDIC ASPIRIN-BASED EUTECTIC SOLVENTS FOR ESTERIFICATION OF FREE FATTY ACID

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ABSTRACT

The combination of high free fatty acid (FFA) palm oil and non-edible microalgae oil presents a promising avenue for sustainable biodiesel production. Efficient catalyst development and innovative technologies are crucial, particularly for the esterification reaction in feedstock pre-treatment processes. In this study, two novel active pharmaceutical ingredients (API)-based deep eutectic solvents (DESs) were introduced. They derived from p-toluenesulfonic acid monohydrate (PTSA) and benzenesulfonic acid (BZSA) combined with aspirin (ASA), encapsulated within medical capsules for safe handling and storage. These DESs effectively reduce FFA content from 11% to less than 2% under optimum conditions (1 wt% of DES catalyst dosage, 10:1 methanol-to-oil molar ratio, 60°C esterification reaction temperature and 60 min reaction time). The activation energies of the reaction using ASA:PTSA and ASA:BZSA based DES were determined as 53.11 and 39.20 kJ mol⁻¹, respectively, according to the pseudo first order reaction rate. Furthermore, FFA to fatty acid methyl ester (FAME) conversion rates remained above 50% for both DESs even after three consecutive recycling runs. The utilisation of API-based DESs enhances catalyst physical properties, recyclability for FFA esterification reactions, underscoring their potential in advancing biodiesel production processes.

Keywords: deep eutectic solvent, encapsulation, esterification, free fatty acid, microalgae oil.

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INTRODUCTION

In the continuous efforts to reduce carbon emissions and advance renewable energy, diesel fuel derived from depletable fossil fuel sources may be substituted with renewable and environmentally benign biofuels such as biodiesel (Wang et al., 2023). Edible, refined oil crops such as soybean, rapeseed and palm oil are the primary feedstocks used in the present biodiesel production market. The utilisation of edible oil crops for biodiesel manufacturing may pose threats to food supply competition and create an economic imbalance (He et al., 2024; Mukhtar et al., 2022). Consequently, there has been a notable increase in research attention towards non-edible second-generation oil crops, such as jatropha oil, *Koelreuteria integrifolia* oil, mahua oil and *Pongamia pinnata* seed oil as alternative sources of feedstock for biodiesel production (Brahma et al., 2022;

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Sangeetha & Baskar, 2024; Zheng et al., 2024). Low commercial oil sources, such as waste cooking oil, animal fats and sludge oils have similarly been explored and demonstrated for biodiesel generation (Pongraktham & Somnuk, 2023; Rocha-Meneses et al., 2023; Singh et al., 2024). However, the lack of well-established waste oil reusability efforts, for example, collecting systems and supply chains, in many countries impedes waste oils from being fully utilised as raw materials for sustainable biodiesel. Thus, non-edible microalgae oil has emerged as a potential raw material to produce third- and fourth-generation biodiesel (Huang et al., 2023; Patnaik & Mallick, 2021).

Catalyst development and technologies play a significant role in the efficient conversion of the feedstocks, particularly for the esterification reaction in the feedstock pre-treatment process for biodiesel production. The use of mineral acids (such as sulphuric acid and hydrochloric acid) as homogeneous catalysts is required in the esterification reaction process due to the high free fatty acids (FFA) nature of the low-value feedstocks. However, their use is associated with high costs for separation and purification, along with concerns about equipment corrosion and the inability to recycle the catalyst (Dai et al., 2020). A viable alternative in homogeneous catalysts for the esterification reactions may be through the development of ionic liquids (ILs) and deep eutectic solvents (DESs) as catalysts (Bian et al., 2021; Li et al., 2021; Zhou et al., 2023). Compared to ILs, DESs offer several valuable advantages such as cost-effectiveness, biodegradability, environmental friendliness and can potentially incorporate natural ingredients (Mamtani et al., 2021). DESs are usually prepared as a homogeneous mixture by combining the hydrogen bond acceptor (HBA) and hydrogen bond donor (HBD) where the melting point is reduced significantly when combining the HBA and HBD. Generally, DESs are composed of organic halide salts and complexing organic agents such as amines, alcohols, carboxylic acids and amides (Al-Farsi & Hayyan, 2023). Due to their unique properties, DESs have received increased research focus and have been investigated for applications in biomass conversion and bioenergy production, such as for the esterification and transesterification of low-value feedstocks for biodiesel production, conversion and synthesis of 5-hydroxymethylfurfural and lignocellulosic treatment/valorisation (He et al., 2023; Liu et al., 2020; Tan et al., 2023; Zhou et al., 2023). Additionally, using *p*-toluenesulfonic acid monohydrate (PTSA) and benzenesulfonic acid (BZSA) as eutectic mixtures for acid-catalysed esterification offers several benefits and can reduce the hygroscopicity of the acid and the potential to reuse the catalyst. Moreover, PTSA is readily available and has

been effectively used as a catalyst in protein and biodiesel production.

Recently, active pharmaceutical ingredients (APIs) such as paracetamol, ibuprofen and phenylacetic acid have been reported to be effective HBAs/HBDs in the formation of DESs, enabling the multifunctional applications of DESs such as in drug delivery, drug solubilisation and catalysts (Abbott et al., 2017; Hayyan et al., 2023; Shah et al., 2023). The use of APIs in the formation of DESs may have the potential to address the issues of highly abundant unused or expired drugs by repurposing their active ingredients. The increase in the production and consumption of pharmaceuticals has generated high volumes of pharmaceutical waste in the form of unused, unwanted or expired medicines (Freitas & Radis-Baptista, 2021). Improper disposal of excess medicine causes water resource contamination and toxicity exposure to the environment and public health (Aluko et al., 2022). To solve the negative impacts of excess waste drugs, studies have suggested several viable applications, such as the recovery and reutilisation of active pharmaceutical contents in waste drugs as reagents for organic syntheses (Pratama et al., 2020; Puzanova et al., 2019). Hence, the development of API-based DESs is prospective in advancing the applications of DESs while enabling the recovery of resources.

In this study, acidic API-based DESs were prepared from aspirin mixed with PTSA and BZSA for acid-catalysed esterification. The high-viscosity DESs were encapsulated in medical capsules to ensure safe handling and storage. Incorporating a catalyst within a confined space such as medical capsules can induce reagent molecules to interact under specific geometric constraints. This can result in reaction products exhibiting regio- and stereoselectivity that differs from typical solution conditions. The blending of microalgal oil with palm oil was performed to produce a non-edible oil with lower viscosity and cost. The resulting oil mixture underwent pre-treatment through acid-catalysed esterification to decrease the FFA content and convert it into FAME. Through batch esterification, the optimum conditions for each variable, in particular, DES catalyst dosage, methanol molar ratio to oil, esterification reaction temperature and esterification reaction times were investigated. Additionally, reaction kinetic studies were conducted to examine the reaction order, and recyclability studies were carried out to assess the catalyst's reusability.

MATERIALS AND METHODS

Materials and Chemicals

As raw material to produce FAME, an untreated high acidity oil mixture was prepared by blending

15 wt% microalgal oil with low grade palm oil (denoted as LGPO-MO). Acetylsalicylic acid (aspirin) ($\geq 99\%$) and two types of acidic catalysts, including *p*-toluenesulfonic acid monohydrate (PTSA) ($\geq 98.5\%$), 8-hydroxyquinoline-5-sulfonic acid monohydrate (98%), 1-amino-2-hydroxy-4-naphthalenesulfonic acid ($\geq 96\%$) and benzenesulfonic acid ($\geq 90\%$) were purchased from Merck (Malaysia) Sdn. Bhd., for the analysis of FFA, potassium hydroxide pellets ($>85\%$) were procured from Merck (Malaysia) Sdn. Bhd.

Preparation of Aspirin-based DESs

Aspirin-based DES were prepared by adding aspirin to PTSA or BZSA at a molar ratio of 1:3 (denoted as ASA:PTSA and ASA:BZSA respectively in this study). The DESs were prepared under a moisture controlled environment, where the mixture was stirred at 400 rpm at temperatures of 80°C - 90°C for 2 hr. The resultant DESs were viscous liquids at elevated temperatures and formed a gel-like appearance when cooled down to room temperature (28°C). The visual appearances of the ASA:PTSA and ASA:BZSA DESs were brownish-purple and chalk white, respectively.

Encapsulation of DES Catalysts

The prepared API-based DES catalysts with the desired dosage were transferred and encapsulated in the medical capsules using a syringe as represented in Figure 1 (Hayyan et al., 2023). To reduce viscosity, DESs were pre-heated to 45°C prior to encapsulation. Then, the encapsulated DES catalyst was stored for use in the esterification. Gelatine-based medical capsules have limited solubility in non-aqueous solvents like methanol due to their protein-derived

nature from collagen. Heating accelerates the dissolution of gelatine capsules by breaking down their structure, allowing the encapsulated DES catalysts to be released into the oil within 20-30 min. As a result, before initiating the esterification reaction, the encapsulated DES catalyst was added to untreated oil and preheated to 60°C to fully release the catalysts. Methanol was subsequently added, and the batch esterification was performed under magnetic stirring.

Analysis of FFA Content

The initial FFA content of the untreated LGPO-MO was recorded as 11%. The acceptable FFA concentration in the treated product was limited to below 2%. According to the American Oil Chemists' Society Ca 5a-40 method, the standard method for determining FFA in untreated oil and treated oil is the acid-base titration technique with KOH as the base component and phenolphthalein as an indicator. The initial FFA concentration was calculated from the titrated volume of KOH and the weight of oil as detailed by the Ca 5a-40 method (AOCS, 2017). Subsequently, the FFA conversion was calculated using Equation (1).

$$\text{Conversion} = \frac{\text{FFA}_{\text{initial}} - \text{FFA}_{\text{final}}}{\text{FFA}_{\text{initial}}} \times 100\% \quad (1)$$

where, $\text{FFA}_{\text{initial}}$ and $\text{FFA}_{\text{final}}$ are the initial and final FFA contents (%), respectively.

Experimental Setup for FFA Esterification Reaction

Before the esterification pre-treatment process, the LGPO-MO which appears in a semi-solid

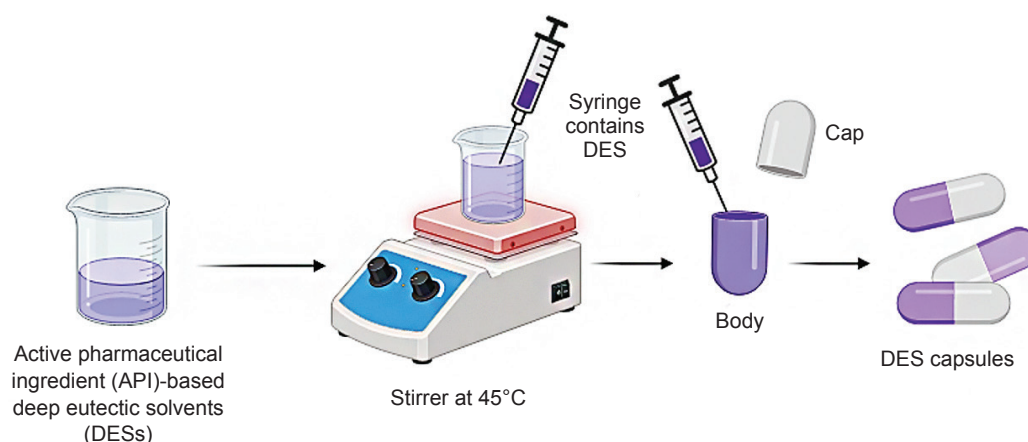


Figure 1. Preparation of active pharmaceutical ingredient (API)-based deep eutectic solvent (DES) catalysts with the desired dosage and encapsulated in medical capsules using a syringe.

state at room temperature was preheated until a homogeneous phase was achieved. Methanol and an untreated LGPO-MO were then added into a conical flask, forming a solution of not more than 50 mL based on the molar ratio required. The esterification reaction of the oil was conducted with a magnetic stirrer at 400 rpm over a specific reaction time period whilst restricting the temperature to below 80°C.

Using single-factor optimisation, the optimum operating condition of esterification was studied using different values of parameters. The experiment was repeated by varying the catalyst dosage (0.25-1.50 wt%), methanol-to-oil mixture molar ratio (4:1-12:1), reaction temperature (40°C-80°C), and reaction time (40-80 min). At the optimum conditions, validation experiments were carried out by repeating the esterification reaction three times to validate the optimum operating conditions. Furthermore, the recyclability of the DESs as catalysts was studied. The separation and recovery of the DESs were carried out by centrifugation for 5 min at 3,000 rpm. After centrifugation, the product mixture underwent phase separation by gravity, forming two distinct layers: The top layer consisted of excess methanol mixed with the DES catalyst, while the bottom layer consisted of the treated oil. The top layer was recovered and transferred to treat a fresh batch of LGPO-MO mixture under the optimal conditions for esterification reaction without purification. The reaction was performed repeatedly, employing the recycled DES catalyst, to evaluate its reusable effectiveness.

RESULTS AND DISCUSSION

Screening of Acidic Catalyst

The FFA content of the treated oil at the end of the esterification reaction using different types of acidic catalysts were demonstrated in *Table 1*. The use of PTSA and BZSA as catalysts led to a significant reduction in FFA in the treated oil, decreasing from 11.00%-0.87% and 0.66%, respectively. This demonstrates that both catalysts have high catalytic activity while 1-amino-2-hydroxy-4-

naphthalenesulfonic acid and 8-hydroxyquinoline-5-sulfonic acid monohydrate did not show any significant reduction in the FFA content.

PTSA and BZSA are strong organic acids that have high solubility in organic solvents such as methanol, making them effective for serving as HBDs in the formation of DESs. Besides, they are considered homogeneous catalysts, leading to a more uniform distribution and interaction with the reactant compared to the heterogeneous catalysts. Additionally, homogeneous PTSA and BZSA with a low melting point (38°C and 51°C, respectively) can easily be prepared as DES. Therefore, PTSA and BZSA catalysts are selected for the formation of the DES due to their promising properties and high efficacy in reducing FFA levels to below 1%.

Effect of DES Catalyst Dosage

Catalyst loading is crucial for completing a chemical reaction and generating the desired product. To study the optimal amount of catalyst required, the dosage of DESs as acid catalysts was investigated at 0.25-1.50 wt%. As shown in *Figure 2*, the increase in DES catalyst dosage promotes a higher reduction and conversion of FFA, which is attributed to the larger area of contact between the reactants and catalysts provided by a higher catalyst dosage facilitating the esterification reaction. At 1.00 wt% of ASA:PTSA and ASA:BZSA DES catalyst dosages, the FFA content was decreased from 11.00% to final levels of 1.77% and 1.71%, respectively. Hence, the optimum dosage of DES catalyst using ASA:PTSA and ASA:BZSA DES in FFA esterification reactions was observed at 1.00 wt%, aiming to achieve FFA levels below 2.00%, which serves as a benchmark for FFA reduction. This consideration also takes into account the associated costs related to catalyst consumption if the DES catalyst dosage is further increased. In comparison, the optimal catalyst dosage for pre-treating highly acidic crude palm oil using methanesulfonic acid as the homogeneous acid for FFA esterification was determined at 1.00 wt%, while requiring a reaction temperature of 60°C and a 30 min reaction time (Hayyan et al., 2012).

TABLE 1. SCREENING OF DIFFERENT TYPES OF ACIDIC CATALYST

Catalyst	Phase in oil	FFA (%)	Conversion (%)
1-amino-2-hydroxy-4-naphthalenesulfonic acid	Homogeneous	8.10	25.24
8-hydroxyquinoline-5-sulfonic acid monohydrate	Homogeneous	6.20	42.78
PTSA	Homogeneous	0.87	91.97
BZSA	Homogeneous	0.66	93.88

Note: PTSA - p-toluenesulfonic acid monohydrate; BZSA- benzenesulfonic acid.

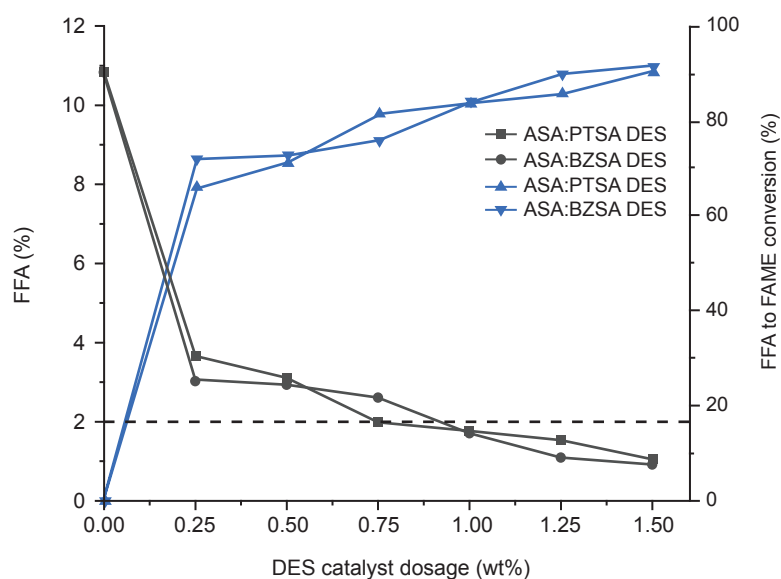


Figure 2. Effect of DES catalyst dosage on FFA content reduction at a temperature of 60°C and a 30 min reaction time for the esterification of LGPO-MO.

Effect of Methanol-to-oil Molar Ratio

Methanol requirement is commonly expressed as a molar ratio to the raw material (untreated oil) used in biodiesel production. Although the stoichiometric equation requires a 1:1 methanol to oil molar ratio in the esterification reactions, an excess amount of methanol is usually used in practice to ensure continuous reaction and to maximise the yield. The experiments were repeated by testing the methanol to oil molar ratio from 4:1 to 12:1, whilst keeping other variables constant. As presented in Figure 3, at the low methanol molar ratio of 4:1, both the ASA:PTSA and ASA:BZSA DES

catalysts exhibit low catalytic activity, resulting in a low reduction of FFA content to 7.19% and 3.41%, respectively. The FFA content of LGPO-MO mixture was decreased to below 2% when utilising a 10:1 molar ratio for both DES catalysts. There was no significant change in FFA conversion and reduction observed when the amount of methanol used exceeded a 10:1 molar ratio, suggesting that the optimal molar ratio for the esterification pretreatment process of FFA was determined. Likewise, an optimal methanol-to-oil molar ratio of 10:1 was reported for the acid-catalysed esterification of acidic crude palm oil at a catalyst dosage of 3 wt% MTPB:PTSA DES (Hayyan et al., 2022).

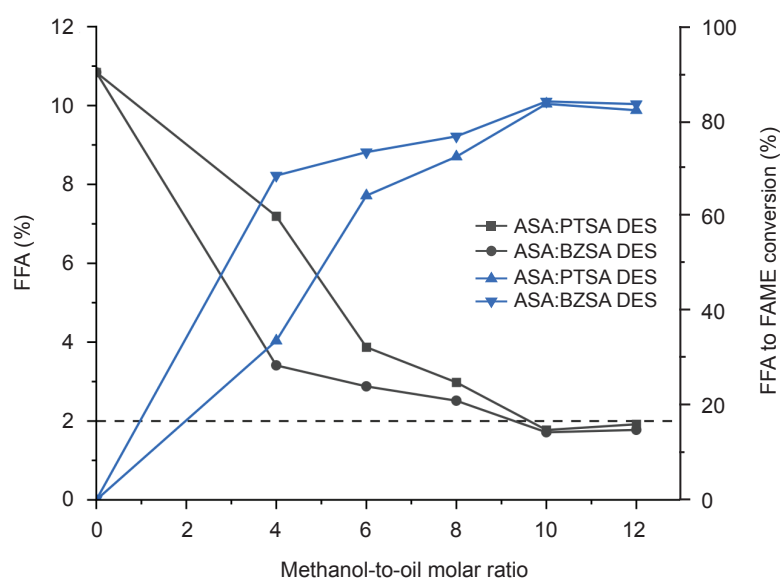


Figure 3. Effect of methanol to oil molar ratio on FFA content reduction at a temperature of 60°C and a 30 min reaction time for the esterification of LGPO-MO at DES dosage of 1 wt%.

Effect of Reaction Time

Esterification reaction time is a crucial factor in kinetic analyses and the reactor design, and optimal contact time between the catalyst and the reactant should be provided to ensure an effective esterification reaction. Thus, the duration for batch esterification reactions is important in achieving an efficient process and cost-effective scaling up in an industrial application. The optimal reaction time was investigated within the range of 20–80 min and presented in Figure 4. The increase in reaction time improves the FFA conversion and reduction as a prolonged reaction time allows the protonation during the reaction, aiding in overcoming the activation energy required for the esterification process to take place. The FFA concentration of the treated LGPO-MO product drops to below 2% within the 60 min reaction time mark achieved by the ASA:PTSA and ASA:BZSA DESs. Hence, the optimal esterification reaction time for the pre-treatment process employing ASA:PTSA-DES and ASA:BZSA-DES were found to be 60 min to ensure the maximum formation of the desired product within a minimal timeframe.

Effect of Reaction Temperature

Methanol as a key component in the esterification reaction, tends to have a high evaporation rate at a higher temperature. When reaction temperatures rise, energy consumption for industrial-scale manufacturing will also increase, driving up overall process running costs. To conduct the optimisation test for the reaction temperature, the optimised catalyst dosage of 1 wt%, methanol to oil molar ratio of 10:1 and esterification reaction time of 60 min (determined previously) were performed

constantly while the reaction temperature was adjusted from 40°C to 80°C. As illustrated in Figure 5, at a reaction temperature of 60°C, the FFA levels decrease to the acceptable level of <2%. However, beyond 60°C, the FFA conversion rate decreases for both the DESs. Elevating the temperature resulted in decreased formation of FAME due to the production of gaseous methanol above its boiling point of 65°C. This caused a decline in the amount of methanol adsorbed on the catalyst surface. Therefore, a 60°C esterification reaction temperature is selected as the optimum for the esterification process with a yield of 84.50%. For comparison, the esterification of sludge palm oil using sulphuric acid catalyst resulted in a yield of 83.72% under the same optimum process conditions of a 10:1 molar ratio of methanol to oil, a reaction temperature of 60°C, and a reaction time of 60 min (Hayyan et al., 2011).

Validation of Reaction Conditions and Catalyst Recyclability

The recyclability performances of the DESs were investigated at the optimal operating conditions. The catalysts were reused without adding fresh catalysts for multiple successive runs. Results in Figure 6 indicated that both catalysts maintained good catalytic performance in the first run, achieving a reduction of FFA content down to 1.58% and 2.02% using ASA:PTSA and ASA:BZSA, respectively. In the second and third runs, lower but considerable catalytic activity was observed, with FFA content ranging from 3.00%–5.00%. However, during the fourth run, the recycled catalyst's effect was insignificant. Thus, the acceptable reusability cycle of both DESs as catalysts were three cycles. Losses of DES catalyst during recycling and unseparated catalyst remaining in the treated oil

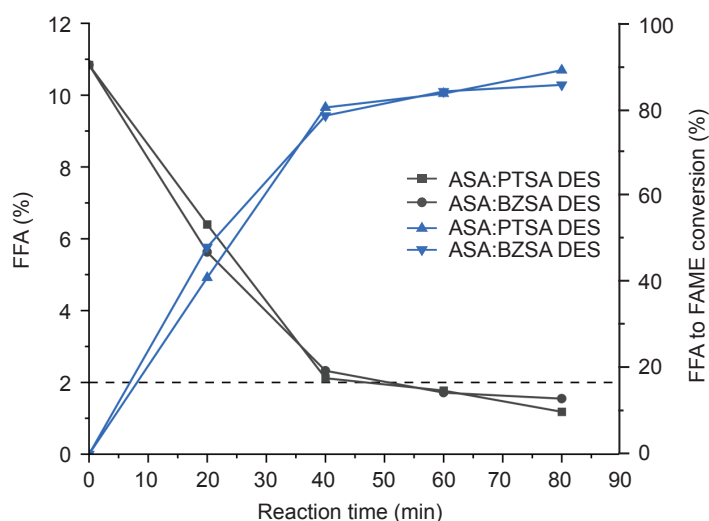


Figure 4. Effect of reaction time on FFA content reduction at a temperature of 60°C with 10:1 molar ratio for the esterification of LGPO-MO at a DES dosage of 1 wt%.

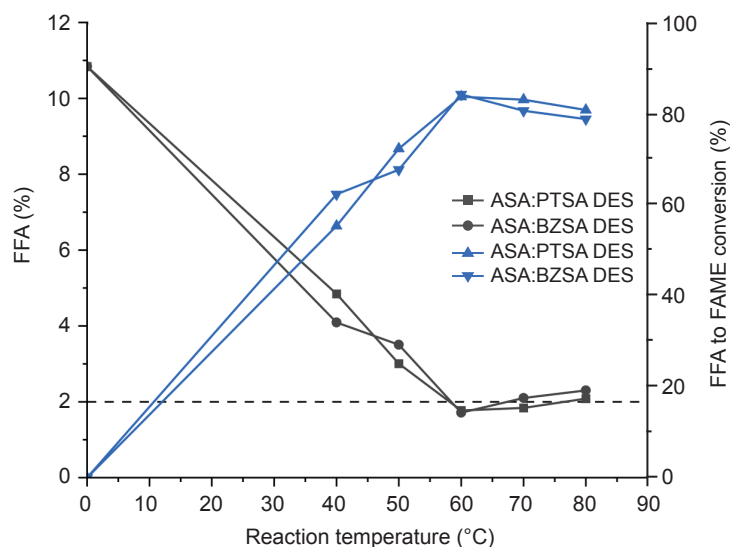


Figure 5. Effect of reaction temperature on FFA content reduction at 30 min reaction time, 10:1 molar ratio for the esterification of LGPO-MO at a DES dosage of 1 wt%.

were potential reasons for the reduced conversion rates. To achieve desired FFA levels, extending the reaction time or increasing methanol concentration in each reuse of the DES catalyst could be explored. In comparison, the recyclability of choline chloride-based DESs employed for the esterification treatment of high acidic crude palm oil was similarly recorded for a maximum recyclability of three cycles (Hayyan et al., 2013).

Evaluation of Reaction Kinetics

The FFA esterification reaction kinetics of the ASA:PTSA and ASA:BZSA DES catalysts were evaluated by correlating the empirical data with the pseudo first order model according to prior

literature, where the reverse rate of reaction could be neglected due to the excess use of methanol in enhancing the forward reaction (Chai et al., 2014). The linearised pseudo first order model is presented in Equation (2).

$$\ln [FFA]_t = (-k)(t) + \ln [FFA]_0 \quad (2)$$

where, k is the reaction rate constant, t is the reaction time, $[FFA]_t$ is the FFA concentration at a specific time and $[FFA]_0$ is the initial FFA concentration. The experimental data was fitted to Equation (2) to yield the corresponding k values at the specific temperatures with a satisfactory coefficient of determination (R^2) as presented in Figure 7.

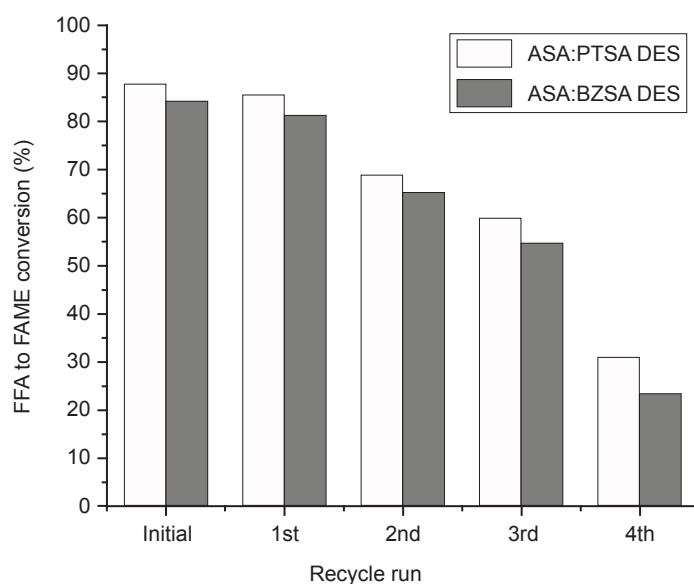


Figure 6. FFA conversion at different recycle run at a temperature of 60°C, 30 min reaction time with 10:1 molar ratio for the esterification of LGPO-MO at a DES dosage of 1 wt%.

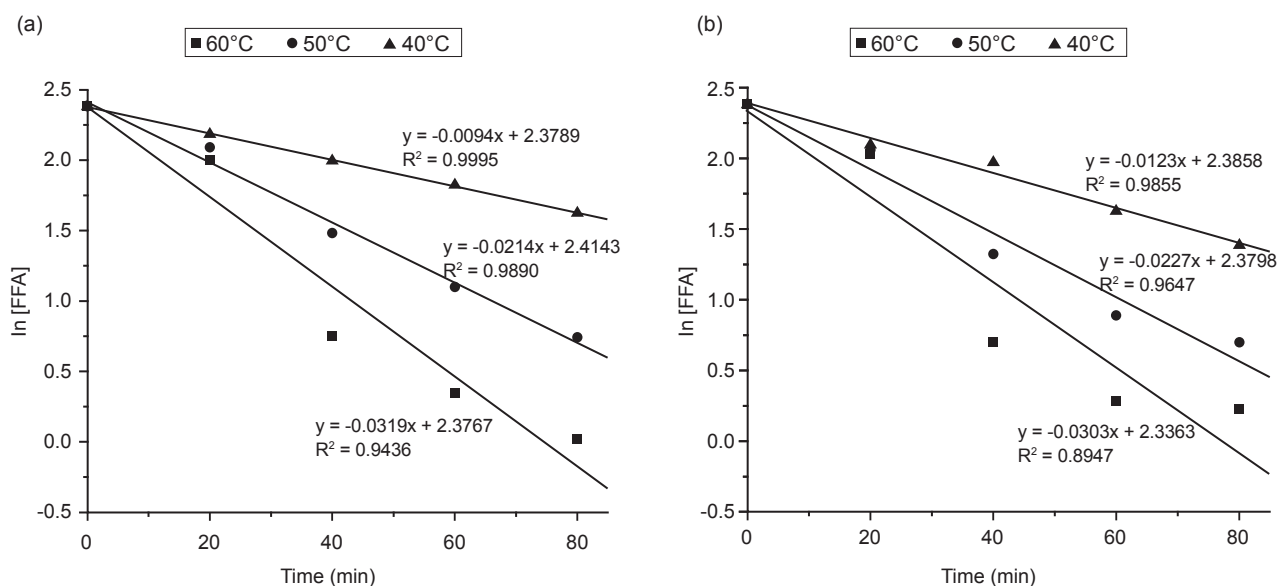


Figure 7. Temperature plot of the first order kinetic modelling using (a) ASA:PTSA DES, and (b) ASA:BZSA DES.

Subsequently, the k values were fitted to the linearised form of the Arrhenius equation [Equation (3) and Equation (4)] to compute the activation energy (E_a) and frequency factor (A) values. In the Arrhenius equation, R is the gas constant ($8.314 \text{ J mol}^{-1}\text{K}$) and T is the absolute temperature (K).

$$k = Ae^{-\frac{E_a}{RT}} \quad (3)$$

$$\ln k = -\frac{E_a}{RT} + \ln A \quad (4)$$

As presented in Figure 8, the activation energy was calculated as 53.11 and 39.20 kJ mol^{-1} with frequency factor values of 7.32 and 4.48 min^{-1} for

ASA:PTSA and ASA:BZSA DESs, respectively. The results imply that higher energy input is required to overcome the activation energy for the reaction to occur when using ASA:PTSA-DES compared to ASA:BZSA-DES. Additionally, the frequency factor of the reaction using ASA:PTSA-DES is significantly higher than that of ASA:BZSA-DES, suggesting that ASA:PTSA-DES promotes a greater number of successful collisions between reactant molecules (LGPO-MO and methanol). The activation energies of the DES catalysts were comparable to existing values reported in the literature (38.82–59.58 kJ mol^{-1}) for the esterification of various low value oils such as animal fats, non-edible seed oils and waste cooking oils (Encinar et al., 2021; Guo et al., 2021; Nayak et al., 2021).

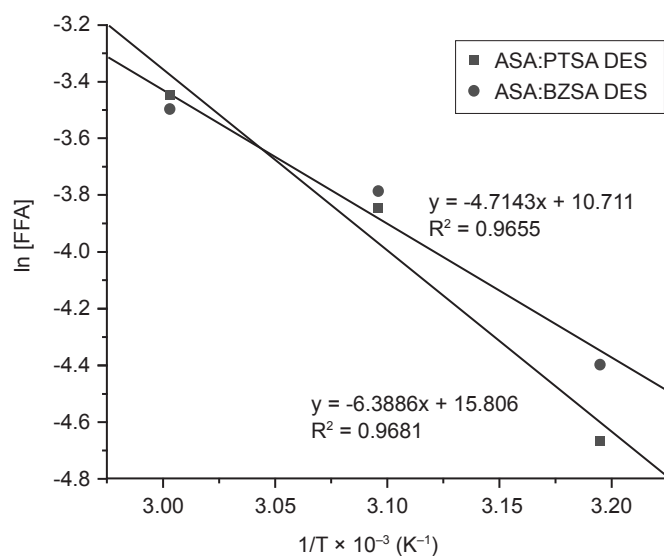


Figure 8. Arrhenius plot for FFA esterification using ASA-based DESs.

CONCLUSION

In this study, two novel encapsulated API-based DESs catalysts (ASA:PTSA and ASA:BZSA) were prepared for the acid-catalysed esterification pre-treatment of LGPO-MO mixture with high FFA content. Under optimised conditions, both DES catalysts exhibited high catalytic activity in the acid-catalysed esterification reaction, leading to a significant reduction in the FFA content of the LGPO-MO mixture from 11% to below 2%. Both the ASA:PTSA-DES and ASA:BZSA-DES gave the optimal performance with the following reaction conditions: DES catalyst to oil ratio of 1 wt%, methanol to oil molar ratio of 10:1, reaction temperature of 60°C, and reaction time of 60 min. The DESs were successfully recycled and reused three times with considerable catalytic activity. The reaction kinetics of the DESs were examined where the activation energies for ASA:PTSA-DES and ASA:BZSA-DES were 53.11 and 39.20 kJ mol⁻¹ with frequency factor values of 7.32×10^6 and 4.48×10^4 min⁻¹, respectively. Overall, the introduced encapsulated ASA:PTSA and ASA:BZSA DES catalysts in this study offered benefits and superior catalytic activity performance in terms of safe handling during pre-reaction procedures. Thus, the formation of DESs from API is worth being investigated for the pre-treatment process of high acidity feedstock and for advancing biodiesel production.

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INVESTIGATION ON MECHANICAL AND CORROSION CHARACTERISTICS OF ASME SA 516 GRADE 70 CARBON STEEL USED IN A BIODIESEL STORAGE FACILITY

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ABSTRACT

In this experimental study, the impact of B100 biodiesel corrosion on the 15 years old ASME SA 516 Grade 70 carbon steel storage tank was investigated. This study provides novel insights into the material's performance and reliability of 15 years old ASME SA 516 Grade 70 carbon steel plate material under prolonged B100 biodiesel storage. Various analytical techniques, including ultrasonic thickness gauge measurements, scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS), were used to assess corrosion behaviour and morphology. Tensile tests were conducted to evaluate the mechanical properties. The findings showed significant deterioration in both mechanical properties and corrosion resistance due to B100 biodiesel corrosion beyond the API 653 tolerance; hence, replacement is required to ensure mechanical integrity. The study also examines surface characteristics using SEM and EDS analyses, showing significant alterations in the metal's morphology and composition due to B100 biodiesel exposure. Additionally, the article discusses corrosion measurement and the remaining life of tanks, indicating a need for regular maintenance and repair. The mechanical performance of the steel is also assessed, showing a decrease in tensile strength and ductility. This indicates the potential risk B100 biodiesel corrosion poses to the carbon steel storage tank's performance and reliability.

Keywords: biodiesel corrosion, carbon steel storage tank, corrosion rate, mechanical properties, specific energy absorption.

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INTRODUCTION

Biodiesel is a type of fuel that has attracted much interest recently due to its lower carbon emissions and renewable nature (Aatola et al., 2009). The Malaysian government, through the Malaysian Palm Oil Board (MPOB), has stressed its commitment to be a carbon-neutral nation by 2050 and biodiesel will play a significant role in this direction. According to Parveez et al. (2021), in 2020, there were 20 operational oleochemical plants and 19 biodiesel plants in Malaysia, with processing

capacities of 2.63 million tonnes and 2.23 million tonnes, respectively. The majority of these facilities are situated in Selangor and Johor, with eight oleochemical plants in Selangor, seven in Johor, and six biodiesel plants in each of these states. However, using biodiesel poses challenges in designing and maintaining storage tanks, particularly concerning corrosion. Biodiesel's corrosive properties are well documented in the literature (Kugelmeier et al., 2021). Biodiesel contains free fatty acids (FFA) and other compounds that can facilitate the formation of corrosive compounds, resulting in storage tank failures. Due to the formation of iron soaps and other corrosion products, carbon steel tanks, commonly used in storage tank construction, can exacerbate the corrosive nature of biodiesel (Rocha et al., 2019). The corrosive nature of biodiesel, more so than

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traditional diesel, can lead to material degradation, sludge formation and microbial growth. While biodiesel offers benefits, its widespread use is hampered by several factors: Its high cost, potential competition with food resources, environmental concerns like deforestation for crop production, and technical issues such as fuel filter blockages and injector contamination. Additionally, biodiesel's lower oxidative stability makes it more prone to fuel oxidation and its reduced heating value affects engine performance in terms of torque and power. Its oxidative instability, poor performance in cold temperatures and solvent properties also contribute to its fewer desirable characteristics (Aatola et al., 2009; Bocha et al., 2007; Fazal et al., 2012; Gunstone, 2009; Samuel & Gulum, 2019).

The impact of biodiesel on storage tank integrity has been the subject of several previous studies. In a study conducted by Fazal et al. (2011), the authors examined the influence of biodiesel on the corrosion behaviour of carbon steel. Their investigation revealed that exposure to biodiesel led to elevated corrosion rates and the formation of iron soaps. The study also found that the corrosive properties of biodiesel were more severe at higher temperatures and in the presence of water. In a similar vein, a comprehensive investigation conducted by Kugelmeier et al. (2021) examined the corrosive properties of biodiesel on a range of storage tank materials, including carbon steel, stainless steel and aluminium. The study found that biodiesel exposure resulted in significant corrosion and pitting on all materials tested, with carbon steel being the most severely affected. Yung et al. (2018) investigated the compatibility of palm biodiesel blends (B7, B10 and B15) with automotive fuel tank materials, focusing on *terne* sheets. The research found that when high-quality fuels were used, palm biodiesel blends did not cause corrosion or compatibility issues with *terne* sheets, as demonstrated by scanning electron microscopy analysis and the absence of heavy metal leaching.

The mechanical properties of storage tanks can also be compromised by biodiesel corrosion. Corrosion can lead to reduced tensile strength, and hardness, increasing the risk of storage tank failures (Samuel & Gulum, 2019). Consequently, it is crucial to comprehensively assess the long-term performance of storage tanks by evaluating their mechanical properties when exposed to biodiesel. Furthermore, the impact of biodiesel corrosion on the fatigue behaviour of storage tanks remains an underexplored area, necessitating further research.

The literature also suggests several strategies to mitigate the effects of biodiesel corrosion on storage tanks. These include the use of corrosion inhibitors (Alam et al., 2022; Arunkumar et al., 2018; Atikpo et al., 2022; Chen & Sun, 2022; Diaz-Ballote et al., 2022; Fernandes et al., 2021a, 2021b;

Jiang et al., 2019; Martins et al., 2020; Ouarga et al., 2022; Patil et al., 2018; Touazi et al., 2020; Yi et al., 2018). However, the effectiveness of these strategies may vary depending on the specific properties of the biodiesel and the storage tank materials. In addition, some of these strategies may have negative environmental impacts and may not be sustainable in the long run. Moreover, it is essential to consider the economic implications of biodiesel corrosion on storage tank maintenance and replacement costs. Storage tank failures due to biodiesel corrosion can lead to significant environmental, economic and safety consequences (Wei et al., 2021). Hence, there is a pressing need to develop cost-effective and sustainable strategies aimed at mitigating the harmful effects of biodiesel corrosion on storage tank integrity.

The impact of biodiesel on storage tank integrity has significant economic, environmental and safety implications. Storage tank failures can result in environmental damage, economic loss and threats to public safety. Therefore, it is critical to understand the mechanisms of biodiesel corrosion and to identify strategies to mitigate its effects. While previous studies have investigated the corrosive properties of biodiesel, there is still a need for more comprehensive studies that evaluate the mechanical properties of storage tanks exposed to biodiesel (Batista et al., 2019; Cursaru et al., 2018; Fernandes et al., 2019, 2021b; Kugelmeier et al., 2021; Martins et al., 2020; Pusparizkita et al., 2020; Serqueira et al., 2021). The mechanical properties of storage tanks can be compromised by corrosion, leading to reduced tensile strength, ductility, and fracture toughness. Therefore, it is crucial to evaluate the mechanical properties of carbon steel exposed to biodiesel to assess their long-term performance. This study aims to bridge this research gap by thoroughly investigating the corrosion and mechanical properties of carbon steel tanks exposed to biodiesel. The assessment will employ weight loss analysis and electrochemical tests to determine the corrosion rate of the tank. Tensile testing and hardness testing will be conducted before and after exposure to biodiesel to evaluate the mechanical properties of the tank. This study unveils comprehensive insights into the corrosion mechanisms affecting 15 years old ASME SA 516 Grade 70 carbon steel in B100 biodiesel in comparison to the virgin carbon steel plate, emphasising the critical importance of implementing tailored maintenance and inspection systems specific to industry and product requirements. The outcomes of this study will provide invaluable insights into the impact of B100 biodiesel storage on the performance and reliability of 15 years old ASME SA 516 Grade 70 carbon steel storage tanks.

MATERIALS AND METHODS

Carbon Steel

The carbon steel specimens utilised in this investigation were obtained from a tank that has been in service since 2007, conforming to the dimensions of 11.4 m outer diameter, 5.0 mm thickness and 7.2 m length, as represented in *Figure 1*. The storage tank is constructed from ASME SA 516 Grade 70 carbon steel, designed for a pressure of 19.2 bar and operating at 10.0 bar, with a design temperature of 50°C and an operating temperature of 40°C. The steel's chemical composition, as presented in *Table 1*, contributes to its mechanical properties, ensuring a well-balanced combination of strength, ductility and corrosion resistance suitable for the specified conditions. The specimens were cut from the corroded shell plate sized 20 x 20 mm in accordance with ASTM E8/E8M. Then, the sample testing was conducted using the Universal Testing Machine (UTM), the Instron 3800, with a load frame capacity of 300 kN. During the test, the specimen is loaded with tension until it fractures. The machine measures and records various data points, such as the force applied and the elongation of the specimen. The ultimate tensile strength (UTS), yield strength and elongation were calculated from these data.

TABLE 1. OPERATING DETAILS, MECHANICAL PROPERTIES AND CHEMICAL COMPOSITION FROM MANUFACTURER

Description	Details
Product	Palm oil methyl-ester (PME)
Year of service	2007-2023
Material	ASME SA 516 Grade 70
Design pressure	19.2 bar
Operating pressure	10.0 bar
Design temperature	50°C
Operating temperature	40°C
Parameters	Values
Yield strength (MPa)	345
Tensile strength (MPa)	510
Elongation (%)	28.5
Hardness (HRB)	76.5
Element	Carbon steel (wt%)
Carbon	0.200
Silicon	0.220
Manganese	0.430
Phosphorus	0.020
Sulphur	0.011
Chromium	0.160
Nickel	0.190
Copper	0.020
Molybdenum	0.001
Vanadium	0.010

The biodiesel employed in this study was obtained from a local biodiesel producer, and its chemical composition was determined using standardised test methods, as tabulated in *Table 2*.

Table 1 describes the biodiesel storage tank operating details and the mechanical properties of the ASME SA 516 Grade 70 carbon steel. Based on the parameters listed in *Table 2*, it can be concluded that monitoring and controlling water content, oxidative stability, sulphur content and acid value are essential to minimise the corrosive impact of biodiesel on carbon steel storage tanks. Extreme temperature fluctuations during storage can lead to condensation inside the storage tanks, introducing moisture into the palm oil methyl-ester (PME). If PME is stored in a humid environment or in containers that are not properly sealed, moisture can enter the biodiesel. Moisture can be introduced into the biodiesel through poor handling or storage conditions. Water can lead to the hydrolysis of the esters in the biodiesel, forming FFA, which can be corrosive. On the other hand, PME contains sulphur compounds, which can react with moisture during storage to form hydrogen sulphide and other corrosive sulphur-containing compounds. Furthermore, exposure to air can lead to oxidation of the unsaturated components in the biodiesel, forming peroxides and acids, which can be corrosive too. Moreover, in the presence of water and at certain temperatures, microbial growth can occur in stored PME. Microorganisms can produce acidic byproducts, leading to corrosion.

Scanning Electron Microscopy (SEM) and Energy-Dispersive X-ray Spectroscopy (EDS)

The morphology and composition of the corrosion products on the carbon steel specimens were examined using scanning electron microscopy (SEM) and dispersive X-ray spectroscopy (EDS). SEM and EDS are widely employed techniques for the examination and analysis of elemental surface components in samples related to corrosion studies. SEM examination allows for the visualisation of metal surface morphology, deposits and corrosion products, which can range from crystalline to amorphous. In this study, the SEM Zeiss Evo MA10 with medium resolution, a solid-state secondary electron detector and a backscattered electron detector were utilised for multi-sample inspections with a resolution of 100 nm. Prior to mounting the specimens on SEM stubs, they underwent a thorough cleaning process with acetone and subsequent drying. High-resolution field emission scanning electron microscopy (FESEM) was utilised to capture SEM images, while EDS analysis was conducted using an EDS detector attached to the microscope. EDS analysis provides valuable chemical information about the features

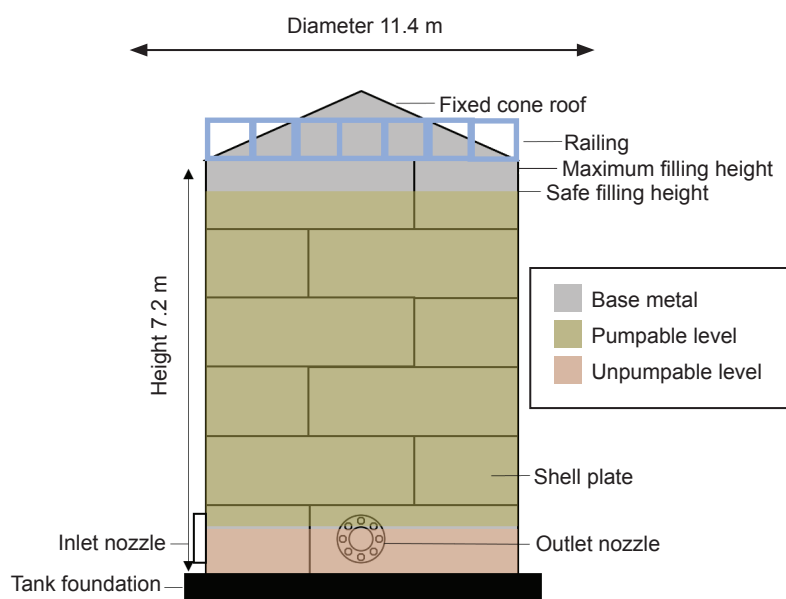


Figure 1. The biodiesel storage tank diagram and its dimension details.

TABLE 2. CHEMICAL COMPOSITION OF THE B100 PALM OIL METHYL-ESTER (PME) BIODIESEL USED IN THIS STUDY

Property	Unit	Limits		Test methods	Test result
		Min	Max		
Ester content	% (m/m)	96.5	-	EN 14103	99.7
Density @ 15°C	kg/m ³	860.0	900.0	ISO 3675	875.2
Viscosity @ 40°C	mm ² /s	3.500	5.000	ISO 3104	4.423
Flashpoint	°C	120	-	ISO 3679	150
Sulphur content	mg/kg	-	10.00	ISO 20846	<1.00
Cetane number	-	51.0	-	ISO 5165	72.1
Sulfated ash content	% (m/m)	-	0.020	ISO 3987	<0.001
Water content	mg/kg	-	500	ISO 12937	272
Total contamination	mg/kg	-	24	EN 12662	6
Copper strip corrosion (3 hr at 50°C)	Rating	-	1	ISO 2160	1a
Oxidation stability, 110°C	hr	10.0	-	EN 14112	23.5
Acid value	Mg KOH/g	-	0.50	EN 14104	0.25
Iodine value	g iodine/100 g	-	110.00	EN 14111	52.63
Linolenic acid methyl-ester	% (m/m)	-	12.0	EN 14103	0.10
Polyunsaturated methyl-esters (4 double bonds)	% (m/m)	-	1.00	EN 15779	<0.10
Methanol content	% (m/m)	-	0.20	EN 14110	0.03
Monoglyceride content	% (m/m)	-	0.70	EN 14105	0.51
Diglyceride content	% (m/m)	-	0.20	EN 14105	0.10
Triglyceride content	% (m/m)	-	0.20	EN 14105	0.01
Free glycerol	% (m/m)	-	0.020	EN 14105	0.019
Total glycerol	% (m/m)	-	0.25	EN 14105	0.17
Group I metal (Na + K)	mg/kg	-	5.000	EN 14108/14109	<1.000
Group II metals (Ca + Mg)	mg/kg	-	5.0	EN 14538	<1.0
Phosphorus content	mg/kg	-	4.0	EN 14107	<1.0
CFPP	°C	-	15	EN 116	+11

Source: Yusoff et al. (2021).

examined via SEM, enabling the identification and quantification of major, minor and trace elements present in the samples. SEM and EDS can provide valuable insights into the microstructure and chemistry of virgin and corroded samples.

Ultrasonic Thickness and Corrosion Rate Measurement

Ultrasonic testing thickness gauge (UTT) is a non-destructive testing method commonly employed to determine the thickness of metals, including ship hulls, piping and structural steel. The method complies with ISO 9712 requirements at levels one, two and three (Purschke, 2013). The thickness range of the Olympus 45MG is 0.080-635.000 mm, the velocity range is 0.508-18.699 mm/s and the standard resolution is 0.010 mm. In many industries, software applications for monitoring corrosion, erosion and damage are critical. This technique calculates the round-trip transit time of an ultrasonic pulse from the gauge to the first back wall echo. By measuring the time, it takes for the pulse to travel through the material and reflect off the back wall, the gauge can determine the metal thickness. Based on this time interval and the speed of sound in the material, the thickness of the material can be calculated, as illustrated in Figure 2. Equation (1) was used to calculate the nominal thickness of the steel plate d_{steel} :

$$d_{steel} = \frac{V_{steel} \times t_{steel}}{2} \quad (1)$$

where, V_{steel} is the sound travel velocity (mm/μs), t_{steel} denotes the time required for the soundwave to return (μs). V_{steel} is computed into Equation (2) to calculate the corrosion rate, C_r (mm/yr).

$$C_r = \frac{d_0 - d_{steel}}{Yr} \quad (2)$$

where, d_0 represents the initial plate thickness (mm) and Yr denotes the duration of plate exposure with the service fluid (Years). On the other hand, minimum thickness of the plate, d_{min} (mm) is calculated using Equation (3):

$$d_{min} = \frac{66.04 (H - 0.3) DG}{SE} \quad (3)$$

where, D is the steel rolling diameter, in feet (ft) while H is the plate length, in feet (ft). In tank design, G represents the highest specific gravity of the tank contents, S denotes the maximum

allowable stress in lbf/in² and E signifies the original joint efficiency of the tank. The values for the parameters listed are presented in Table 3.

TABLE 3. THE STEEL ROLLING DIAMETER (D), THE HIGHEST SPECIFIC GRAVITY OF THE TANK CONTENTS (G), THE MAXIMUM ALLOWABLE STRESS (S), AND THE ORIGINAL JOINT EFFICIENCY OF THE TANK, (E)

Parameters	D (m)	G (kg/m ³)	S (N/mm ²)	E
Values	34.20	0.780	172	1.0

The C_r and V_{steel} are used to calculate the remaining life, RL (years) through Equation (4):

$$RL = \frac{d_{steel} - d_{min}}{C_r} \quad (4)$$

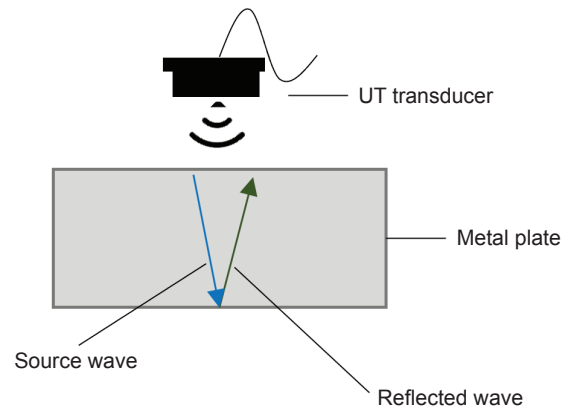


Figure 2. The schematic diagram of the working principle of UTTG.

Corrosion rate and remaining life calculations serve as valuable tools in assessing the current condition and future prognosis of carbon steel storage tanks. By measuring the corrosion rate and estimating the remaining life, the effectiveness of corrosion prevention and mitigation methods such as coating, cathodic protection and corrosion inhibitors can be evaluated. Moreover, one can also plan for maintenance, repair, or replacement activities based on the corrosion rate and remaining life data. This system can help optimise the operational cost and extend the service life of carbon steel storage tanks. The thickness values were taken at the top, middle and bottom locations to represent the tank's biodiesel exposure level.

Tensile Test

To assess its structural integrity and durability, it is critical to investigate the mechanical properties of a corroded steel plate immersed in biodiesel. Tensile, hardness, testing, fatigue, toughness and impact tests can all be used to characterise corroded

steel plates immersed in biodiesel. Strength, ductility, hardness, fatigue life, fracture toughness and impact resistance are all mechanical properties that can be measured on corroded steel plates using these methods. Throughout their service lives, storage tanks experience forces in the axial and hoop directions. The tangential or hoop stress acting on the wall thickness is observed, as shown in Equation (5) (Jerome & Ross, 1997).

$$\sigma_H = \frac{P}{A} = \frac{pDL}{2Lt} = \frac{pD}{2t} \quad (5)$$

where, L denotes the length of the cylindrical structure, D denotes the internal diameter, t denotes the wall thickness and p denotes the fluid pressure inside the structure. Equation (6) is used to determine the longitudinal stress (Jerome & Ross, 1997).

$$\sigma_A = \frac{P}{A} = \frac{\pi D^2 p}{4\pi Dt} = \frac{pD}{4t} \quad (6)$$

Combining Equation (3) and Equation (4), the hoop stress is greater than the axial stress. Hence, studying stress in the hoop direction compared to the axial direction is crucial. The outcomes of these tests provide valuable insights into the extent and severity of corrosion damage, the impact of corrosion on the mechanical behaviour of steel plates, as well as potential failure modes and mechanisms associated with corroded steel plates. ASTM E8 tensile testing was conducted, where the specimens were subjected to a UTM at a crosshead speed of 2 mm/min until failure occurred (Kardak & Sinclair, 2020). The stress-strain curves derived from the test results were utilised to determine various mechanical parameters, such as the yield point and tensile strength.

RESULTS AND DISCUSSION

These findings showed that exposing carbon steel tanks to B100 biodiesel caused significant corrosion and deterioration of mechanical properties. Weight loss analysis and electrochemical tests were used to assess the corrosion rate of the tanks. Weight loss analysis revealed a corrosion rate of 0.26 mm/yr, which is higher than the oil and gas industry's recommended maximum corrosion rate of 0.1 mm/yr for carbon steel tanks. The findings of this study are consistent with previous research on the corrosive properties of biodiesel (Dharma et al., 2023; Diaz-Ballote & Genesca, 2021; Nguyen & Vu, 2019; Samuel & Gulum, 2019). Biodiesel has been linked to a variety of corrosion processes, including uniform corrosion, pitting corrosion and stress corrosion cracking. The exact mechanism of

biodiesel corrosion is unknown, but it is thought to be caused by the presence of water, oxygen, and FFA in biodiesel, which promote the formation of corrosive compounds.

Surface Characteristic

SEM can reveal the surface features of the samples, such as cracks, pits, scales, films, and deposits. There are iron (III) oxide deposits on the surface of the corroded specimen. As previously reported by Kugelmeier et al. (2021) and Fazal et al. (2018), the EDS analysis revealed the presence of carbon and oxygen at 64.11 wt% and 15.92 wt%, respectively, indicating the formation of corrosion products such as Fe_2O_3 . This observation suggests that the material was exposed to a less corrosive environment with lower oxygen concentrations, indicating the resistance of carbon steel when in contact with biodiesel blends (Maru et al., 2009). By comparing the SEM images and EDS spectra of virgin and corroded samples, it can be inferred that the corrosion affects the morphology and composition of the samples. According to Fazal et al. (2013), the existence of fatty acid compounds, oxygen, and water content contributed to the higher degradation of the metal surface. As illustrated in Table 4 and Figure 3, the element weights exhibited variations between the virgin and corroded samples. The decrease in the molecular weight of iron and oxygen ions corresponds to an increase in carbon content. This phenomenon can be attributed to the reduction in iron ion weight caused by the formation of rust, which is deposited as a thin layer on the corroded sample surface, as illustrated in Figure 4. The red dots represent the EDS spots from which the measurements, in Table 4, were taken. Following that, the weight of the element oxygen decreases because the sample is corroded due to oxidation, whereas the weight of carbon increases because the carbon chain is breaking down and forming more carbon chains [Equation (7)].



This reaction explains that the virgin sample had 4 mol of Fe and 8 mol of O_2 before corrosion occurred. This explains why the virgin sample contains the least amount of each component in EDS. The corroded metal has 6 mol of Fe, and all the component values have increased, indicating that the layer of corrosion has occurred.

Corrosion Measurement and Remaining

As illustrated in Figure 5 and Table 5, reading #2 constitutes the highest C_r of 0.322 mm/yr and has the lowest RL of 7.4 years. This finding can be

TABLE 4. THE WEIGHT AND ATOMIC PERCENTAGE COMPARISON IN BETWEEN VIRGIN AND CORRODED SPECIMENS

Item	Virgin		Corroded	
	Weight (%)	Atomic (%)	Weight (%)	Atomic (%)
C	14.05	29.04	64.11	72.54
O	29.55	45.87	15.92	13.52
Fe	56.40	25.08	11.67	8.35

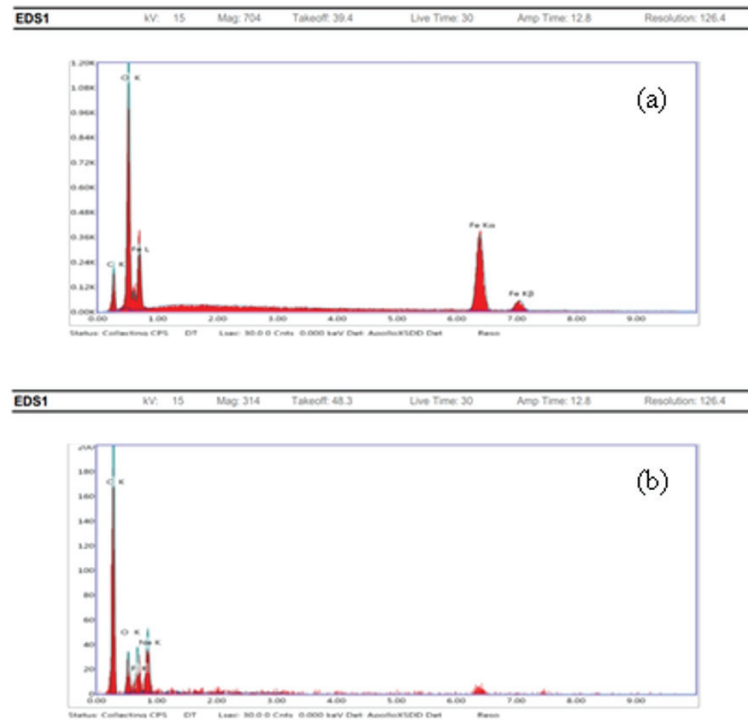


Figure 3. The EDS analysis of the (a) virgin, and (b) corroded specimens.

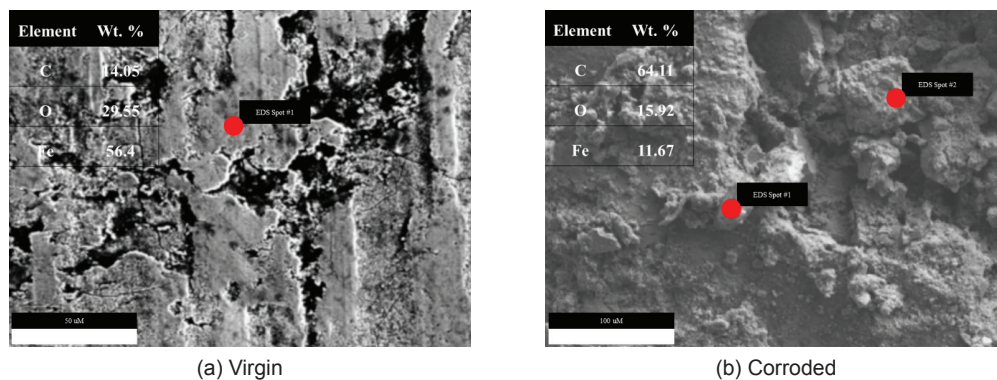


Figure 4. The SEM images of (a) virgin, and (b) corroded samples after exposure to B100 biodiesel for 15 years.

attributed to the total exposure of the biodiesel throughout the service life. Compared to other shell courses, the first shell course has complete immersion and exposure at an unpumpable level, which contains the deadstock. As the biodiesel product is highly hygroscopic, water formation will settle at the bottom level of the first shell course.

This finding aligns with the research conducted by Komariah et al. (2021), which investigated the corrosion behaviour of steel tanks upon contact with palm-based biodiesel. Their study focused on the corrosion type and behaviour in different zones within the fuel tank, with varying coating elements. The primary outcomes highlighted

those tanks made with various protective layers exhibited distinct types of corrosion, and the potential for leaks was higher at the base compared to the walls and roof of the storage tank. The increased corrosion rate observed in various metals in palm biodiesel can be attributed to the presence of oxygen and moisture absorption. Palm biodiesel typically contains 10-12 wt% oxygen, whereas conventional diesel does not contain oxygen. These findings are consistent with previous studies on the corrosion of metals in palm oil-based biodiesel blends conducted by (Fazal et al., 2019; Sgroi et al., 2005). As per API 653:2014, a storage tank must be externally inspected at least once every five years and internally inspected at least once every 20 years (American Petroleum Institute, 2014). Maintenance and repair should be conducted based on the corrosion rate (CR) and remaining life (RL) of the tank. This requirement aligns with Malaysia's Customs Act 1967, which mandates that subsidised storage tanks, including those for biodiesel products, must be calibrated within a 15 year duration (Parliament of Malaysia, 1967). Since readings #1 and #2 have remaining life durations lower than 15 years, the shell plate is required to be replaced.

Mechanical Performance

As presented in *Figure 6* and *Table 6*, the elastic region for the virgin specimen was maintained up to the yield point. However, the corroded specimen likely had a lower modulus of elasticity, as indicated by a reduced slope due to the changes in thickness and microstructure. The tensile test results showed that the yield point of the corroded specimens decreased by 30% compared to the virgin specimens, indicating that the corroded specimen begins to plastically deform at a lower stress level. The tensile strength decreased by 38%, indicating a compromised strength through a loss of ductility and toughness. The corroded sample also had a higher elongation at break than the virgin sample, suggesting that the material underwent plastic deformation before fracture. This finding is consistent with the observation that the material becomes more ductile after corrosion, meaning it can stretch further before breaking despite the loss of tensile strength. The finding is comparable to the finding by Samuel and Gulum (2019), which indicated that the brass tensile strength after exposure to B100 biodiesel for 960 hr was 70.77%.

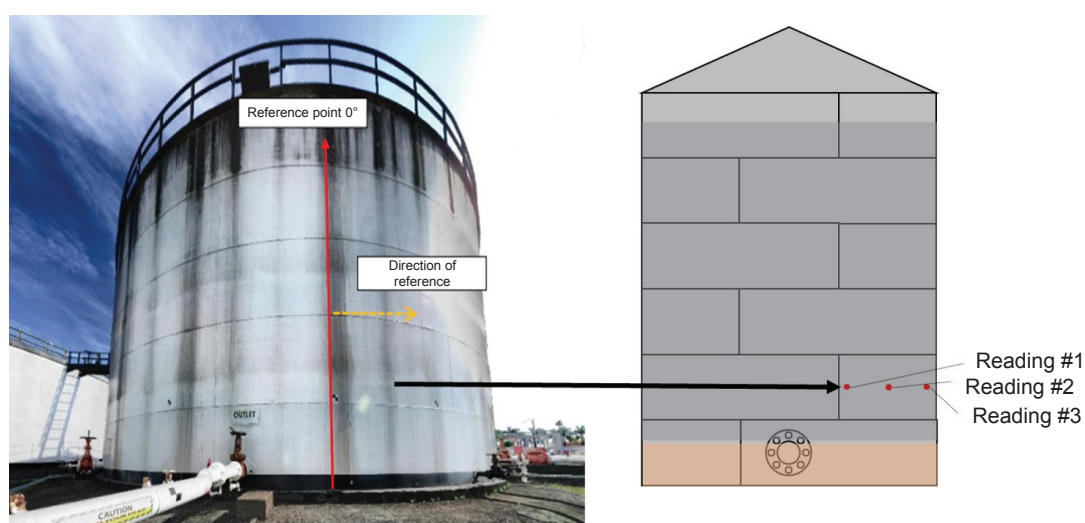


Figure 5. The illustration of the B100 biodiesel tank and its diagram showcasing the reading points.

TABLE 5. THE LOCATION READINGS AND THEIR INITIAL THICKNESS, MEASURED THICKNESS, MINIMUM THICKNESS, CORROSION RATE AND REMAINING LIFE

Reading	Initial thickness, d_0 (mm)	Measured thickness, d_{steel} (mm)	Minimum thickness, d_{min} (mm)	Corrosion rate, C_r (mm/yr)	Remaining life, RL (yr)
#1	7.11	5.61	2.80	0.2503	11.20
#2	7.11	5.18	2.80	0.3220	7.40
#3	7.11	6.08	2.80	0.1720	19.10
Average	7.11	5.62	2.80	0.2481	12.57

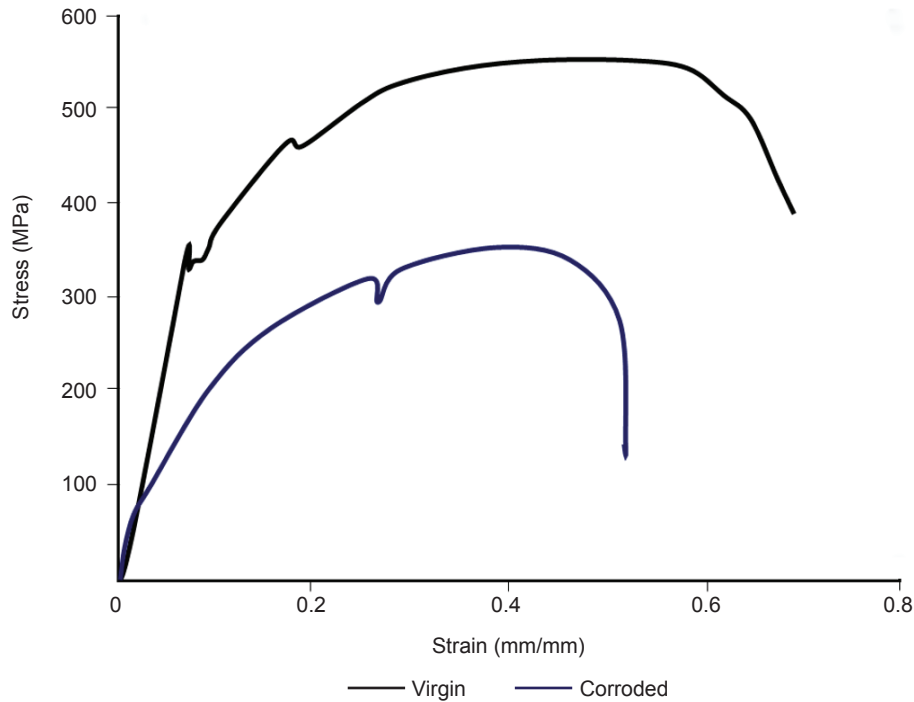


Figure 6. The load displacement curve and damaged specimen.

TABLE 6. THE SPECIMEN DIMENSION AND TENSILE TEST RESULTS

Specimen label	Thickness (mm)	Width (mm)	Maximum load (kN)	Tensile stress at maximum load (MPa)	Tensile strain at maximum load (mm/mm)	Tensile extension at maximum load (mm)
#1	4.4	10.6	16.0	350.4	0.4	11.9
#2	4.4	10.6	16.5	360.3	0.4	12.4
#3	4.4	10.6	17.0	370.2	0.4	12.9

CONCLUSION

In conclusion, this study focused on the impact of B100 biodiesel corrosion on carbon steel storage tanks. The corrosion testing results revealed significant weight and thickness loss in the carbon steel specimens after 15 years of immersion in biodiesel. The UTM results indicated a substantial decrease in corrosion resistance following exposure to B100 biodiesel. SEM and EDS analyses revealed the presence of rust compounds on the specimen surfaces. Tensile test results demonstrated the significant influence of B100 biodiesel corrosion on the mechanical properties of the specimens. These findings highlight the substantial impact of B100 biodiesel corrosion on the performance and reliability of carbon steel storage tanks, emphasising the need for further research in developing effective corrosion prevention and mitigation strategies. It can be suggested that future work should focus on

developing more advanced corrosion mitigation and prevention strategies, specifically tailored to the unique challenges posed by biodiesel. It also recommends exploring the efficacy of different materials and coatings, such as glass fibre reinforced polymers (GFRP), to enhance the longevity and reliability of storage tanks instead of a total shell plate replacement. The limitations of the current study include its relatively narrow scope on a specific type of carbon steel and the conditions under which the tests were conducted, which may not fully replicate all operational environments, such as biodiesel blends, exposure time, and temperature. The new knowledge contributed by this study includes detailed insights into the corrosion mechanisms of 15 years old ASME SA 516 Grade 70 carbon steel immersed in a B100 biodiesel environment, highlighting the critical need for industry- and product-specific maintenance and inspection systems.

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INSIGHT INTO FATTY ACID-AMINO ACIDS INTERACTIONS IN LIPASE ENZYME CATALYSIS OF TRANSESTERIFICATION REACTION OF PALM OIL USING DENSITY FUNCTIONAL THEORY B3LYP 6-31G(D,P)

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ABSTRACT

This study elucidates the effects of various triacylglycerol (TAG) fatty acid constituents consist of palmitoyl, oleoyl and linoleoyl on the enzymatic transesterification of palm olein and discusses the synergistic effect between substrates and lipase (amino acid). Quantum mechanical calculations of Gaussian09 and density functional theory (DFT) at the B3LYP 6-31G(d,p) level were employed to evaluate their physical parameters including total electronic energy, high-occupied molecular orbital (HOMO), lowest-unoccupied molecular orbital (LUMO), energy band gap, hardness, softness, global electronegativity, dipole moment and interaction energy. The results showed that the interaction energy is the main contributor to the performance of the triacylglycerol constituents. Significantly higher interaction energy values of the complexes with amino acids suggest a prolonged half-life of the TAG-amino acid systems. The enhanced compatibility between 1,2,3-tripalmitoyl-glycerol (PPP) and amino acids is attributed to the similarity of the dipole moment between these species, resulting in the formation of more stable complexes compared to other combinations. The higher the value of the dipole moment, the more electrically polar the molecule and the more it can polarise the target molecule. These bonds are important to the stabilisation of the transition state of the enzymatic reaction and will have a great impact on overall TAG transformation.

Keywords: DFT, interaction energy, palm oil, transesterification, theoretical approach.

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INTRODUCTION

Due to recent developments in computational calculations, density functional theory (DFT) approaches have become a main tool for calculations involving various compounds and complexes. Because of its efficiency, the DFT/B3LYP approach has typically been chosen to investigate the molecular geometry and other characteristics of chemical compounds. It provides a great balance between chemical precision and computing

expenses. Molecular orbital computation is an aspect of chemistry that is rapidly spreading and has become a useful tool in studying molecular systems prior to their synthesis in the laboratory. It aids in understanding biochemical processes such as enzymatic reactions where it assists with specific properties and leads to the discovery of structure-property-reactivity relationships. As is widely known, the influence of one molecule basis functions augmenting the other molecule causes errors in *ab initio* and DFT calculations of the energetics of intermolecular interactions in complexes. The error arises from the fact that the basic functions of the additional species are typically not included in the calculation of individual molecule energetics. This error known as the basis set superposition

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error (BSSE), leads to an incorrect lowering of the complex's total energy. BSSE occurs when the atoms of the interacting molecules or different parts of the same molecule (intermolecular BSSE) approach one another, thus causing their basic functions to overlap.

Over the last 10 years, there has been a growing application of molecular modelling methods to examine the molecular basis of enzyme catalysis. According to recent advancements in the field of computational calculations over the past few years and the availability of more suitable methods, DFT approaches have been employed as one of the primary methods for calculation on various compounds and complexes. Theoretical methods are employed to determine the physical-chemical properties of compounds. The analysis encompasses the examination of molecular geometry, the high-occupied molecular orbital (HOMO)-lowest-unoccupied molecular orbital (LUMO) energy gap, hardness and total energy. The B3LYP computational method has been employed to optimise the structure of $[\text{Cu}(\text{H}_2\text{PDC})(\text{H}_2\text{O})_2]$ (1) complex at various bases sets, including 6-31G(d), 6-31G*, 6-31G** and 6-31++G** (Haghighi et al., 2022). Analysing the energy difference (energy gap) between the HOMO and LUMO is crucial for understanding the characteristics of molecular electrical transport. Numerous computational studies based on quantum mechanics or molecular mechanics approaches have been applied to lipase-catalysed reactions and have helped to elucidate reaction mechanisms or evaluate the role of catalytic amino acids in influencing bioconversion. Increased demand for biodiesel production proves that biodiesel is highly used and has gained the most attention among biofuels as an alternative to conventional fuel (Muhammad et al., 2021). In the context of edible oil transesterification, utilising lipase enzymes as catalysts provides numerous benefits over traditional chemical reactions. Lipase-catalysed biodiesel production holds significant potential for the advancement of an ecologically sustainable and economically viable fuel origin. Triacylglycerols (TAGs) are the main component of vegetable oils. TAGs are molecules made up of one glycerol molecule and three extended hydrocarbon chains connected to it (Long et al., 2021). The composition of the fatty acids, either saturated

or unsaturated is important as it affects the fuel properties of biodiesel made from vegetable oil (Khan et al., 2019). In the stoichiometric reaction of transesterification, one mole of TAGs reacts with three moles of alcohol in three consecutive steps, resulting in the formation of fatty acid alkyl esters and glycerol, as shown in Figure 1.

A catalyst presence is essential for accelerating the reaction, thereby minimising the formation of intermediate phases, and promoting the efficient formation of the desired product (Figure 2).

Based on the literature, it is found that both edible and non-edible oils have been used as feedstocks to produce biodiesels like palm oil, coconut oil, soybean oil, corn oil, sunflower oil, peanut oil, jatropha oil, karanja oil, linseed oil and cottonseed oil, used cooking oil, tallow fat and microalgal (Singh et al., 2019). In countries that produce the most palm oil like Indonesia, Malaysia, Thailand and Colombia it is used as feedstock for biodiesel (Bautista et al., 2019; Kapor et al., 2017; Zahan & Kano, 2018). Palm oil is an attractive feedstock due to its high yield per hectare and low cost to produce biodiesel (Boonrod et al., 2017). The fatty acid profile for vegetable oil can be very complex; for instance, palm oil constituents are mainly palmitic acid (C16:0) and oleic acid (C18:1) (Table 1). There are almost equal proportions of saturated and unsaturated fatty acids in palm oil which are palmitic acid (39%-48%), oleic acid (36%-44%) and linoleic acid (9%-12%) (Singh et al., 2020).

In the process of converting vegetable oil into biodiesel, *Candida antarctica* Lipase B (CALB) enzyme was discovered to work well with secondary alcohol to produce branched alkyl esters. This lipase was initially isolated from the yeast *C. antarctica* and is a member of the alpha/beta hydrolase-fold family. CALB catalytic activity is attributed to a serine-histidine-aspartate catalytic triad (Li et al., 2010). Enzymatic catalysis works under ambient pressure and mild conditions. Besides, a fundamental advantage of these enzymes is linked to high substrate specificity, selectivity, regioselectivity and enantioselectivity, where little or no side products are formed, enhancing reaction yields. When compared to other conversion methods, biodiesel and glycerol produced via enzymatic conversion have demonstrated a

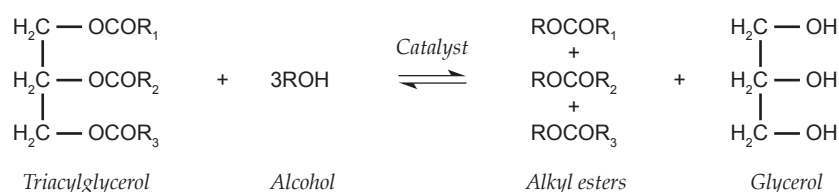


Figure 1. The general chemical reaction depicting the transesterification of TAGs.

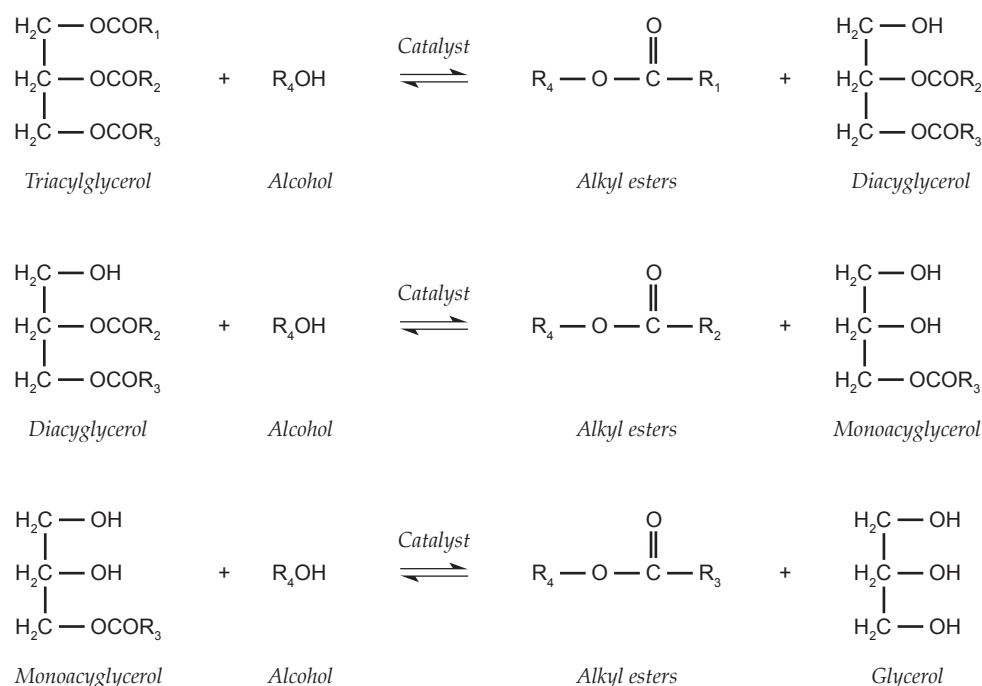


Figure 2. Reversible pathways in the transesterification of vegetable oil form the diacylglycerol and monoacylglycerol intermediates.

TABLE 1. TRIACYLGLYCEROL COMPOSITION (%) OF MALAYSIAN PALM OIL

No double bond (%)		One double bond (%)		Two double bonds (%)		Three double bonds (%)		Four double bonds (%)		Five double bonds (%)	
PPP	6.91	POP	20.02	POO	20.54	PLO	6.59	PLL	1.08	OLL	0.56
		PPO	7.16	PLP	6.36	OOO	5.38	OLO	1.71	LOL	0.14
				OPO	1.86	POL	3.39	OOL	1.76		
				PPL	1.17	OPL	0.61				

Note: P - palmitic acid; O - oleic acid; L - linoleic acid.

greater level of purity. As global energy demands continue to escalate, identifying alternative fuels represents the most effective approach to fulfil this increasing need. It is essential to identify sustainable and environmentally-friendly alternative fuels to support the go green movement and environmental protection. Computes quantum chemistry based on the DFT calculations Gaussian basis set 6-31G** were carried out in these enzyme-substrates complexes indicating three flavonoids (rutin, isoquercitrin and rutin) that are docked in the catalytic cavity of the lipase B from *C. antarctica* (De Oliveira et al. 2010). The CALB enzyme, known for its high enantioselectivity, catalytic activity, substrate versatility and thermal stability, finds widespread use in industrial applications and scientific research. CALB active sites comprise Ser105, His224 and Asp187, with the hydrogen bonds between Ser105 and His224, as well as between Asp187 and His224 playing a crucial role in stabilising the transition state of the enzymatic

reaction (Li et al., 2010). Lipases are the most useful sources of the enzymes used in the processes to produce biodiesel (César et al., 2017). Comparing this type of enzyme to others, microbial enzymes can be produced easily and at a low cost due to their abundance, seasonal insensitivity and ease of development, expansion and immobilisation on inexpensive media. Furthermore, compared to other enzymes, microbial-based lipases are more stable and safe for the environment (eco-friendly) (Ureta et al., 2021).

Nevertheless, there is still limited understanding and exploration of the kinetic and physical behaviour of the enzyme-substrate environment. Previous studies have focused on experimental investigations without establishing a correlation between the physical parameters of TAG constituents and the enzymatic transesterification rate of palm olein. To address this gap, this study employs a quantum mechanical approach utilising Gaussian09 software with a DFT level of *ab initio*

molecular simulation to predict the reaction mechanism between the enzyme (specifically lipase) and the substrate in the transesterification process, for biodiesel production. The advancement of computational calculations, particularly DFT methods, has made them highly suitable for predicting the behaviour of different compounds and complexes.

MATERIALS AND METHODS

The Gaussian09 software package programme was employed to do DFT calculations utilising hybrid B3LYP (Frisch et al., 2016). Quantum mechanical calculations were employed using DFT at B3LYP/6-31 G (d,p) level of theory. DFT/ B3LYP produces more precise values although it is similar in computational cost to Hatree-Fock. According to Zabarnick and Phelps (2006), considering the relatively large reaction systems, the DFT technique produced the best results between computational cost and accuracy. The basis set 6-31G (d,p) was used because it was a reasonable compromise between speed and accuracy. For this study, structure drawings were done with GaussView 5.0 (Dennington et al., 2009). The optimisation was done to study the interaction between amino acids and TAG of palm olein. The DFT approach was used to minimise gradients and provide optimised geometries without any symmetry constraints. Eighteen representative TAG fatty acid constituents consisting of palmitoyl, oleoyl and linoleoyl had been selected representing triacylglycerol in palm

oil and were used as the neutral molecule, while five amino acids [aspartic acid (Asp), histidine (His), isoleucine (Iso), methionine (Met) and serine (Ser)] represent the enzyme's active sites in *C. antartica* were considered (Kamal et al., 2015). In the cluster approach, the characteristics and reaction mechanism of the enzyme are studied by including a small portion of the enzyme in the model.

RESULTS AND DISCUSSION

The molecules of TAG constituents were first optimised to minimum potential energy and then were forced to form complexes with amino acids (active sites). Quantum chemical models of enzyme active sites have been shown to be highly effective for studying different active site properties as well as reaction mechanisms. The active site is the part of the enzyme which the substrate binds to. Active sites can be extremely sensitive to changes in the enzyme's environment because they are finely tuned to facilitate a chemical reaction. Interaction between H_β TAG substituents and amino acids (active sites) was studied theoretically using Gaussian09. Self-consistent field (SCF) is an initial set of orbitals used to generate a new set of orbitals and the process continues until a set of convergence requirements is met. SCF energy and interaction energy readings between H_β TAG substituents and amino acids (active sites) were collected, listed and ranked according to their efficiency in Table 2.

TABLE 2. INTERACTION ENERGY AND DIPOLE MOMENT COMPLEXES OF THE TAG CONSTITUENTS AND AMINO ACIDS

No.	System	E _{int} (uncorr), kJ/mol	E _{int} (corr), kJ/mol	Dipole moment complexes of amino acids with TAG constituents (Debye, D)
1.	PPP + amino acid	-343.925	-307.505	2.7083
2.	OOP + amino acid	-330.437	-293.226	3.6326
3.	POP + amino acid	-326.957	-288.855	2.9827
4.	OPO + amino acid	-292.166	-255.710	2.8498
5.	OLO + amino acid	-292.487	-254.819	3.4720
6.	OPP + amino acid	-292.479	-254.322	2.9560
7.	LPL + amino acid	-292.092	-253.794	3.0931
8.	LPO + amino acid	-290.302	-252.054	2.8766
9.	LPP + amino acid	-289.935	-252.046	3.0081
10.	LLO + amino acid	-288.812	-250.966	3.5016
11.	OLP + amino acid	-287.405	-250.957	3.7051
12.	LOL + amino acid	-288.694	-250.607	3.0487
13.	LLL + amino acid	-288.895	-250.294	3.1448
14.	OOO + amino acid	-288.804	-250.253	3.0456
15.	LLP + amino acid	-287.399	-250.050	3.7485
16.	PLP + amino acid	-287.677	-249.006	3.3503
17.	LOO + amino acid	-286.360	-247.509	2.9831
18.	LOP + amino acid	-284.211	-246.079	3.7310

Note: P - palmitic acid; O - oleic acid; L - linoleic acid.

Results from the calculation showed that the interaction energy for complexes of TAG tripalmitin or 1,2,3-tripalmitoyl-glycerol (PPP) are higher compared to TAG triolein or 1,2,3-trioleoyl-glycerol (OOO) and TAG trilinolein or 1,2,3-trilinoleoyl-glycerol (LLL). The performance of more saturated, TAG PPP has good compatibility with higher interaction energy of -343.925 kJ/mol compared to other TAG ($E_{\text{int LLL}} + \text{amino acid} = -288.895$ kJ/mol and $E_{\text{int OOO}} + \text{amino acid} = -288.804$ kJ/mol). The higher value of interaction energy of the complexes is due to the compatibility of the species in the complexes. As we know, saturated fatty acids are more stable and have better stability than polyunsaturated fatty acids. A high concentration of polyunsaturated fatty acid can enhance the level of unsaturation of the product and can lead to low cetane number values and oxidation stability of biodiesel (Carvalho et al., 2018). Mainly, palm oil is characterised by a higher content of palmitic acid ester (41.8-46.8 wt%), oleic acid ester (37.3-40.8 wt%) and in minor proportion of linoleic acid ester (9.1-11.0 wt%) (Jiménez-Cruz et al., 2021). In this study, the interaction between TAG and amino acids was examined through two distinct methods: Simple energy calculations and counterpoise corrected energy calculations (BSSE). The BSSE-corrected energy calculations are particularly valuable as they account for errors arising from the superposition of finite basis sets

when the species interact. BSSE is related to the incomplete and unbalanced quantum chemical description of the molecules in clusters or complexes. The common method to eliminate the BSSE is the counterpoise (CP) method whereby the BSSE is established using means of mixed basis sets and by performing all the computations again and again, then the error is subtracted recursively from the uncorrected energy. The value of interaction energy was obtained through the computation of interaction energy, and the following formula is shown in Equation (1).

$$\Delta E_{\text{Interaction (corrected)}} = \Delta E_{\text{Interaction (uncorrected)}} + \Delta E_{\text{BSSE}} \quad (1)$$

After correcting for BSSE, the interaction energies exhibit a revised range of -246.079 to -307.505 kJ/mol. The higher value of interaction energy of the complexes is due to the compatibility of the species in the complexes. Saturated fatty acids are generally more stable and have better stability than polyunsaturated fatty acids. The DFT-optimised geometries of the complexes are shown in Figure 3.

Analysis of the calculated interaction energies reveals that the complex between 1,2,3-tripalmitoyl-glycerol (TAG PPP) and amino acids exhibits the highest interaction energy among all the 18 types

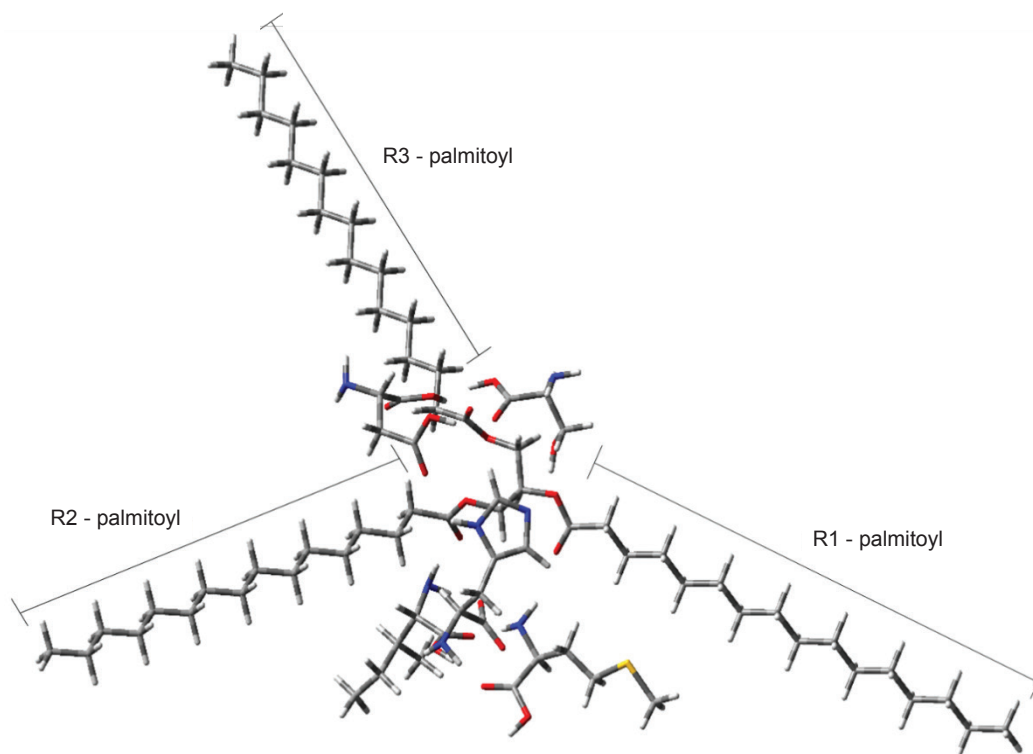


Figure 3. DFT-optimised geometries of the complex 1,2,3-tripalmitoyl-glycerol (TAG PPP) and amino acids (active sites) ($E_{\text{int (corr)}} = -307.505$ kJ/mol).

of TAGs, with a value of -307.505 kJ/mol. This is followed by the complexes of 1,2-dioleoyl-3-palmitoyl-glycerol (TAG OOP) and amino acids (-293.226 kJ/mol) and 1,3-dipalmitoyl-2-oleoyl-glycerol (TAG POP) and amino acids (-288.855 kJ/mol). The interaction energies of the remaining complexes are approximately around -250 kJ/mol. The complexes formed between TAG PPP, TAG OOP and TAG POP with amino acids exhibited remarkable compatibility and increase stability compared to the other complexes studied. This enhanced compatibility is expected to result in synergistic effects between the substrate (PPP) and the enzyme (amino acid). Palmitic and oleic acids are the main fatty acids in palm oil. The observed high compatibility between PPP and amino acids can be attributed to the similarities in dipole moments between the species within the complex. Specifically, the dipole moments of PPP ($\mu_{PPP} = 2.7083$ D) and the amino acids ($\mu_{Iso} = 1.4702$ D; $\mu_{Asp} = 1.9976$ D; $\mu_{Ser} = 2.4280$ D; $\mu_{Met} = 3.0373$ D; $\mu_{His} = 8.3957$ D) align closely, facilitating stronger interactions and a more stable complex formation. As a result, the PPP molecule forms a more stable complex with amino acids compared to the other complexes studied.

A higher dipole moment value suggests that the molecule has higher electrical polarity and can polarise the target molecule considerably. This prediction will contribute to the interaction

of the complexes of TAG constituents and amino acids. These findings align with previous study which has demonstrated how the reaction rates of protease-catalysed transesterification reactions increase with decreasing chain length of the acyl donor and increasing size of the acyl acceptor. Specifically, previous research has indicated that *C. antarctica* lipase exhibits higher activity towards short and medium chain fatty acids, while its activity decreases for long chain fatty acids (Pedersen et al., 2002). The findings reveal that the complex formed between TAG POL and amino acids displays the lowest interaction energy (-246.079 kJ/mol), corresponding to its lowest dipole moment of 3.7310 D (Figure 4). The hydrolysis of triacylglycerols by lipases involves a two-step catalytic mechanism: Acylation and deacylation. These reactions are facilitated by the presence of a catalytic triad consisting of three crucial amino acid residues: Ser, His and Asp.

The quantum chemical properties of molecules enable us to determine their energy states utilised in molecular electronics. Kinetic energy is the result of molecules moving in space which is derived from chemical bonds. In addition to the chemical reaction, which is the rearranging of atoms, other chemical energy sources include the vibrations and rotations of molecules. The chemical reactivities of TAG molecules

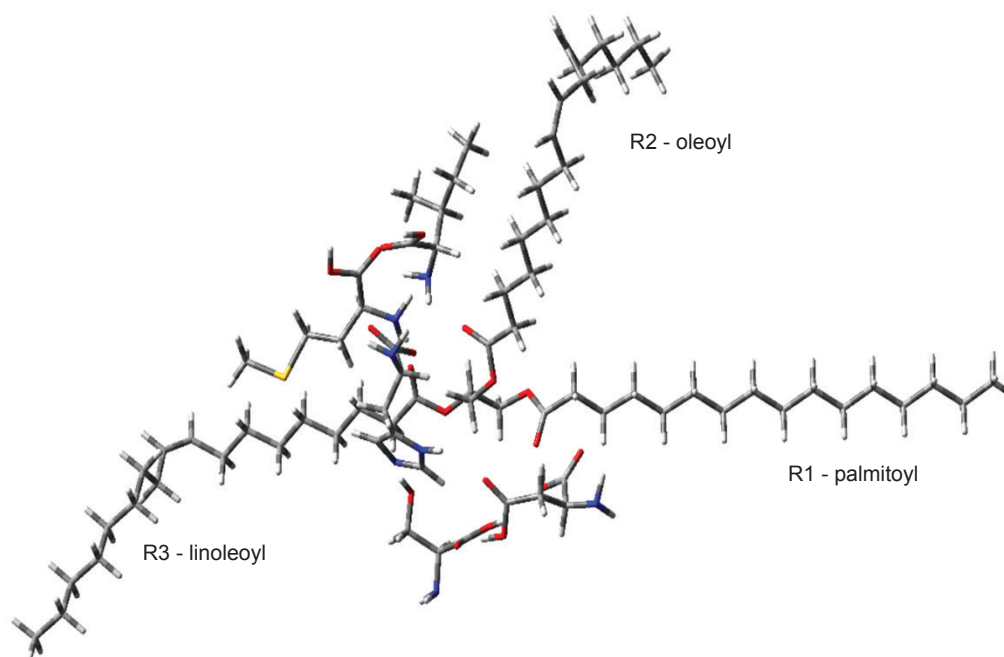


Figure 4. DFT-optimised geometries of the complex 1-palmitoyl-2-oleoyl-2-linoleoyl-rac-glycerol (TAG POL) and amino acids (active sites) ($E_{int(corr)} = -246.079$ kJ/mol).

and complexes were accessed through various electron density-based molecular properties. These properties include E_{HOMO} , E_{LUMO} , chemical hardness, chemical softness, and global electronegativity as tabulated in Table 3. HOMO and LUMO energies are quantum mechanical descriptors crucial in understanding a wide range of chemical reactions which indicate the molecules' electron affinity and ionisation potential, respectively. These energies serve as indicators of the molecule's reactivity and can predict the regions of atomic electrophiles and nucleophiles (Rebaz et al., 2020). The HOMO value provides insight into the molecule's ability to donate electrons. In this study, the HOMO-LUMO frontier orbital compositions for TAG constituents and complexes of TAG constituents with amino acids were calculated using the DFT B3LYP 6-31G (d,p) model chemistry.

Table 3 also shows that the HOMO values of TAG constituents are highly similar, with an average value of approximately -0.23 a.u., except for PPP, which exhibits a slightly lower value of -0.26468 a.u. Higher HOMO values indicate a greater tendency of electron transfer to acceptor molecules (Sayin & Karakas, 2013). The LUMO values of the TAG constituents were examined, and it was observed that OOP and PPO exhibited the lowest value of 0.00090 atomic units compared to the other constituents. It is known that only the TAG constituent palmitoyl (PPP) and TAG constituents containing both palmitoyl and oleoyl groups display LUMO values around 0.001 a.u., while TAG constituents consisting of linoleoyl exhibits LUMO values exceeding 0.007 a.u. This finding aligns with the research by Keypour et al. (2015), which suggests that a lower value for the LUMO orbital energy indicates a higher propensity to accept electronic density. Furthermore, it is well-established that the binding ability of an inhibitor to its target molecule increases with an increase in the HOMO energy and a decrease in the LUMO energy of the complex. An additional important parameter to consider is the energy band gap between the HOMO and LUMO orbitals of the complexes. The energy band gap serves as an indicator of the chemical stability of a molecule, as it represents the difference in energy between these frontier orbitals. In this study, the energy band gaps were calculated for both the TAG constituents alone and the complexes formed with amino acids, using the difference in HOMO and LUMO energies obtained from the completed optimisation process. Generally, the energy band gaps of the TAG constituents alone exhibit higher values compared to the complexes formed with amino acids. Specifically, the energy band gap of PPP is computed as 0.26578 atomic units, while OOP and PPO share the same value of 0.23336

atomic units. This indicates that PPP has a higher energy band gap compared to OOP and PPO, which possess the lowest energy band gap values. Prior research by Rebaz et al. (2020) has indicated that a lower energy band gap corresponds to easier electron donation and greater reactivity. Upon combination with amino acids, the energy band gaps of the complexes differ from those of the TAG constituents alone. Notably, the complex of PPO with amino acids exhibits the highest energy band gap with a value of 0.19710 atomic units, while the complex of LLO and amino acids displays the lowest energy band gap with a value of 0.19521 atomic units.

In addition to the energy band gap (E_{gap}), the HOMO and LUMO values offer valuable insights into other important parameters such as chemical hardness (η), softness (σ) and electronegativity (χ). The chemical hardness for a large HOMO-LUMO gap means a hard molecule which is related to the stability of the molecule. A molecule with a large HOMO-LUMO gap indicates that it is less reactive, while a small HOMO-LUMO gap refers to a soft molecule, which is more reactive. PPP exhibits a hardness value of 0.13, slightly higher than other molecules. Chemical hardness, the measure of conversion efficiency, apparently looks like other complexes with a value of 0.12. Intriguingly, upon complex formations between TAG and amino acids, the hardness values of the molecules decrease from 0.12-0.09, while the softness values increase from approximately 8.30-10.20. Among the molecules, PPP exhibits the lowest softness value of 7.53. This implies that soft molecules, characterised by smaller HOMO-LUMO gaps, possess enhanced reactivity compared to hard molecules, particularly when electron transfer or rearrangement is necessary for the reaction. Soft molecules are highly polarisable, with strong chemical reactivities and low kinetic stabilities (Madkour & Elshamy, 2016). Similarly, global electronegativity accounts for the electronic interactions of molecules with their biological targets. The ability of an atom or a functional group to draw electrons or electron density towards itself is known as global electronegativity. Global electronegativity serves as an important parameter for quantifying the affinity of a species to attract electrons, providing valuable insights into the chemical behaviour of molecules [Equation (2)]. The highest global electronegativity value is obtained for complexes of OLO and amino acids (0.11597), whereas it is observed to be the lowest for complexes of PPP and amino acids (0.11447) as listed in Table 3.

$$\text{Global electronegativity, } \chi = \frac{-(E_{\text{HOMO}} + E_{\text{LUMO}})}{2} \quad (2)$$

TABLE 3. HOMO, LUMO OF TAG CONSTITUENTS AND COMPLEXES OF AMINO ACIDS WITH TAG CONSTITUENTS AND OTHER PARAMETERS CALCULATED BY HOMO AND LUMO

No.	Symbol	HOMO (a.m.u)	LUMO (a.m.u)	Energy band gap (a.u) (E_{gap})	Hardness (η)	Softness (σ)	Global electronegativity (χ)
1.	PPP	-0.26468	0.00110	0.26578	0.13289	7.52502	0.13179
	PPP + amino acid	-0.21293	-0.01601	0.19692	0.09846	10.15641	0.11447
2.	OPO	-0.23428	0.00793	0.24221	0.12111	8.25730	0.11318
	OPO + amino acid	-0.21400	-0.01727	0.19673	0.09837	10.16622	0.11564
3.	OOO	-0.23422	0.00795	0.24217	0.12109	8.25866	0.11314
	OOO + amino acid	-0.21382	-0.01747	0.19635	0.09818	10.18589	0.11565
4.	POL	-0.23420	0.00693	0.24113	0.12057	8.29428	0.11364
	POL + amino acid	-0.21325	-0.01663	0.19662	0.09831	10.17191	0.11494
5.	LLP	-0.23391	0.00719	0.24110	0.12055	8.29531	0.11336
	LLP + amino acid	-0.21392	-0.01690	0.19702	0.09851	10.15125	0.11541
6.	PLO	-0.23391	0.00745	0.24136	0.12068	8.28638	0.11323
	PLO + amino acid	-0.21394	-0.01695	0.19699	0.09849	10.15280	0.11545
7.	PPL	-0.23388	0.00809	0.24197	0.12098	8.26549	0.11289
	PPL + amino acid	-0.21368	-0.01688	0.19680	0.09840	10.16260	0.11528
8.	OLO	-0.23379	0.00762	0.24141	0.12071	8.28466	0.11308
	OLO + amino acid	-0.21418	-0.01776	0.19642	0.09821	10.18226	0.11597
9.	LLL	-0.23338	0.00680	0.24018	0.12009	8.32709	0.11329
	LLL + amino acid	-0.21275	-0.01726	0.19549	0.09775	10.23070	0.11501
10.	LOL	-0.23334	0.00701	0.24035	0.12018	8.32119	0.11317
	LOL + amino acid	-0.21383	-0.01740	0.19643	0.09822	10.18174	0.11562
11.	LPL	-0.23322	0.00732	0.24054	0.12027	8.31463	0.11295
	LPL + amino acid	-0.21372	-0.01733	0.19639	0.09819	10.18382	0.11553
12.	LPO	-0.23307	0.00742	0.24049	0.12025	8.31635	0.11283
	LPO + amino acid	-0.21392	-0.01727	0.19665	0.09833	10.17035	0.11560
13.	PLP	-0.23296	0.00765	0.24061	0.12031	8.31221	0.11266
	PLP + amino acid	-0.21405	-0.01711	0.19694	0.09847	10.15538	0.11558
14.	LLO	-0.23293	0.00706	0.23999	0.11999	8.33368	0.11294
	LLO + amino acid	-0.21273	-0.01752	0.19521	0.09761	10.24538	0.11513
15.	OOL	-0.23288	0.00787	0.24075	0.12038	8.30737	0.11251
	OOL + amino acid	-0.21360	-0.01717	0.19643	0.09822	10.18174	0.11539
16.	POP	-0.23263	0.00108	0.23371	0.11686	8.55761	0.11578
	POP + amino acid	-0.21360	-0.01697	0.19663	0.09832	10.17139	0.11529
17.	OOP	-0.23246	0.00090	0.23336	0.11668	8.57045	0.11578
	OOP + amino acid	-0.21324	-0.01640	0.19684	0.09842	10.16054	0.11482
18.	PPO	-0.23246	0.00090	0.23336	0.11668	8.57045	0.11578
	PPO + amino acid	-0.21387	-0.01677	0.19710	0.09855	10.14713	0.11532

Note: P - palmitic acid; O - oleic acid; L - linoleic acid.

CONCLUSION

In conclusion, several molecular properties can be utilised after the optimisation such as SCF energy, molecular geometry, dipole moment, HOMO and LUMO, energy band gap, hardness, softness, global electronegativity and interaction energy (complexes). Due to the BSSE, the interaction energies exhibit a revised range of -246.079 to -307.505 kJ/mol. Interaction energies reveal that the complex between TAG PPP and amino acids exhibits the highest interaction energy among all the eighteen types of TAGs, while other TAGs have remaining complexes that are approximately around -250.000 kJ/mol. A variety of variables

affect biodiesel characteristics, such as the number and position of the double bonds, the degree of unsaturation, molecular weight, and hydroxyl branch groups. Complex PPP molecules form a more stable complex with amino acids than the other complexes studied. The performance of more saturated fatty acid constituents such as TAG PPP is very effective compared to other TAG constituents. These findings align with previous research that has demonstrated how the reaction rates of protease-catalysed transesterification reactions increase with decreasing chain length of the acyl donor and increasing size of the acyl acceptor. The changes in the dipole moment are associated with the hydrogen bonding between

the TAG constituents and amino acids, which is the hydrogen bond between Ser and His and the hydrogen bond between Asp and His.

Much work is still required to understand the kinetics and reaction mechanisms for improving catalyst activity. Since transesterification is well-known as the most efficient biodiesel processing methodology, new catalytic transesterification technology that can consolidate transesterification processes is essential to reduce the gap between the cost of production and biodiesel price. Furthermore, extensive collaboration should be considered to explore the synergistic relationship towards the advancement of biodiesel production in terms of designing and developing of effective catalyst that is suitable at low temperatures, with improved regeneration and high stability. Therefore, we reasoned that to understand the transesterification pathways and intricate transesterification mechanism of the oil with enzymes, comprehensive theoretical calculations of the transesterification would be necessary.

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EFFECTS OF DRYING TECHNIQUES ON PHENOLICS CONTENT AND ANTIOXIDANT ACTIVITY OF WATER-SOLUBLE PALM FRUIT EXTRACT

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ABSTRACT

Water-soluble palm fruit extract (WSPFE) is produced from the vegetation liquor of palm oil milling. It comprises water-soluble phenolic acids including protocatechuic acid (PCA), *p*-hydroxybenzoic acid (*p*-HBA) and three isomers of caffeoyl-shikimic acid (3-O-CSA, 4-O-CSA and 5-O-CSA). WSPFE has been shown to exhibit a range of bioactive properties both *in vitro* and *in vivo*. The present study investigated the effects of freeze and spray drying, on the phenolic content, antioxidant capacity, organoleptic properties, hygroscopicity and moisture content of WSPFE. To quantify the concentrations of PCA, *p*-HBA and 3-O-CSA within WSPFE, an isocratic HPLC-UV method was developed and validated. The HPLC method employed a mobile phase system consisting of 20% methanol and 80% water, with a flow rate of 0.8 mL min⁻¹, a running time of 40 min and UV detection at 280 nm. Comparative analysis revealed that freeze drying maintained a marginally higher retention of phenolic compounds. Spray drying resulted in WSPFE with lower hygroscopicity and moisture content. Spray drying showed a slightly higher concentration of PCA ($23 \pm 0.3 \mu\text{g mL}^{-1}$) compared to freeze drying ($22 \pm 0.1 \mu\text{g mL}^{-1}$). No significant differences were found in the concentrations of *p*-HBA acid and 3-O-CSA between freeze drying and spray drying.

Keywords: HPLC-UV, phenolics, quantification, validation, water-soluble palm fruit extract.

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INTRODUCTION

Palm oil is extracted from the pulp of the fruit of *Elaeis guineensis*, a tropical monocotyledon tree (Sundram et al., 2003). During the palm oil extraction process, large volumes of vegetation liquor are produced as a by-product which contains valuable hydrophilic phenolics (Sambanthamurthi et al., 2011).

The Malaysian Palm Oil Board (MPOB) has implemented environmentally friendly techniques, without the use of solvents, to recover water-soluble palm fruit extract (WSPFE) from this vegetation liquor of palm oil production. WSPFE has exhibited numerous *in vitro* and *in vivo* bioactive effects. These effects include anti-diabetic, anti-cancer, anti-hypertensive, anti-obesity, cardioprotective and neuroprotective properties (Leow et al., 2013, 2022a). Moreover, phase I clinical trials conducted on healthy subjects have confirmed the safety of WSPFE (Fairus et al., 2018). The primary bioactive components of WSPFE are phenolic acids, namely protocatechuic acid (PCA), *p*-hydroxybenzoic acid (*p*-HBA) and three distinct isomers of caffeoylshikimic acid (3-O-CSA, 4-O-CSA and 5-O-CSA) (Leow et al., 2022a). Potential health benefits of WSPFE could be attributed to its phenolic content (Goleniowski et al., 2013; Sambanthamurthi et al., 2011; Saxena et al., 2012; Syarifah-Noratiqah et al., 2019).

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After the extraction procedures, plant extracts are usually dried using different techniques to remove the extraction solvents and to get a dry mass of the plant extract. The drying process is also performed to enhance the shelf life and stability of the plant extracts. However, the quality and quantity of the dried plant extracts are influenced by the drying technique and conditions. The drying process could also affect the concentration and activity of bioactive compounds present in the plant extracts (Belwal et al., 2022; Nakbanpote et al., 2019). In addition, the drying process might affect the physicochemical and organoleptic properties of the dry products. Thus, it is necessary to optimise the drying conditions for each plant extract. Energy sources, physical properties of the product, duration of the drying process and the purpose of the drying should be considered before choosing the optimum drying method (Langford et al., 2018).

Plant extracts could be dried by different techniques such as spray drying, freeze drying, oven drying, vacuum drying, fluid bed drying and sun-drying (ElNaker et al., 2021; Singh et al., 2013). Spray drying is a widely used method to dry plant extracts and natural products, particularly in the food, nutraceutical and pharmaceutical industries. It is a simple, fast and cost-effective technique that is ideal for large-scale productions (Gallo et al., 2015). On the other hand, freeze drying, also known as lyophilisation, is a common drying technique for plant extracts that contain thermolabile bioactive compounds. This method operates at low temperatures, making it suitable for such compounds. Before drying, the extract solution should be frozen and aqueous. During freeze drying, the frozen product is dehydrated under a vacuum sublimation process (Belwal et al., 2022; Pudziuvyte et al., 2020).

The primary objective of this research was to determine the optimal drying method for WSPFE by conducting a comparative analysis of the phenolics content and properties of the extract after freeze-drying and spray-drying. Freeze-drying and spray-drying were selected as the preferred methods for drying WSPFE due to their ability to maintain the integrity of bioactive compounds and produce high product quality. Freeze drying involves sublimation at low temperatures which is suitable to dry heat-sensitive compounds. Meanwhile, spray drying produces a fine, uniform powder with excellent flowability and minimal thermal degradation of compounds (Shishir & Chen, 2017; Sollohub & Cal, 2010). These techniques are studied over traditional methods such as hot air drying, which may compromise bioactive compounds due to high temperatures (Hii et al., 2009), or vacuum and microwave drying, which can result in uneven drying and potential loss of volatile substances (Mujumdar, 2014; Zhang et al., 2006).

Freeze and spray-dried WSPFE were investigated previously to evaluate their potential anti-diabetic mechanism (Leow et al., 2022b). However, these two drying methods were not compared regarding their ability to retain bioactive phenolic compounds. The current study aims to fill this gap by evaluating and comparing the effects of freeze drying and spray drying on the total phenolic content, major phenolic constituents, product quality and antioxidant properties of WSPFE.

Considering the potential development of WSPFE into nutraceutical or pharmaceutical products, accurate quantification of phenolic acids is important for ensuring product quality and efficacy. High-performance liquid chromatography (HPLC) is a widely used method for phenolic separation and quantification due to its high resolution and sensitivity (Çayan et al., 2020; Kalili & De Villiers, 2011; Spáčil et al., 2008). HPLC analysis of WSPFE was previously carried out using a gradient HPLC method which involved methanol, acetonitrile and sodium sulphate as a mobile phase system and a running time of 60 min (Sambanthamurthi et al., 2011). For this reason, a novel and efficient isocratic HPLC-UV method was developed and validated to accurately quantify the concentrations of PCA, *p*-HBA and 3-O-caffeoylshikimic acid (3-O-CSA) in freeze and spray-dried WSPFE. Isocratic elution involves using a constant mobile phase composition throughout the entire process, which simplifies method development and operation. This consistency can result in more reliable retention times and peak areas, making it easier to attain consistent results across various runs and batches of samples (Snyder et al., 2011). The selection of *p*-HBA and 3-O-CSA was based on their high abundance in WSPFE, as indicated by previous studies (Sambanthamurthi et al., 2011). PCA, on the other hand, has demonstrated significant antioxidant properties even at low concentrations, along with various therapeutic effects documented in different studies (Kakkar & Bais, 2014; Semaming et al., 2015).

The method validation process was carried out according to the ICH Expert Working Group (2005) guidelines for analytical procedures including system suitability, accuracy, specificity, precision, robustness, linearity, limits of detection (LOD) and limits of quantification (LOQ) (Branch, 2005). This proposed method provides a simple, cost-effective and practical approach for detecting phenolic compounds with minimal usage of organic solvents and acids. To the best of our knowledge, no previous isocratic reversed-phase HPLC (RP-HPLC) method has been presented for measuring phenolics in WSPFE. Besides, the impact of freeze and spray drying techniques on the quality and quantity of WSPFE was not reported previously.

MATERIALS AND METHODS

Materials and Chemicals

Analytical standards of *p*-HBA and PCA were obtained from Thermo Fisher Scientific (USA), while 3-O-CSA was obtained from ChemFaces (China). Methanol (HPLC grade), dimethyl sulphoxide (DMSO), and formic acid were procured from Thermo Fisher Scientific (USA). The WSPFE samples were provided by MPOB in liquid form and were frozen and spray-dried before analyses.

Freeze Drying of WSPFE

WSPFE solution (200 mL) was passed through a 0.45 µm filter paper and collected in a container that was weighed beforehand. The collected solution was then frozen at -20°C for two days. After that, the frozen sample was lyophilised using a freeze dryer (Labconco, USA) at -50°C for three days. The vacuum pressure was kept at 0.06 mBar during the process. The dried sample was collected from the drying vessel and kept in a freezer before the analysis procedures.

Spray Drying of WSPFE

The process of spray drying WSPFE involved using a mini-spray dryer (Büchi B-290, Büchi, Flawil, Switzerland). A 200 mL solution of WSPFE was first filtered using a 0.45 µm filter paper and then used as a feeding solution for spray drying. The feeding solution was fed into the drying chamber at a rate of 2 mL min⁻¹ while maintaining an inlet temperature of 85°C and an outlet temperature of 75°C. The aspirator value was kept constant at 100%. Dry WSPFE was obtained from the collection vessel and kept in a desiccator before analyses.

Instrumentation and Chromatographic Conditions

The chromatographic analysis was conducted using a Shimadzu Prominence HPLC System (Shimadzu Corporation, Kyoto, Japan), which includes a dual pump system (LC-20AD), an autosampler, a column oven, an on-line degasser and a UV-Vis detector. Data processing and analysis were performed using the Shimadzu Lab-Solution software. Chromatographic separation of the phenolics was achieved on an RP-HPLC C-18 ODS column (200 × 4.6 mm) (Thermo Fisher Scientific, USA), maintained at 25°C. The flow rate was set at 0.8 mL min⁻¹, and the injection volume was 20 µL with UV detection occurring at 280 nm. The mobile phase consisted of a mixture of 80:20 deionised (DI) water and methanol (v/v), with 0.1% formic acid added to adjust the pH to 2.5 in the aqueous phase. Prior to use, mobile phase solutions were filtered

through a 0.45 µm Millipore filter and degassed by ultrasonication for 20 min. The total runtime for each chromatographic run, for both the standard solutions and WSPFE samples were 40 min.

Standards and Sample Preparation

Stock solutions of the analytical standards were prepared by dissolving 1 mg of *p*-HBA and PCA in 1 mL methanol and 1 mg of 3-O-CSA in 1 mL dimethyl sulfoxide (DMSO). Solutions of phenolics were diluted using a 1:1 v/v ratio of deionised water and methanol. A series of dilutions were then made to produce phenolic standard solutions at various concentrations, specifically 6.25, 12.50, 25.00, 50.00 and 100.00 µg mL⁻¹. Samples of WSPFE, both freeze-dried and spray-dried, were dissolved in deionised water to achieve a concentration of 5 mg mL⁻¹. These solutions were subsequently filtered through a 45 µm filter prior to being injected. Three injections were carried out for each WSPFE sample, and the average peak absorbance was recorded.

Method Validation

The specificity of the method was assessed by subjecting the WSPFE solution to both acid and alkaline hydrolyses using 0.1 M hydrochloric acid (HCl) and 0.1 M sodium hydroxide (NaOH), respectively. Additionally, various parameters were monitored to confirm the system's suitability for the intended analysis, including the tailing factor of the peaks, the number of theoretical plates (NTP) and the resolution between peaks. An average of twelve replicates of each phenolic standard solution was used to measure system suitability. Linearity was established using three replicates across six concentrations of a standard mixture of phenolic acids (*p*-HBA, PCA, 3-O-CSA). Calibration curves were created by plotting the ratio of peak area against the concentrations of the phenolics (6.25, 12.50, 25.00, 50.00, 100.00 µg mL⁻¹). Linear regression analysis was performed using Microsoft Excel to determine the intercept, slope and correlation coefficient (*r*²) for each phenolic compound. Standard solutions were prepared within the concentration range observed in WSPFE samples. The LOD and LOQ were calculated based on the signal-to-noise (S:N) ratio of the lowest concentration samples, with LOD expected to produce a S:N ratio of 3-10, while LOQ should fall within a S:N ratio of 10-20.

The precision of the developed method was evaluated both intra-day and inter-day for three phenolic standard solutions at different concentrations. For intra-day precision, the relative standard deviation (RSD%) was measured across three replicates at three concentrations (6.25, 25.00, 100.00 µg mL⁻¹) within a single day, resulting in nine total measurements, in accordance with ICH

guidelines for the development of analytical methods (Kazusaki et al., 2012). To assess inter-day precision, the three concentrations of the phenolics (6.25, 25.00, 100.00 $\mu\text{g mL}^{-1}$) were tested three times per day over four days. The accuracy of the method was verified by calculating the average recovery of phenolic acid standards that were deliberately added to the WSPFE sample at the same three concentrations. Furthermore, accuracy was also tested by spiking these concentrations into distilled water to serve as a blank sample. Three replicates were injected for each concentration. The recovery of phenolics was calculated using the following Equation (1).

$$\text{Recovery} = \frac{\text{Conc. spiked sample} - \text{Conc. unspiked sample}}{\text{Conc. of added standard}} \times 100 \quad (1)$$

Note: Conc. – concentration.

The robustness of the method was tested through slight alterations in the methodological conditions. The robustness was evaluated by analysing three different concentrations of the phenolic solutions using altered mobile phase ratios of 22:78 and 18:82 methanol to water (v/v), and by adjusting the pH of the mobile phase from the standard 2.5 to both 2.3 and 2.7. Additionally, the experiments were conducted at temperatures of 23°C and 27°C, deviating from 25°C. The peak responses under these varied conditions were recorded and compared with those obtained under the standard conditions to assess any impacts.

Organoleptic Properties of Freeze and Spray-Dried WSPFE

The sensory attributes including taste, colour, odour and appearance of the dried WSPFE samples were observed and recorded. Taste and odour were tested by human sensory evaluation using tasting by tongue and smelling.

Antioxidant Effects of Freeze and Spray-Dried WSPFE

The antioxidant properties of the dry WSPFE samples were evaluated using the DPPH free radical scavenging method. To prepare the stock solution, 2.4 mg of DPPH was dissolved in 10.00 mL of methanol. This stock was then diluted with an additional 45.00 mL of methanol to create the working solution. A sample of the extract, 0.15 mL, was mixed with 2.85 mL of the working solution. Methanol served as both the blank and the negative control, while ascorbic acid (vitamin C) was employed as the positive control. The samples were placed in a 96-well plate and incubated in the dark at room temperature for 1 hr. Absorbance readings

were taken at a wavelength of 515 nm using a UV-vis microplate reader, with six replicates recorded for each sample. The inhibition of DPPH was calculated using the following Equation (2).

$$\frac{(\text{Abs control} - \text{Abs sample})}{(\text{Abs control})} \times 100 \quad (2)$$

Note: Abs - absorbance.

Phenolic Content of Freeze and Spray Dried WSPFE

The total phenolic content of the dry WSPFE samples was determined using the Folin-Ciocalteu method (Singleton & Rossi, 1965). For each analysis, 100 μL of sample was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7.00 mL of distilled water. After allowing the mixture to stand for 5 min, 1.5 mL of 7.5% sodium carbonate solution was added. The resulting mixture was then left to incubate for 2 hr in the dark at room temperature before measurement. Each sample was tested in six replicates. A calibration curve was established using serial dilutions of gallic acid (0.50, 0.25, 0.10, 0.050, 0.025 mg mL^{-1}). Methanol served as the blank for the experiments. Absorbance was measured at 765 nm using a UV-VIS microplate reader. Total phenolic content (TPC) was calculated following the Equation (3).

$$\text{TPC} = \frac{C \times V}{M} \quad (3)$$

where, C is the calculated concentration from the calibration curve, V is the volume used in mL and M is the extract weight in g.

Hygroscopicity of Freeze and Spray-Dried WSPFE

Hygroscopicity was assessed using a method outlined in the European Pharmacopoeia (Council of Europe, 2013). Initially, an empty glass vessel was weighed. Subsequently, a dry WSPFE sample was added to this vessel, which was then weighed again. The vessel containing the sample was placed in a stability chamber (Clean Tech, Malaysia) maintained at $25 \pm 1^\circ\text{C}$ and $80 \pm 2\%$ relative humidity. After 24 hr in the stability chamber, the vessel was weighed once more. The percentage increase in mass was calculated using the Equation (4).

$$\text{Hygroscopicity} = \frac{m_3 - m_2}{m_2 - m_1} \times 100 \quad (4)$$

where, m_1 represents the weight of the empty glass vessel, m_2 is the combined weight of the glass vessel and the sample at the start, and m_3 is the combined weight after exposure to humidity for 24 hr.

Drying-induced Loss of Freeze and Spray Dried WSPFE

Moisture content was evaluated through the loss on drying method. A gram of the dry WSPFE samples was placed into a glass container that had been pre-weighed. This container was then positioned inside an oven and maintained at 100°C for 3 hr. After heating, the sample was weighed again, and the moisture content was determined by calculating the decrease in weight of the sample.

RESULTS AND DISCUSSION

HPLC Method Development and Optimisation

Various mobile phase compositions and concentrations were tested to refine the separation of phenolic acids. Initial trials using acetonitrile and water, with and without additives such as formic acid and acetic acid, yielded poor peak shape and resolution. Subsequently, methanol was experimented in varying proportions of 20:80, 50:50 and 80:20 with water. The best separation and peak resolution, exceeding a resolution factor of 2, was achieved with a 20:80 methanol to water ratio, incorporating 0.1% formic acid at a pH of 2.5. Moreover, the optimal wavelength for detecting the phenolic acid standards was identified as 280 nm after testing at wavelengths ranging from 200–320 nm. A temperature setting of 25°C was selected to prevent potential thermal degradation

of the phenolic acids. The most effective flow rate determined was 0.8 mL, maintaining a stable column pressure of 12.5 MPa and facilitating efficient separation. With an isocratic mobile phase system of 20% methanol and 80% water, this method represents a simple approach with reduced solvent consumption compared to the previously used gradient RP-HPLC method for WSPFE analysis. The only reported method for WSPFE analysis has used a gradient system composed of a mixture of methanol, acetonitrile and sodium sulphate with 60 min of running time (Leow et al., 2022a; Sambanthamurthi et al., 2011), while the currently developed method involves 40 min of running time. The isocratic system is reproducible with less complex equipment and does not require precise gradient mixing capabilities, which reduces the cost and maintenance (Habyalimana et al., 2017; Snyder et al., 2011).

As shown in Figure 1, PCA eluted first with a retention time between 6.9 and 7.0 min, followed by *p*-HBA at 11.5 min, and 3-O-CSA at 26.6 min. The separation and elution order of the three phenolics aligns with that of the previously reported method. The intensity of the PCA peak in the WSPFE sample is low which is due to the low concentration of the compound in the extract compared to *p*-HBA and 3-O-CSA (Sambanthamurthi et al., 2011). PCA eluted earlier than *p*-HBA, possibly due to higher polarity and less hydrophobicity (Robbins, 2003), while 3-O-CSA, a more complex compound, eluted at the end likely due to higher molecular size and more possible interactions with the stationary phase.

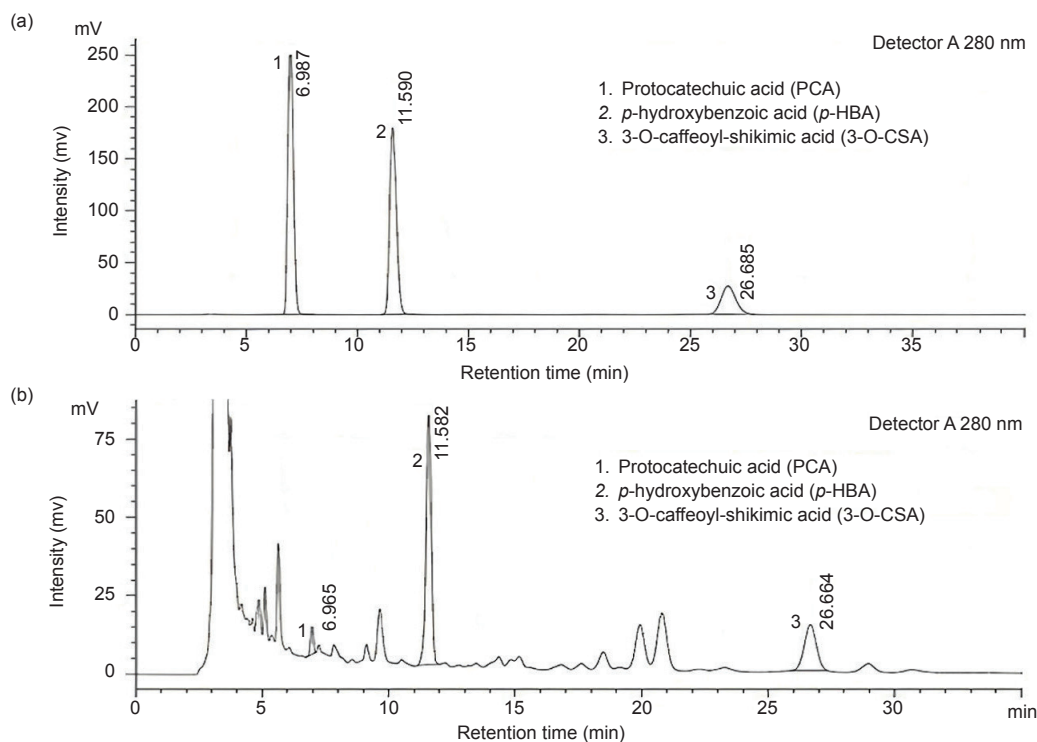


Figure 1. Chromatograms of PCA, *p*-HBA and 3-O-CSA in (a) standard solutions, and (b) WSPFE sample at 280 nm.

Method Validation

Specificity and system suitability. Specificity is described as the capacity of an analytical method to distinguish and measure compounds distinctly in the presence of other potential analytes or impurities (Branch, 2005). This method's specificity was proven by successfully separating and distinguishing the peaks in the standard solution and WSPFE sample despite the existence of other compounds. Additionally, after undergoing acid and alkaline hydrolyses using hydrochloric acid and sodium hydroxide, PCA, *p*-HBA and 3-O-CSA were separated and detected clearly, further confirming the method's specificity (Figure 2). System suitability tests were performed to confirm the effectiveness of the equipment, electronics and the designated analytical method under specified conditions (Aminu et al., 2019; Shabir, 2003). Table 1 presents the system suitability parameters for the three phenolic compounds. All compounds met the recommended criteria with a resolution greater than 2, the number of theoretical plates (NTP) exceeding 2,000 and a tailing factor less than 2.

Linearity, calibration curves and sensitivity.

Linearity indicates a proportional response between the concentration of analytes and their analytical signals (Kazusaki et al., 2012). The correlation coefficient (r^2) for each phenolic standard was derived from the regression lines of their calibration curves. These curves were established by plotting concentrations ranging from 6.25-100.00 $\mu\text{g mL}^{-1}$ against the mean peak areas. Figure 3 illustrates the calibration curves for each phenolic compound. Table 2 confirms that the correlation coefficients for all three compounds were ≥ 0.999 , demonstrating robust linearity within the tested range. LOD, represents the smallest concentration of an analyte detectable by a specific method, whereas LOQ, is the minimum concentration at which an analyte can be reliably quantified with suitable precision and accuracy (Aminu et al., 2019; Shabir, 2003). Table 2 reveals that the LODs for PCA, *p*-HBA and 3-O-CSA were 0.16, 0.22 and 1.65 $\mu\text{g mL}^{-1}$ respectively, while the LOQs were 0.48, 0.65 and 5.00 $\mu\text{g mL}^{-1}$. These results confirm the method's sensitivity for detecting and quantifying these phenolics at low concentrations.

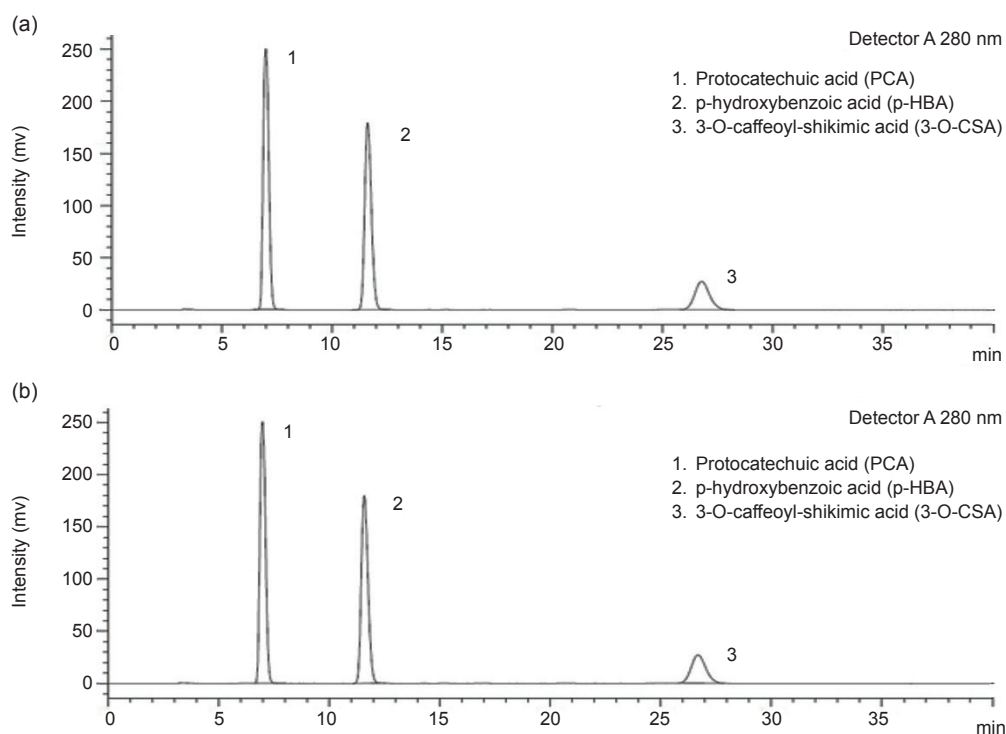


Figure 2. Chromatograms of standard solutions containing (1) PCA, (2) *p*-HBA and (3) 3-O-CSA after (a) acid, and (b) alkaline treatments.

TABLE 1. SYSTEM SUITABILITY PARAMETERS

Parameters	PCA (n=12)	<i>p</i> -HBA (n=12)	3-O-CSA (n=12)	Recommended values (Shabir, 2003)
Resolution	>2	>2	>2	>2
NTP	3 680	6 928	7 849	>2 000
Tailing factor	1.154	1.19725	1.116	≤ 2

Note: NTP - Number of theoretical plates.

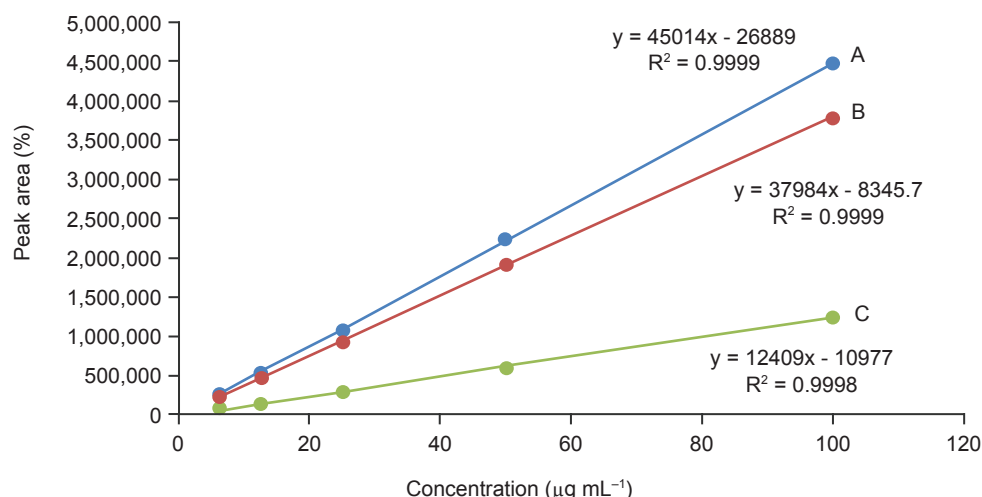


Figure 3. Calibration curves for the phenolic standards (a) PCA, (b) *p*-HBA, and (c) 3-O-CSA (peak area vs. concentration in $\mu\text{g mL}^{-1}$).

Precision and accuracy. Accuracy in an analytical method refers to how closely the measured values align with the true or known values (Kazusaki et al., 2012). The method's accuracy was evaluated by the percentage recovery of phenolic standards that were added to the WSPFE samples at concentrations of 6.25, 25.00 and 100.00 $\mu\text{g mL}^{-1}$ (diluted 1:5). The concentration of the recovered phenolics was determined using the average peak area from three measurements for each concentration. The recovery rates for all standards were within $100 \pm 5\%$, reflecting the high accuracy of the method. Additionally, the repeatability (intra-day) and intermediate precision (inter-day) were assessed using the mean relative standard deviation (RSD%), which remained below 2% across various concentrations for all phenolic standards, demonstrating the reliable precision of the method (Shabir, 2003). Table 3 illustrates the accuracy and precision data for the three phenolics at different concentrations.

Robustness. Robustness was evaluated to determine the method's resilience to minor intentional variations in experimental conditions (Kazusaki et al., 2012; Shabir, 2003). Robustness was tested by making slight adjustments to the mobile phase composition, pH and column temperature for three different concentrations of standard solutions (6.25, 25.00 and 100.00 $\mu\text{g mL}^{-1}$). The peak responses for the three phenolic compounds PCA, *p*-HBA and 3-O-CSA, remained stable and the relative standard deviations (RSD%) were under 2% for all tested concentrations, indicating the method's robustness.

Quantity of the Main Phenolics in Freeze and Spray Dried WSPFE

Spray drying is a process that involves atomising a liquid feed into fine droplets and quickly drying them with hot air, resulting in the formation of a powder. This method is efficient in producing particles of uniform size. Freeze

TABLE 2. LINEARITY EQUATION, CORRELATION COEFFICIENT, LOD AND LOQ FOR THE PHENOLICS IN WSPFE

Compound	Linearity equation (6.25 – 100.00 $\mu\text{g mL}^{-1}$)	r^2	LOQ ($\mu\text{g mL}^{-1}$)	LOD ($\mu\text{g mL}^{-1}$)
PCA	$y = 45014x - 26889$	0.9999	0.48	0.16
<i>p</i> -HBA	$y = 37984x - 8345.7$	0.9999	0.65	0.22
3-O-CSA	$y = 12409x - 10977$	0.9998	5.00	1.65

Note: LOQ - limits of quantification; LOD - limit of detection.

TABLE 3. MEAN RECOVERY (ACCURACY), INTRA- AND INTER-DAY PRECISIONS OF THE THREE PHENOLIC ACIDS

Compound	Recovery % (n=3)			Intra-day precision (RSD%) (n=3)			Inter-days precision (RSD%) (n=12)		
	100	25	6.25	100	25	6.25	100	25	6.25
PCA	104.6	98.0	96.7	0.6	0.7	1.4	0.6	0.7	1.6
<i>p</i> -HBA	98.8	100.5	102.0	0.5	0.9	1.6	0.5	0.9	1.6
3-O-CSA	105.0	101.7	99.4	1.3	0.2	1.5	1.3	1.9	1.5

Note: RSD - Relative standard deviation (RSD%).

drying (lyophilisation) involves freezing the material and then reducing the pressure to allow the ice to sublime directly from solid to gas, preserving the structure and activity of sensitive compounds (Pinto et al., 2021; Santos et al., 2017). As shown in *Table 4*, the concentration of PCA was significantly higher in spray-dried WSPFE. Although higher levels of *p*-HBA and 3-O-CSA were observed in freeze-dried WSPFE, the differences were insignificant. According to Chumroenphat et al. (2021), protocatechuic acid is slightly heat-sensitive, which might explain why it is not affected by the high temperatures of a spray dryer. On the other hand, *p*-HBA was identified as highly sensitive in the same study, which aligns with the result of the current study showing a higher concentration of the compound in the freeze-dried extract. The process of drying is fast in a spray dryer, and the exposure time to the hot air is very short (Shishir & Chen, 2017), which could be the reason for the minimal variation in the phenolic levels between the two methods.

Organoleptic Properties of Dry WSPFE

The effects of freeze drying and spray drying on the organoleptic properties of WSPFE were compared. *Table 5* shows that the taste and odour of the extract remained consistent before and after both drying processes. However, there were slight differences in colour post-drying. WSPFE contains a high amount of fruit sugars, which may account for the sweet taste of the dried extract, as reported by Ibrahim et al. (2020). Spray drying produced a dry sticky powder, whereas freeze drying resulted in a sticky, paste-like product. This stickiness can be attributed to the sugar content, which is hygroscopic and tends to absorb moisture from the environment (Feng et al., 2018). Higher moisture content was linked to a reduction in the glass transition temperature leading to sticky behaviour. Thus, the sticky, paste-like product of the freeze dryer is probably produced due to higher moisture

content (Braga et al., 2019). Freeze dryers have been found to produce voluminous flakes which could not be turned or ground into flowable powder (Febriyenti et al., 2014). On the other hand, a spray dryer is well known for its ability to produce dry powder of plant extract. Gallo et al. (2015) explored spray drying effects on several aqueous extracts, which produced powder with low moisture content and good flow properties.

Antioxidant Effects and Phenolic Content of Dry WSPFE

The antioxidant capacity of dry WSPFE samples was assessed by their ability to scavenge free radicals, using 2,2-diphenyl-1-picrylhydrazyl (DPPH) to estimate the antioxidant effects of both freeze-dried and spray-dried extracts. The DPPH assay, which is stable and cost-effective, measures either hydrogen atom transfer or single electron transfer. The antioxidant properties of WSPFE are linked to their capability to donate hydrogen atoms (Ibrahim et al., 2013; Sambanthamurthi et al., 2011), with the antioxidant potential of the phenolics being proportional to the hydroxyl groups in their structure. As shown in *Table 6*, the IC_{50} value for the freeze-dried WSPFE was slightly lower at $2.40 \pm 0.15 \text{ mg mL}^{-1}$ compared to $2.41 \pm 0.07 \text{ mg mL}^{-1}$ for the spray-dried extract, suggesting a marginally higher antioxidant capacity in the freeze-dried form. However, the difference in antioxidant activity between the two drying methods was minimal, at just 0.01 mg mL^{-1} and likely influenced by the phenolic content of the extracts.

To further understand the impact of drying methods on the phenolics, the Folin-Ciocalteu method was employed, using gallic acid for calibration. The phenolic content was slightly higher in the freeze-dried WSPFE at $42.10 \pm 0.28 \text{ mg g}^{-1}$ GAE compared to $41.49 \pm 0.50 \text{ mg g}^{-1}$ GAE in the spray-dried extract as illustrated in *Table 6*. Freeze drying tends to better preserve bioactive and thermolabile compounds in extracts (ElNaker et al.,

TABLE 4. CONCENTRATION OF MAIN PHENOLICS IN DRY WSPFE SAMPLES

Drying technique	PCA ($\mu\text{g mL}^{-1}$)	<i>p</i> -HBA ($\mu\text{g mL}^{-1}$)	3-O-CSA ($\mu\text{g mL}^{-1}$)
Freeze drying	22 ± 0.1	300 ± 11.0	364 ± 4.6
Spray drying	$23 \pm 0.3^*$	298 ± 4.3	361 ± 2.6

Note: *- $p \leq 0.05$.

TABLE 5. ORGANOLEPTIC PROPERTIES OF FREEZE AND SPRAY-DRIED WSPFE

Drying technique	Taste	Odour	Colour	Appearance
Freeze-dried	Sweet	Caramelised	Dark brownish	Sticky paste-like product
Spray-dried	Sweet	Caramelised	Light brownish	Sticky powder

TABLE 6. ANTIOXIDANT EFFECTS, PHENOLIC CONTENTS, HYGROSCOPICITY AND MOISTURE CONTENT ($\pm$ SEM) OF DRY WSPFE (N = 3)

Drying technique	DPPH IC ₅₀ (mg mL ⁻¹)	TPC (mg g ⁻¹ GAE)	Hygroscopicity	Moisture content (mg g ⁻¹)
Freeze-dried	2.40 $\pm$ 0.15	42.1 $\pm$ 0.3	14.7 $\pm$ 0.2	18.3 $\pm$ 6.0
Spray-dried	2.41 $\pm$ 0.07	41.5 $\pm$ 0.5	12.6 $\pm$ 0.8	15.2 $\pm$ 0.7

2021; Soares et al., 2012). Despite this, the variations in phenolic content post-drying were minor. Even with the higher temperatures involved in spray drying, significant phenolic loss did not occur, possibly due to the brief exposure of particles to the drying gas. Consequently, both drying techniques showed a nearly equivalent effect on the phenolic content of WSPFE.

Hygroscopicity and Loss on Drying of Dry WSPFE

Hygroscopicity is described as the capacity of materials to absorb and adsorb moisture from their surroundings. It influences various properties of materials such as stability, flow characteristics, compression and dissolution rates in pharmaceuticals. Thus, it is critical to assess the moisture absorption capabilities of drug substances (Allada, 2016; Arigo et al., 2019). The hygroscopic nature of WSPFE was evaluated using a standard procedure outlined in the European Pharmacopoeia. Compared to its spray-dried counterpart, the freeze-dried WSPFE exhibited greater hygroscopicity, indicating a stronger propensity to attract ambient moisture Table 6. This characteristic is likely due to the presence of sugars in WSPFE, which are known to absorb moisture (Feng et al., 2018; Muzaffar et al., 2015). It was reported that lower hygroscopicity of spray-dried extracts might be due to higher glass transition than the freeze-dried extract. The glass transition temperature (T_g) of a product has a significant impact on its hygroscopicity. Generally, higher T_g values are associated with lower hygroscopicity (Zhang et al., 2018). Research has shown that spray drying produces powders with a higher T_g compared to freeze drying, resulting in reduced hygroscopicity (Wang et al., 2020). Additionally, freeze-dried WSPFE demonstrated a higher moisture content, which can impact the physical characteristics of the product, such as its flowability and stickiness. In contrast, spray-dried extracts were found to possess lower levels of moisture and hygroscopicity (Feng et al., 2018; Wang et al., 2020). The lower moisture content of spray-dried extract could be due to the higher temperatures used during the drying process with a low feed flow rate. At lower flow rates, there is higher contact time between the feed and the drying air, resulting in efficient heat transfer and higher water evaporation (Tonon et al., 2008).

CONCLUSION

Following the ICH Expert Working Group (2005) guidelines, a new isocratic RP-HPLC method was developed and validated for the separation and quantification of three phenolic acids, namely PCA, *p*-HBA and 3-O-CSA in WSPFE. The proposed method has the advantage of using a low composition of organic solvents, which makes it cost-effective and more environmentally friendly. In addition, the sample preparation process was simple and rapid. The developed method was validated via several tests. It demonstrated acceptable ranges of specificity, linearity, accuracy, precision and robustness. WSPFE was subjected to both spray-drying and freeze-drying methods. The freeze-drying process resulted in a paste-like, sticky form of WSPFE, while spray drying produced a sticky powder. Although the freeze-dried WSPFE exhibited a marginally higher phenolic content and enhanced antioxidant properties, it also involved higher production costs and extended drying times compared to spray drying. Additionally, the spray-dried WSPFE demonstrated lower hygroscopicity and moisture content, making spray drying a more viable option for large-scale and industrial applications of WSPFE.

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DIETARY INCLUSION OF CRUDE PALM OIL, RED PALM OIL, REFINED PALM OIL, PALM KERNEL OIL AND SOYBEAN OIL ON MODULATION OF INTESTINAL BARRIER INTEGRITY AND IMMUNITY IN LAYING HENS

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ABSTRACT

Oils derived from plants have unique and distinct properties based on the compounds present. Prominent chemical compositions in different oils include fatty acids, tocopherols, tocotrienols and carotenoids. Different compounds can affect the regulation of gut immune response and barrier function. Feeds containing either crude palm oil (CPO), red palm oil (RPO), refined palm oil (RBD), palm kernel oil (PKO) and soybean oil (SBO) were fed to 150 laying hens. The feeding trial lasted for 16 weeks. Jejunum mucosa was collected at the end of week 16 to measure the proteins associated with barrier function using ELISA and genes related to immunity and barrier integrity by real-time polymerase chain reaction (PCR). In terms of barrier function proteins, various oils did not affect the concentration of intestinal trefoil factor (ITF) and diamine oxidase (DAO). However, the concentration of IgA was higher in CPO and RPO in contrast to SBO and the alkaline phosphatase (ALP) concentration was higher in RPO and PKO. In terms of gene expression, the downregulation of genes related to immunity and barrier function genes was seen in CPO, RBD, RPO and PKO. Similar trends of lower regulation were observed in CPO and RPO than in other oils. Suppression of genes associated with immunity and barrier function would suggest reducing pathogens' load and exposure to antigens in the gut. In conclusion, different sources of oil affect the regulation of intestinal mucosal immunity and barrier function.

Keywords: crude palm oil, gene expression, layer chickens, red palm oil, refined palm oil.

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INTRODUCTION

The inclusion of lipids in poultry feed is important because it provides energy at a lower cost due to its high energy density and simultaneously supplies essential fatty acids. Lipids in poultry feed can originate from either animal or plant sources. Oils of plant origin are abundant in unsaturated fatty acids (USFA) and phytonutrients such as squalene, polyphenols, vitamins and carotenoids (Zhou et al., 2020). Palm, soybean, linseed and rapeseed (canola) oils are commonly used plant-based oils in poultry feed. Figure 1 shows the distinctive properties of fatty acids, vitamin E and

carotenoids in crude palm oil (CPO), red palm oil (RPO), refined palm oil (RBD), palm kernel oil (PKO) and soybean oil (SBO). Palm oil extracted from the mesocarp of palm fruit has a balance of saturated to USFA; saturated fatty acids are mainly palmitic acid (C16:0) at around 50%, and USFA are mainly oleic acid (C18:1) at around 40%. PKO is abundant in saturated fatty acids, with about 80% contributed by mainly lauric acid (C12:0). SBO has around 80% USFA, mainly contributed by linoleic acid (C18:2).

The gut immunity and barrier function-related pro and anti-inflammatory proteins are kept in balance in a healthy condition, exposure to foreign antigens such as pathogens will induce the immune response and elevate the levels of the related proteins. Fatty acids and phenolic compounds in the oils may affect the regulation of immune response and gut barrier function at the gut mucosa. The carotenoids and vitamin E are potent antioxidants and have been shown to reduce oxidative stress and enhance intestinal immunity and integrity (Eroglu et al., 2022; Lewis et al., 2019). Carotenoids and vitamin E have been proven to display antioxidant properties that reduce oxidative stress, prevent inflammation by lowering tumour necrosis factor alpha (TNF α) and Interleukin 6 (IL6), enhance intestinal integrity by upregulating tight junctions and mucous secretion and modulate the immune system by upregulating Immunoglobulin A (IgA) and toll-like receptors (TLR) (Eroglu et al., 2022; Lewis et al., 2019). Measurement of proteins related to gut immunity and barrier functions can be accessed by measuring the proteins and genes (messenger RNA) through ELISA and real-time PCR, respectively.

We recently reported a study on the dietary influence of SBO, RPO, CPO, RBD and PKO on egg production, egg quality and β -carotene, tocopherol and retinol deposition (Izuddin et al., 2022), antioxidant enzyme protein and gene expression

(Izuddin et al., 2023a), blood lipid profiles and fatty acid profiles and metabolism (Izuddin et al., 2023b) in laying hens. Izuddin et al. (2022) reported the different sources of the aforementioned oils could not alter egg production performance and egg quality. Still, dietary CPO and RPO increased the feed's β -carotene levels, leading to greater concentrations of β -carotene in the egg yolk and liver (Izuddin et al., 2022). Thus, including CPO and RPO provides better protection against oxidation and the reduction of antioxidant enzyme production from antioxidants provided by vitamin E and carotenoids (Izuddin et al., 2023b). The inclusion of oils in the feeds influences fatty acid composition. Dietary supplementation of the oils also affects fatty acid concentration in the egg yolk, serum and liver (Izuddin et al., 2023a).

There have been no reports on the effects of palm oil and palm kernel oil on the gut barrier function and immune response in poultry. Palmitic acid has been linked to the induction of inflammation and the destruction of epithelial integrity (Ghezzal et al., 2020). Even though palm oil contains high levels of palmitic acid, it also has lower levels of oxidation-prone USFA, along with high levels of antioxidants such as carotenoids and vitamin E. PKO contains a great amount of medium-chain fatty acids (MCFA) (Izuddin et al., 2023b), which are linked with reducing inflammation and maintaining the intestinal mucosal barrier function in monogastric animals (Jia et al., 2020). The addition of MCFA into the feed reduced the number of pathogenic microbes and contributed to the performance and gut health of broiler chickens (Gomez-Osorio et al., 2021). Dietary supplements containing polyunsaturated fatty acids (PUFA) have been shown to improve immune response and gut health in chickens (Attia et al., 2020). No research has investigated the potential of palm oil and PKO on gut health related to the immune response in laying hens. Hence, this

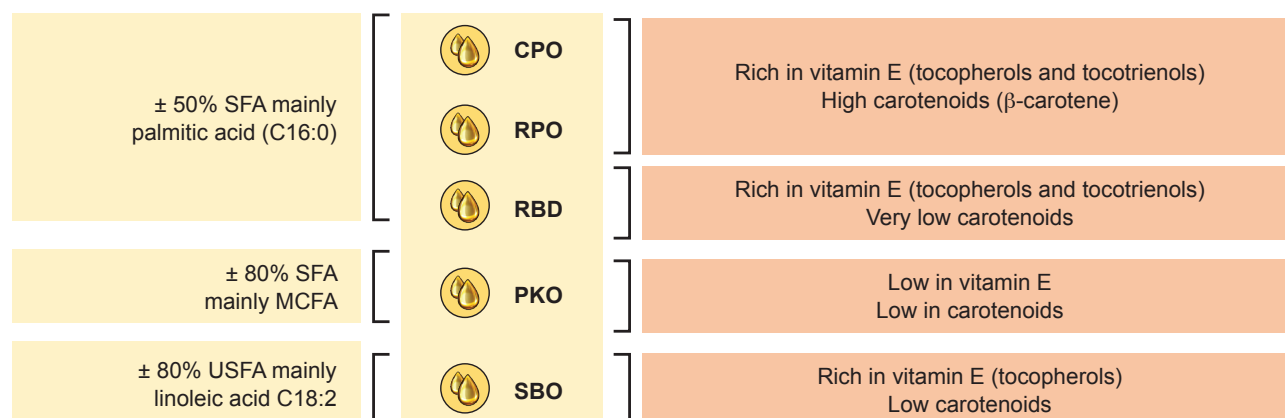


Figure 1. Key properties of the oils.

study aimed to explore the impacts of the inclusion of SBO, RPO, CPO, RBD and PKO on intestinal immunity and intestinal barrier function in laying hens.

MATERIAL AND METHODS

Ethics, Animal Management and Dietary Treatments

The Institutional Animal Care and Use Committee (IACUC) of UPM approved the care and use of commercial Hisex Brown laying hens for this experiment under AUP No.: UPM/IACUC/AUP-R013/2020. The ISO laying hens at the 22nd week of age were divided into five dietary groups. Each treatment comprised six biological replicates, with five hens per biological replicate, totalling 30 hens per treatment. Each hen was placed in an A-type battery cage with dimensions of 40 cm in height, 30 cm in width and 50 cm in depth. The lighting programme consisted of 16 hr of total light (11 hr of natural light with an additional 5 hr of LED lighting) and 8 hr of total darkness in an open house system. Five treatment groups were given isocaloric and isonitrogenous diets [2,790 kcal kg⁻¹ metabolisable energy (ME), 17% crude protein] in mash form containing either 3% SBO, RPO, CPO, RBD, or PKO. The feed formulation is as detailed by Izuddin et al. (2022). Each hen was offered 120 g of feed daily in the morning and had unrestricted access to water through nipple drinkers. The feeding trial for laying hens lasted 16 weeks.

Slaughtering and Sample Collection

Six hens per treatment (one hen from each replicate) were randomly picked for slaughter and sample collection. During evisceration, approximately 3 cm of the middle section of the jejunum was excised, drenched with PBS buffer (pH 7.4) to eliminate digesta, and then cut to expose the mucosa. The exposed mucosa was then scrapped with a glass slide, collected in a microcentrifuge tube (Eppendorf) and snap-frozen.

Intestinal Mucosa IgA, ALP, DAO and ITF

The mucosal IgA was detected using a Chicken IgA ELISA kit (Qayee-Bio, Shanghai, China). Mucosal concentrations of alkaline phosphate (ALP), diamine oxidase (DAO) and intestinal trefoil factor (ITF) were determined using the Chicken ALP, DAO and ITF ELISA Kit, respectively, from Sunlong Biotech, Hangzhou, China. The ELISA procedure was conducted according to the provided

protocols. The target protein concentration was determined using an equation derived from a standard curve of known concentrations of ALP, DAO and ITF (ng mL⁻¹) plotted against absorbance measured at 450 nm.

Liver and Intestinal Mucosa Immunity and Barrier Function Gene Expression

The ribonucleic acid (RNA) extraction, reverse transcription, qPCR protocol and quantification of gene expression were detailed by Azizi et al. (2023). The NucleoSpin® RNA plus kit (Machery Nagel, Dueren, Germany) was used to extract total RNA. The RNA was transcribed using a cDNA Synthesis Kit (Biotechrabbit, Berlin, Germany). The quantification of genes was performed on a qPCR machine (LightCycler® 480, Roche, Basel, Switzerland) using qPCR Green Master Mix (Biotechrabbit, Berlin, Germany). The information about the genes is summarised in *Table 1*.

Experimental Design and Statistical Analysis

The study was performed based on a completely randomised design. Statistical analysis software was used to analyse the data (SAS 9.4, SAS Inst. Inc., Cary, NC, USA). The normality of the data distribution was confirmed using PROC UNIVARIATE and the Shapiro-Wilk. The mean difference among different oils was determined using a one-way analysis of variance (ANOVA). The significant difference was confirmed at $P < 0.05$.

RESULTS AND DISCUSSION

Intestinal Mucosa Barrier Integrity Proteins

There was a significant difference in IgA and ALP levels, but no significant difference in ITF and DAO between the oils (*Figure 2*). Greater concentrations of mucosal IgA antibodies were found in CPO compared to SBO and PKO, but there was no significant difference between RBD and RPO. The SBO had lower IgA concentrations than the CPO and RPO but did not differ from the RBD and PKO. There was no significant difference in IgA levels between RPO, RBD and PKO. The PKO and RPO displayed significantly greater levels of ALP compared to other oils. The CPO, RBD and SBO did not show significant differences from each other.

The impact of the different oils on the regulation of intestinal mucosa immunity and integrity proteins was investigated by measuring protein biomarkers such as ITF, secretory IgA, ALP and DAO proteins using the ELISA approach. The intestinal mucosa is constantly exposed to external environments, including commensal and non-commensal

TABLE 1. THE INFORMATION OF GENES

Gene	Forward and reverse primers	Accession No.	Product size (bp)	Functions
<i>GAPDH</i>	CTGGCAAAGTCCAAGTGGTG AGCACCACCCTTCAGATGAG	NM_204305.1	275	Housekeeping gene to normalise gene expression
<i>IL17</i>	TATCAGCAAACGCTCACTGG AGTTCACGCACCTGGAATG	NM_204460.1	110	Pro-inflammatory cytokine to induce immune response
<i>IL10</i>	TAACATCCAACCTGCTCAGCTC TGATGACTGGTGCTGGTCTG	NM_001004414.2	135	Anti-inflammatory cytokine
<i>IL6</i>	GCTCGCCGGCTTCGA GGTAGGTCTGAAAGGCGAACAG	NM_204628.1	71	Pro-inflammatory cytokine to induce immune response
<i>IL1β</i>	TGCTTCGTGCTGGAGTCACCC GGCCGGTACAGCGCAATGTT	NM_204524.1	98	Pro-inflammatory cytokine to induce immune response
<i>TLR4</i>	TCTTTCAAGGTGCCACATCCA AGCGACGTTAAGCCATGGAA	NM_001030693.1	132	Recognition of foreign antigens and maintenance of epithelial barrier function
<i>TGFβ1</i>	GCCGACACGCAGTACACCAAG GCAGGCACGGACCACCATATTG	NM_001318456.1	168	Immune homeostasis to suppress harmful proinflammatory responses
<i>SIGA</i>	ACCACGGCTCTGACTGTACC CGATGGTCTCCTTCACATCA	XM_003642969.5	1,446	IgA class of antibody with secretory function that recognises specific antigens
<i>TNFα</i>	GCTGTTCTATGACCGCCAGTT AACAACCAGCTATGCACCCCA	NM_204267.1	140	Pro-inflammatory cytokine and principle mediator of immune response
<i>MUC2</i>	TTCATGATGCCTGCTCTTGTTG CCTGAGCCTTGGTACATTCTTGT	XM_040673077.1	93	Forming mucous barrier to prevent direct contact between epithelial cells and luminal content
<i>CLDN1</i>	CATACTCCTGGGTCTGGTTGGT GACAGCCATCCGCATCTTCT	NM_001013611.2	100	Transmembrane protein of tight junction barrier between cells
<i>OCLD</i>	ACGGCAGCACCTACCTCAA GGGCGAAGAAGCAGATGAG	NM_205128.1	123	Transmembrane protein of tight junction barrier between cells
<i>ZO1</i>	CTTCAGGTGTTTCTCTCCTCCTC CTGTGGTTTCATGGCTGGATC	XM_040680632.1	131	Transmembrane protein of tight junction barrier between cells

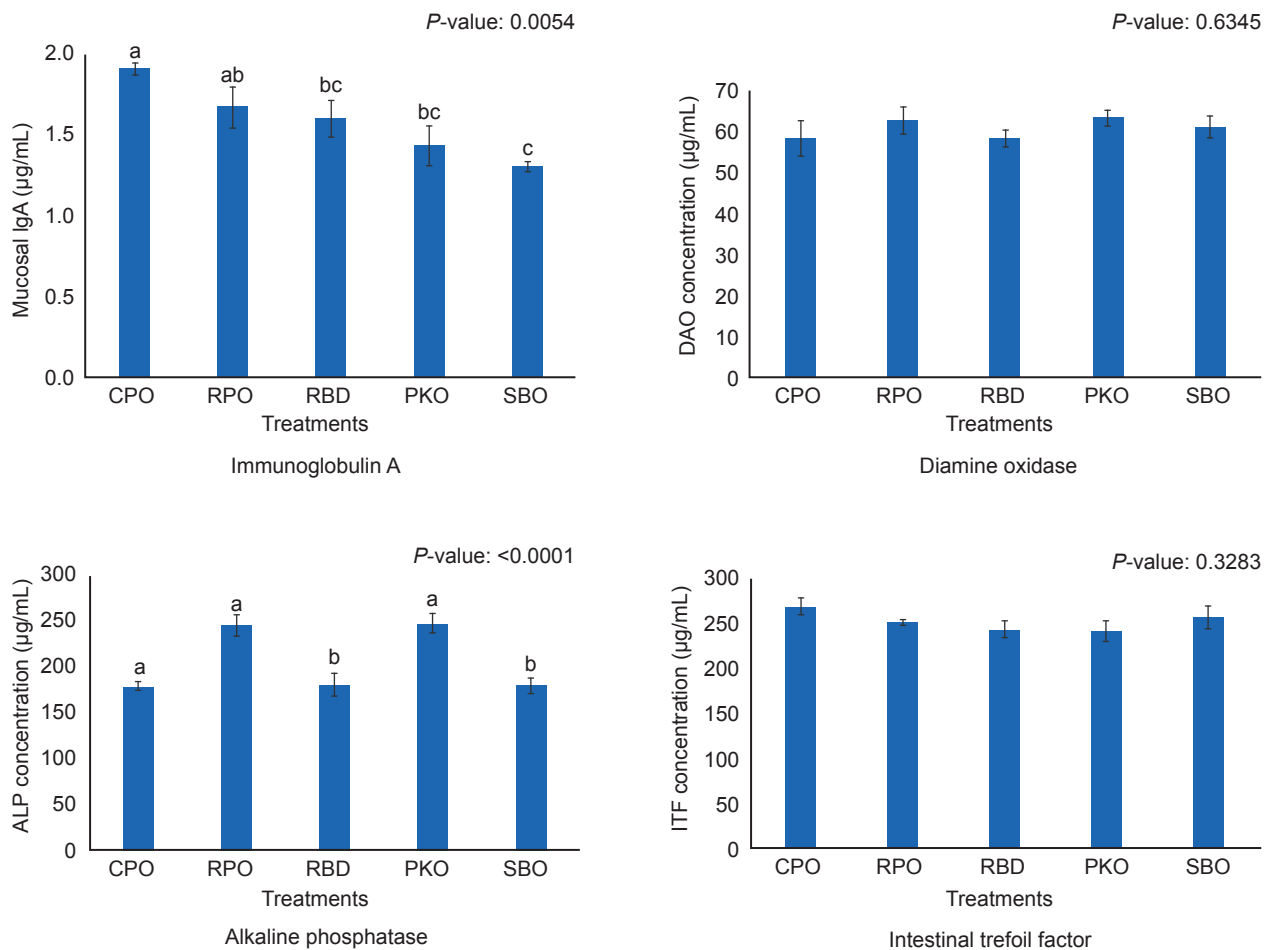
Note: *GAPDH* - glyceraldehyde 3-phosphate dehydrogenase; *CLDN1* - claudin 1; *IL17* - interleukin 17; *IL10* - interleukin 10; *IL6* - interleukin 6; *IL1 β* - interleukin 1-beta; *MUC2* - mucin 2; *OCLD* - occluding; *SIGA* - secretory immunoglobulin 1; *TLR4* - toll-like receptor 4; *TNF α* - tumour necrosis factor-alpha; *TGF β 1* - tumour growth factor-beta 1; *ZO1* - zona occludens 1.

microorganisms in intestinal content, mainly from dietary intake and it needs to balance the inflammatory response (Spencer & Belkaid, 2012). Therefore, it is essential to maintain the integrity of mucosal tissue and immune response to defend the tissues from invading non-commensal pathogenic microorganisms and other antigens.

In maintaining good gut condition, trefoil factors are essential components to mucin at the mucosal surfaces, contributing to mucosal integrity, healing and barrier function. Similarly, the apical layer of intestinal mucosa contains DAO, which is secreted into the lamina propria from the intestinal epithelia and binds to the basolateral membrane for the maintenance of mucosal tissue barriers (Wollin et al., 1998). The current study discovered no differences in ITF and DAO concentration of jejunal mucosa

between different oils. The differences in the oil properties, such as fatty acid profiles, vitamin E and carotenoid content, did not contribute to ITF and DAO production. It indicates no adverse impacts on feeding oil containing high palmitic acid (C16:0) in palm oil or MCFA in PKO to laying hens.

Another important peptide, secretory IgA is the major class of antibodies in the mucous membrane of the intestinal mucosa. It acts as a passive immunity and first line of defence that protects the intestinal barrier from the adhesion and penetration of pathogenic microorganisms (Pietrzak et al., 2020). In this study, a greater concentration of secretory IgA was seen in CPO and RPO. Higher secretory IgA indicates stronger protection of mucosal surfaces against various antigens. The induction of higher production of secretory IgA in mucosal



Note: SBO - soybean oil; PKO - palm kernel oil; RBD - refined palm oil; RPO - red palm oil; CPO - crude palm oil.
a,b,c with different letters between means on a row denote a significant difference ($P < 0.05$).

Figure 2. Immunoglobulin A, diamine oxidase jejunal mucosa, alkaline phosphatase and intestinal trefoil factor concentrations.

can be attributed to the presence of a palmitic acid (C16:0)-rich diet contributed by palm oils (RPO and CPO) compared to MCFA-rich PKO and PUFA-rich SBO. In a mice model, intestinal IgA was greater in mice receiving palm oil than in soybean and coconut oils, but rapeseed oil did not show an increment in intestinal IgA production despite having a comparable amount of oleic acid (C18:1) as palm oil (Kunisawa et al., 2014). The same study suggested that coconut oil contains higher SFA in lauric (C12:0) and myristic (C14:0) acids but did not give effects similar to palm oil containing higher palmitic acid (C16:0). Kunisawa et al. (2014) then enriched the SBO with palmitic acid (C16:0) and they discovered an increase in the faecal IgA concentration in mice fed palmitic acid (C16:0) enriched SBO, suggesting that palmitic acid (C16:0) is a crucial fatty acid in enhancing intestinal IgA production.

Intestinal ALP is essential in promoting intestinal homeostasis and health by regulating the intestinal microbiota through a relationship between mucosa, microbiota and diet. The ALP protects the host by detoxifying harmful microbial

ligands (Estaki et al., 2014). Our study suggests that RPO and PKO contribute to the higher production of ALP in the jejunal mucosa. The presence of palmitic acid (C16:0) and carotenoids in RPO and MCFA-rich PKO seems to induce the regulations of intestinal ALP and leads to better intestinal health.

Intestinal Mucosa Immunity Gene Expression

All target genes showed differences in expression between the oils (Table 2). The expression of *TNFA* was lower in palm oils (RBD, CPO and RPO) and PKO, with no significant differences observed among them. In palm oils (RBD, CPO and RPO) and PKO, the expression of *TLR4*, *IL1β*, *IL6*, *IL10* and *IL17* was downregulated. The CPO and RPO showed lower fold changes than PKO but showed no difference compared to RBD. In palm oils (RBD, CPO and RPO) and PKO, the expression of *TGFβ1* was downregulated, with the lowest expression in CPO and no difference in expression between RPO, RBD and PKO.

TABLE 2. JEJUNAL MUCOSA IMMUNITY GENE EXPRESSION

Parameters	CPO	RPO	RBD	PKO	SBO	SEM	P-value
<i>TNFα</i>	0.280 ^b	0.307 ^b	0.383 ^b	0.490 ^b	1.000 ^a	0.075	<0.001
<i>TLR4</i>	0.005 ^c	0.040 ^c	0.182 ^{bc}	0.304 ^b	1.000 ^a	0.101	<0.001
<i>IL1β</i>	0.010 ^c	0.070 ^c	0.246 ^{bc}	0.386 ^b	1.000 ^a	0.100	<0.001
<i>IL6</i>	0.002 ^c	0.024 ^c	0.170 ^{bc}	0.253 ^b	1.000 ^a	0.101	<0.001
<i>IL10</i>	0.004 ^c	0.027 ^c	0.169 ^{bc}	0.253 ^b	1.000 ^a	0.101	<0.001
<i>IL17</i>	0.003 ^c	0.023 ^c	0.124 ^{bc}	0.261 ^b	1.000 ^a	0.101	<0.001
<i>TGFβ1</i>	0.032 ^c	0.136 ^{bc}	0.189 ^b	0.216 ^b	1.000 ^a	0.094	<0.001

Note: SBO - soybean oil; PKO - palm kernel oil; RBD - refined palm oil; RPO - red palm oil; CPO - crude palm oil; SEM - standard error of means; *TLR4* - toll-like receptor 4; *IL1 β* - interleukin 1-beta; *IL10* - interleukin 10; *TNF α* - tumour necrosis factor-alpha; *IL6* - interleukin 6; *IL17* - interleukin 17; *TGF β 1* - tumour growth factor-beta 1. ^{a,b,c} with different letters between means on a row denote a significant difference ($P < 0.05$).

Cytokines are small-size signalling proteins or peptides secreted by immune cells as a signal to activate and regulate the immune system and inflammatory responses. The current study explored the anti-inflammatory *IL10* and several pro-inflammatory cytokines such as *TNF α* , *IL1 β* , *IL6*, *IL17* and pattern recognition receptor *TLR4*. The further elucidation of the regulation of mucosal immunity and barrier functions was determined by quantifying the expression of those cytokine genes.

Oxidative stress contributes to fibrogenesis by inducing cytokines belongs to pro-inflammatory groups such as *TGF β 1*, *IL6* and *TNF α* (Casas-Grajales & Muriel, 2015). *TNF α* , *IL1 β* and *IL6* are important mediators and potent pro-inflammatory cytokines for host defence in response to infection and injury. Stimulating molecules from bacterial cell wall products such as peptidoglycans, lipopolysaccharides and other bacterial toxic products in the intestinal lumen activate macrophages and T lymphocytes to secrete strong proinflammatory cytokines such as *TNF α* , *IL1* and *IL6* (MacDermott, 1996). *TGF β 1* also contributes to the inflammation response in which the presence of *IL6* stimulates the production of T helper 17 (Th17) cells to induce additional inflammation (Sanjabi et al., 2009). The *IL17* involves host defence against pathogens in the mucosa. It is known to contribute to inflammatory response, but *IL17* also contributes to the fine balance between immunity tolerance and inflammatory responses in the mucosa (Guglani & Khader, 2010).

Proinflammatory cytokines upregulation responds to antigens or lipid peroxidation products that induce cell oxidative stress. The lipid sources in animal feed may influence the level of lipid peroxidation products, which are affected by the fatty acid profile, storage conditions and period and processing of the lipid source (Totani et al., 2007). In this study, the upregulation of

proinflammatory cytokines in the jejunal mucosa of SBO could be associated with higher content of USFA, particularly PUFA. Higher TBARS and PUFA present in the SBO may enhance lipid peroxidation and induce oxidative stress to the mucosal tissues, thus affecting the regulation of proinflammatory cytokines. Tan et al. (2018) conducted a study on dietary supplementations of oxidised SBO in broiler chickens that upregulated the pro-inflammatory *IL22* of ileal mucosa, indicating the products of lipid peroxidation from oxidised oil-induced inflammation.

TLRs are part of the pattern recognition receptor family crucial to the initial protection because of the presence of receptors that recognise pathogen-associated molecular patterns (PAMPs) and gram-negative bacteria's lipopolysaccharide (Molteni et al., 2016). This study disclosed that the *TLR4* of jejunal mucosa was downregulated in PKO and palm oils (RBD, CPO and RPO). *TLR4* expression can be related to the recognition of lipopolysaccharides of Gram-negative bacteria that activate NF κ B, leading to proinflammatory cytokine and chemokine production (Fukui et al., 2001). *TLR4* signalling upon detecting bacterial antigens generates several cytokines such as *IL1 β* , *IL6* and *IL8* (Laurent et al., 2001). The upregulation of *TLR4* in the SBO aligned with activating other proinflammatory cytokines such as *IL17*, *IL6*, *IL1 β* and *TNF α* .

Anti-inflammatory cytokines are immunoregulatory molecules essential to manage the response of proinflammatory cytokines and work in concert with specific receptors and cytokine inhibitors to regulate immune response (Gan et al., 2019). *IL10* plays a vital role as the primary regulator in suppressing the immune system's proinflammatory response. The barrier of the intestine plays a critical role in preventing mucosal damage and promoting barrier integrity by the action of *IL10* on Treg cells or macrophages (Wei et al., 2020). This study revealed the *IL10*

downregulation in palm oils (RBD, CPO and RPO) and PKO. The downregulation of this anti-inflammatory was in concert with the control of proinflammatory cytokines.

Intestinal Mucosa Barrier Function Gene Expression

The *SIGA*, *CLDN1*, *OCLD* and *ZO1* genes differed between oils, except for the *MUC2* gene (Table 3). In *SIGA*, the CPO and RBD were downregulated. The CPO had lower regulation than the RPO and PKO. The expression of *CLDN1*, *OCLD* and *ZO1* genes were downregulated in palm oils (RBD, CPO and RPO) and PKO compared to SBO. The CPO and RPO were lower in the regulation of *CLDN1* compared to PKO, with no difference from RBD. Palm oils (RBD, CPO and RPO) and PKO showed no significant differences in *OCLD*. In *ZO1*, with no difference in CPO compared to RBD and RPO and lesser *ZO1* regulation in CPO than in PKO.

The intestinal barrier comprises a mucous layer lining the epithelial cells that grip together by tight junction proteins on a lamina propria. The mucous membrane lining the epithelial cells is composed of mucin produced and secreted by goblet cells, the high composition of secretory IgA antibodies to defend against foreign antigens and the presence of commensal microbiota recognised as mutual by the host immune system. The secretory IgA presence at the mucous membrane aids in eradicating and blocking pathogenic microorganisms and antigens from the intestinal lumen by entrapping in mucous and eliminating them by mucociliary and peristaltic actions (Mantis et al., 2011).

In the current study, no influence of feeding different oils on the expression of the *MUC2* in the jejunal mucosa. The RPO, PKO and SBO had no difference in *SIGA* regulation, but CPO and RBD were downregulated relative to the SBO. However, it was inconsistent with the higher mucosal secretory IgA protein of CPO and RPO, as palmitic acid (C16:0) was suggested to raise the production of IgA in the intestinal mucosa. Kunisawa et al.

(2014) fed SBO enriched with palmitic acid (C16:0). They discovered an increase in the faecal IgA concentration in mice, suggesting that palmitic acid (C16:0) is crucial in enhancing intestinal IgA production.

The intercellular barrier of epithelial cells is held together by a continuous network of tight junction proteins that secure the paracellular space and manage the permeability of substances across the epithelial tissues. Tight junction protein comprises claudins, occludins, zona-occludens-1 and actin-myosin cytoskeletal proteins (Gil-Cardoso et al., 2016). The current result discovered upregulation of *CLDN1*, *OCLD* and *ZO1* in SBO in contrast to palm oils (RBD, CPO and RPO) and PKO groups. Yan et al. (2016) found that the emulsion of SBO-based increased the intestinal permeability of lipopolysaccharides in Caco-2 cells by downregulating the P-glycoprotein gene that facilitates the permeability of lipopolysaccharides in the *in vitro* cell model. However, our study found the upregulation of barrier function genes in SBO than in other oils. Therefore, the interpretation could be that the higher regulation of tight junction proteins was in response to the upregulation of the proinflammatory cytokines such as *TNF α* , *IL1 β* , *IL6*, *IL17* and *TLR4* to reinforce the intestinal mucosa barrier. The cytokines such as *TNF*, interferon-gamma, *IL-13* and *IL-17* affect the epithelial barrier function through several mechanisms, including cytoskeletal modulation, epithelial apoptosis, kinase activation, membrane trafficking and protein synthesis (Turner, 2009).

It was suggested that higher dietary supplementations of omega-6 (linolenic acid; C18:2n-6) fatty acids induce the production of intestinal proinflammatory mediators such as leukotrienes in mice (Ohtsuka et al., 1997) and increase intestinal epithelium barrier permeability and susceptibility in mice (Deol et al., 2021). Ohtsuka et al. (1997) reported that the jejunal mucosa of mice with a diet less in omega-6 or omega-3 (linolenic acid; C18:3n-3) fatty acids reduced the production of leukotrienes; leukotrienes are produced in response to mucosal damage induced by food-

TABLE 3. JEJUNAL MUCOSA BARRIER FUNCTION GENES EXPRESSION

Parameters	CPO	RPO	RBD	PKO	SBO	SEM	P-value
<i>SIGA</i>	0.168 ^c	0.763 ^{ab}	0.318 ^{bc}	0.789 ^{ab}	1.000 ^a	0.104	0.024
<i>MUC2</i>	0.474	0.219	0.455	0.421	1.000	0.095	0.078
<i>CLDN1</i>	0.004 ^c	0.029 ^c	0.156 ^{bc}	0.495 ^b	1.000 ^a	0.111	0.001
<i>OCLD</i>	0.331 ^b	0.528 ^b	0.516 ^b	0.421 ^b	1.000 ^a	0.073	0.008
<i>ZO1</i>	0.018 ^c	0.082 ^{bc}	0.195 ^{bc}	0.276 ^b	1.000 ^a	0.098	<0.001

Note: SBO - soybean oil; PKO - palm kernel oil; RBD - refined palm oil; RPO - red palm oil; CPO - crude palm oil; SEM - standard error of means; *SIGA* - secretory immunoglobulin A; *MUC2* - mucin 2; *OCLD* - occluding; *CLDN1* - claudin 1; *ZO1* - zona occludens 1. ^{a,b,c} with different letters between means on a row denote a significant difference ($P < 0.05$).

sensitive enteropathy. The current study disclosed that SBO increased the proinflammatory cytokines in the jejunal mucosa as the SBO contains a higher fraction of omega-6 fatty acid composition than palm oils (RBD, CPO and RPO) and PKO.

Dietary supplementations of MCFA can reduce pH in the gastrointestinal, creating an unsuitable medium for some pathogens in the intestinal tract and potentially substituting the antibiotic growth promoter in poultry (Gomez-Osorio et al., 2021). The benefits of MCFA can be seen in dietary coconut oil, which is an oil with a high content of MCFA. Coconut oil contains up to 91% SFA made up of MCFA, mainly lauric acid (C12:0). Braundmeier-Fleming et al. (2020) reported that substituting SBO with coconut oil increases the growth performance and immune functions in pre-weaning and grower pigs without dietary supplementations of antibiotics. Similarly, PKO contributes to higher MCFA in the diet and might positively influence the regulation of pathogens in the intestinal cavity and provide a lesser pro-inflammatory response to the intestinal mucosa.

CONCLUSION

In conclusion, the dietary inclusion of different oils in laying hens did not affect mucosal ITF and DAO protein, but dietary palm oils increased mucosal IgA and ALP protein compared to SBO. In contrast to proteins, all mucosal immunity (*TNF α* , *TLR4*, *TGF β 1*, *IL1 β* , *IL6*, *IL10* and *IL17*) and barrier function (*SIGA*, *MUC2*, *CLDN1*, *OCLD* and *ZO1*) genes were downregulated in palm oil (CPO, RPO and RBD) and PKO, with the lowest regulation observed in CPO and RPO compared to other oils. Lower gene expression associated with proinflammatory cytokines (*TLR4*, *TNF α* , *IL1*, *IL6*, *IL17*) would suggest reduced mucosal exposure to antigens in the gut environment.

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MOLECULAR IDENTIFICATION OF A MEALYBUG SPECIES (HEMIPTERA: PSEUDOCOCCIDAE) INFESTING AN OIL PALM PLANTATION IN TAWAU, SABAH

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ABSTRACT

*Mealybugs are common insect pests that can cause significant damage to oil palms, including reduced yield and overall plant health. The mealybugs feed on the plant sap, which later excretes honeydew, leading to the growth of sooty mold, and impedes photosynthesis. The traditional morphological identification methods for mealybugs are very time-consuming and challenging, due to their diminutive size and similar characteristics between their developmental stages. In this research, we investigated the effectiveness of the Ef-1 α and 28S rRNA genes for molecular identification of a mealybug species infesting oil palm plantations in Tawau, Sabah. The DNA was extracted from the mealybug samples and amplified with M51.9/rcM53-2, D2F/D2R, and D10F/D10R primers. The amplified regions of the identified *Nipaecoccus nipae* Tawau-isolate were sequenced and deposited in GenBank. Our results demonstrate the potential of molecular methods for quick and efficient identification of mealybug species, which can aid in developing appropriate control strategies.*

Keywords: 28S rRNA, Ef-1 α , mealybugs, oil palm plantation.

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INTRODUCTION

Mealybugs (Hemiptera: Pseudococcidae) are common insect pests that thrive on fruits, branches, leaves, roots, and trunks of the oil palm (Pacheco Da Silva et al., 2017). Despite their small size even as adults, they can cause direct harm to their hosts by extracting the plant sap and triggering the formation of sooty mould through their excretion of honeydew, which directly reduces the vigour of the host plants (Park et al., 2010). The black sooty mould fungus impedes photosynthesis by covering the leaves, and stem, thereby causing chlorosis, plant stunting and leaf defoliation, which eventually lead to the possible death of the host plant (Moniruzzaman et al., 2017; Williams & Watson, 1988).

Worldwide, there are about 2,005 species of mealybugs that have been described with 272 genera identified. They are extensively distributed across the tropical and subtropical regions, inflicting damage on various crops, including oil palm (Ahmad & Akhtar, 2016; Morales et al., 2016). For example, Mariau and Biggins (2001) reported that *Dysmicoccus brevipes* live on the roots of oil palms in Ecuador. *Pseudococcus citricutis*, *Palmicultor* sp. and *D. brevipes* were reported to be infesting oil palm in India (Kalidas & Subbana, 2022). In Malaysia, Williams (1969) stated *Geococcus johorensis* as the first reported mealybug infestation in oil palm plantation in Johor. However, recently, there were several mealybug population outbreaks on the East Coast of Sabah, which were morphologically identified as *Nipaecoccus nipae* among the oil palm growers. Despite morphological identification, molecular techniques are crucial for confirming the species identity and detecting any cryptic species

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or genetic variations that might not be evident morphologically. The effect of sooty mould was often neglected until the yield reduction in this region became pronounced. After considering other aspects that can cause yield reduction, such as nutrient deficiencies, the possible effects of sooty mould on photosynthesis rates were explored. The disruption to the photosynthesis rate will gradually lead to the reduction of plant productivity and health. Currently, two treatments are being employed to control the mealybugs: 1) Trunk injection of acephate and 2) spraying of white oil and imidacloprid (Helmay et al., 2023). In addition, the lack of knowledge of mealybug species poses a threat to the implementation of integrated pest management strategies in the area.

Mealybugs have similar morphological characteristics, making it difficult to distinguish between their developmental stages due to their small size, thereby hindering the development of appropriate control strategies (Abd-Rabou et al., 2012). Accurate molecular identification is essential for enhancing the management of mealybugs due to the specificity of their control techniques (Chong & Oetting, 2007). Moreover, the acknowledgement of molecular techniques is increasing, emphasising their significance in discriminating species, especially when morphological identification techniques are ambiguous (Tahir et al., 2018). If the species is mistakenly identified, it can lead to ineffective pest management strategies, the use of inappropriate control methods and eventually leading to potential economic loss due to continued pest damage.

The mitochondrial cytochrome c oxidase subunit I (COI) gene sequence has been widely and extensively utilised as a DNA barcode for animals. Unfortunately, its universality faces challenges, as amplifying the barcode region for the insect family Pseudococcidae has proven to be difficult (Palma-Jiménez et al., 2018; Park et al., 2010, 2011; Sethusa et al., 2014). In contrast, Ef-1 α and 28S ribosomal RNA (28S rRNA) genes have been successfully used for the identification of mealybugs and other insect scales (Puig et al., 2021). Thus, in this research, we examine the effectiveness of the D2 and D10 regions of 28S rRNA gene for species identification of mealybugs in Tawau, Sabah.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

In November 2023, some mealybugs were randomly collected from 14 year old oil palm fronds in KLK Plantation, Tawau, Sabah (4°26'47.5"N and 118°12'33.3"E), from 25 oil palms that were randomly chosen within 2 ha of the

oil palm plantation. The samples were pooled to 0.05 g/tube making it one replicate. The pooled samples were then immersed in RNAlater to retain the DNA quality and kept in a -20°C freezer. Total DNA extraction was carried out on three replicates by using the InnuPREP DNA Mini Kit according to the manufacturer's instructions (Analytikjena) with some modifications involving liquid nitrogen during the cell lysis process. The final resuspension was done by adding 150 μ L of elution buffer, followed by storage at -20°C freezer. Following the extraction, DNA quantification was determined using a NanoPhotometer spectrometer (IMPLEN P300 NanoPhotometer, CA, USA) and the Qubit™ dsDNA BR Assay Kit on Qubit 2.0 Fluorometer (Life Technologies, CA).

Polymerase Chain Reaction (PCR) Amplification of EF-1 α and 28S rRNA Genes

Three samples of mealybugs and three primer pairs, M51.9/rcM53-2, D2F/D2R and D10F/D10R were chosen in this study (Table 1). These primers were selected based on their effectiveness in amplifying EF-1 α and 28S rRNA genes in the mealybug species (Downie & Gullan, 2004; Puig et al., 2021). Each 25 μ L PCR reaction mix contained 2.5 μ L of 10x reaction buffer, 0.3 μ L TransStart Taq DNA Polymerase (TransGen Biotech Co., Ltd, Beijing, China), 1.0 μ L of each primer (0.2 μ M), 0.5 μ L of 2.5 mM dNTPs, 1.0 μ L BSA (Bovine Serum Albumin), 2.5 μ L MgCl₂, 5 μ L DNA template, and 11.2 μ L of ultrapure water. The PCR analysis was performed using Biometra TADVANCE (MATRIOUX) under the following conditions: 4 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 54°C, 1.30 min at 72°C, and 1 min at 72°C and held at 4°C. All PCR products were visualised at 1% agarose gel stained using TransGen GelStain. The bands were excised and purified using EasyPure® Quick Gel Extraction kit (Transgen Biotech, China). The purified DNA samples were sent to Apical Scientific Sdn. Bhd., laboratory for Sanger sequencing.

Sequence Similarity

Nucleotide sequences were assembled and edited where necessary, using Sequence Scanner 2 (ThermoFisher Scientific). The sequence similarity search was conducted using the Basic Local Alignment Search Tool (BLAST) with BLASTn as the database at the National Center for Biotechnology Information (NCBI) and the top match was selected based on the highest percentage identity. The EF-1 α , D2 and D10 amplified region sequences were deposited in Genbank with Accession Number: PP814618, PP158656 and PP158655, respectively.

TABLE 1. PRIMER PAIR SEQUENCES OF ELONGATION FACTOR 1- α AND 28S RIBOSOMAL RNA GENES USED IN SPECIES IDENTIFICATION

Gene	Primer	Sequence (5'-3')	References
Elongation factor 1- α	M51.9/rcM53-2	F: CARGACGTATACAAAATCGG R: CCGCGGCTGCTGGCACCAGA	Downie and Gullan (2004)
28S ribosomal RNA	D2F/D2R	F: AGAGAGAGTTCAAGAGTACGTG R: TTGGTCCGTGTTCAAGACGGG	Puig et al. (2021)
	D10F/D10R	F: GTAGCCAAATGCCTCGTCA R: CACAATGATAGGAAGAGCC	

RESULTS AND DISCUSSION

Considering the morphological identification of the pests is time-consuming due to their small size, identification using molecular techniques offers a promising alternative with quicker and more efficient results (Mwanauta et al., 2021). The cytochrome c oxidase subunit has proven to be difficult to amplify barcode regions in mealybugs (Malausa et al., 2011). The utilisation of molecular identification for insects heavily depends on the availability of reference libraries within the Genbank database.

Nipaecoccus nipae is commonly known as spiked mealybug, nipa mealybug, avocado mealybug, coconut mealybug, sugar apple mealybug, or Kentia mealybug due to the wide host range of this pest (Espinosa et al., 2009) described the size of the adult female mealybug range between 0.059 and 0.98 inches (1.5 and 2.5 mm) long, featuring an oval shape, exhibiting hues ranging from reddish-brown to orange. The surface is coated with yellowish-orange thick wax, with 10-12 pairs of marginal pyramid-shaped wax filaments (Figure 1). The honeydew excretions by this species during feeding can lead to the growth of the black sooty mould, which diminishes photosynthesis and attract ants that eventually protect the mealybugs from predators or parasitoids (Josephraj Kumar et al., 2012). The insect is easily carried by the wind and transported by ants within and between different plant species (Mani et al., 2012). Aside from the COI gene's limitations to molecularly identify *N. nipae*, the ITS gene was also considered uninformative due to the absence of this gene in Genbank. Conversely, with its highly informative and conserved gene, the translation elongation factor 1- α (EF-1 α) was used instead within the diverse insect species (Cho et al., 1995). In addition, gene 28S rRNA gene sequence which is highly conserved also offers a potential utility in distinguishing the mealybugs species (Lang, 2023).

In this study, three primer pairs (M51.9/rcM53-2, D2F/D2R and D10F/D10R) of EF-1 α and 28S rRNA gene produced a clear and single band in all tested replicates (Figure 2). Species identification using BLAST showed that the isolated mealybugs

have a high percentage similarity with EF-1 α and 28S rRNA gene from other *N. nipae*, ranging from 82.90%-92.79% (Table 2). Identification using EF-1 α showed 86.08% identity to *N. nipae* of Genbank Accession Number: AY427245.1, collected from South Africa. Meanwhile, D2 and D10 region amplification showed 86.69% and 92.79%, respectively to *N. nipae* of Genbank Accession Number: JQ651292.1 and AY427421.1, also from South Africa (Sethusa et al., 2014). Therefore, the mealybugs collected in this study were molecularly characterised as *N. nipae*.

In addition, our findings seemed to align with previous research that highlights the challenges of using COI and ITS genes for mealybug identification (Malausa et al., 2011). The consistent amplification and the high identity percentage obtained in this study using EF-1 α and two regions of 28S rRNA genes underscore their utility as reliable markers for *N. nipae* identification. Moreover, expanding the reference libraries in GenBank to include more sequences from diverse geographic regions and species will enhance the accuracy and reliability of molecular identification, facilitating a better pest management strategy. Precise identification allows for targeted control measures, reducing the spread and impact of *N. nipae* in oil palm plantation, particularly.

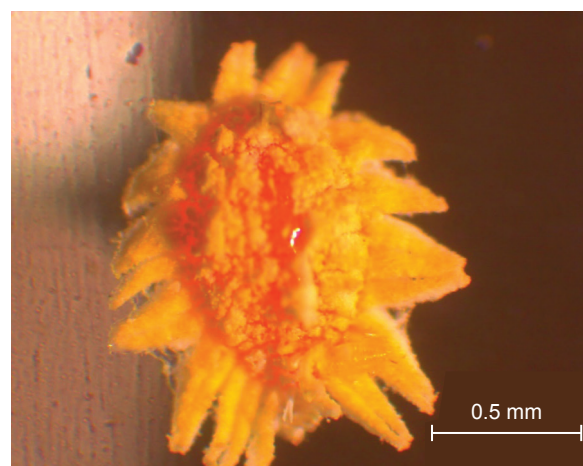


Figure 1. A ventral view of a *Nipaecoccus nipae* isolate from Tawau under a compound microscope at 10 $\times$ magnification.

TABLE 2. BLASTn RESULT FOR EF-1 α AND 28S rRNA GENES AMPLIFICATION SEQUENCED WITH EF-1 α , D2F/D2R AND D10F/D10R PRIMERS

Marker primer	Gene	Scientific name	Genus	Family	Query coverage (%)	Identity (%)	Accession
EF-1 α	Elongation factor 1- α	<i>N. nipae</i>	<i>Nipaecoccus</i>	Pseudococcidae	93	86.08	AY427245.1
		<i>N. nipae</i>	<i>Nipaecoccus</i>	Pseudococcidae	93	85.83	EU188555.1
		<i>N. nipae</i>	<i>Nipaecoccus</i>	Pseudococcidae	89	82.90	AY427245.1
D2	28S ribosomal RNA	<i>N. nipae</i>	<i>Nipaecoccus</i>	Pseudococcidae	94	89.96	JQ651292.1
		<i>N. nipae</i>	<i>Nipaecoccus</i>	Pseudococcidae	94	86.33	JQ651292.1
		<i>N. nipae</i>	<i>Nipaecoccus</i>	Pseudococcidae	94	86.64	JQ651292.1
D10	28S ribosomal RNA	<i>N. nipae</i>	<i>Nipaecoccus</i>	Pseudococcidae	95	92.79	AY427421.1
		<i>N. nipae</i>	<i>Nipaecoccus</i>	Pseudococcidae	95	92.79	AY427421.1
		<i>Nipaecoccus</i> aff. <i>gilli</i> PJG-2003	<i>Nipaecoccus</i>	Pseudococcidae	94	92.53	AY427422.1

These findings highlight the effectiveness of using EF-1 α and 28S rRNA gene for the molecular identification of *N. nipae*. Furthermore, the consistent amplification and success of the molecular approach used in this study suggest the applicability and reliability of these genes in the identification of mealybug species.

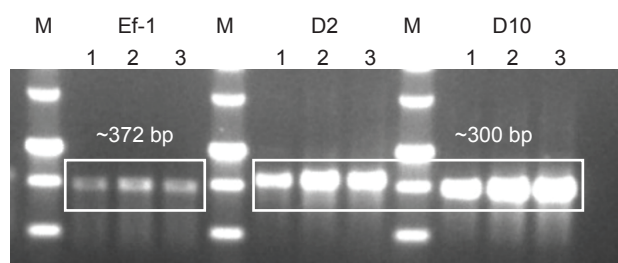


Figure 2. Gel electrophoresis image displaying the amplified products of EF-1 α and 28S rRNA genes from *N. nipae* isolate from Tawau. Lanes M indicate 1Kb ladder, while 1, 2, and 3 represent individual samples.

CONCLUSION

Although the techniques presented in this study were developed for *N. nipae* identification, they can also be utilised to identify mealybug species from other regions or for studying pest populations. The success of these molecular approaches, despite the limitations of the COI and ITS genes, demonstrate that alternative genes in this case, the EF-1 α and 28S rRNA, have the potential to distinguish the mealybug species. To date, among the 2,005 identified mealybug species, 37 recognised species belong to *Nipaecoccus* sp. However, further research is necessary to acquire more DNA markers for the identification of mealybugs, particularly from diverse geographical regions.

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