STUDY OF TECH-FUNCTIONAL PROPERTIES, BIOAVAILABILITY AND BIOLOGICAL ACTIVITIES OF SUPERCRITICAL EXTRACTED PROTEIN AND OIL FROM COMMERCIAL INSECTS FOR COMMERCIALIZATION

NANTAWAN BOONMEE

A Thesis Submitted to the Graduate School of Naresuan University

in Partial Fulfillment of the Requirements

for the Doctor of Philosophy Degree in Food Science and Technology

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Thesis entitled “Study of Tech-functional Properties, Bioavailability and Biological Activities of Supercritical Extracted Protein and Oil from Commercial Insects for Commercialization”

By Miss Nantawan Boonmee

has been approved by the Graduate School as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Food Science and Technology

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Title STUDY OF TECH-FUNCTIONAL PROPERTIES, BIOAVAILABILITY AND BIOLOGICAL ACTIVITIES OF SUPERCRITICAL EXTRACTED PROTEIN AND OIL FROM COMMERCIAL INSECTS FOR COMMERCIALIZATION

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ABSTRACT

Edible insects have emerged as a promising alternative source of high-quality proteins and oils with substantial nutritional and health benefits. They represent a sustainable source of nutrients suitable for both human and animal consumption. This doctoral research aimed to investigate the effects of pretreatment, oil extraction, and protein extraction processes on the physicochemical properties, bioavailability, and biological activities of three commercial edible insects—adult house cricket (*Acheta domesticus*, AHCK), black soldier fly larvae (*Hermetia illucens*, BSFL), and silkworm pupae (*Bombyx mori*, SWP).

In the first phase, the effects of high-pressure processing (HPP) and ultrasonication (US) pretreatments prior to supercritical carbon dioxide (SC-CO_2) extraction were examined with respect to microbial inactivation and oil yield. Raw insect samples were pretreated using HPP at 200, 400, and 600 MPa for 5 min or US at 37 kHz for 5, 10, and 15 min, followed by dehydration, grinding, and sieving through an 18-mesh screen. Oils were extracted using SC-CO_2 and compared

with solvent extraction (SE) as a reference method. HPP significantly reduced total viable counts and yeast and mold levels by 6–8 log cycles, whereas US showed a moderate reduction. Pretreatments enhanced oil yields in most samples by disrupting cellular integrity and facilitating lipid release. For AHCK, US15 (37 kHz, 15 min) followed by SC-CO_2 extraction at 30 MPa yielded the highest oil recovery. In BSFL, optimal yields were achieved using non-pretreated samples at 35 MPa and US10-pretreated samples at 30 MPa, while for SWP, the highest yield was obtained from non-pretreated samples at 35 MPa. The extracted oils were rich in unsaturated fatty acids, particularly ω-3, ω-6, and ω-9, with BSFL oil exhibiting a high lauric acid content (24.11 g/100 g), suggesting its potential as an antimicrobial additive. The resulting protein meals were identified as promising candidates for food and feed formulations.

The second phase focused on the bioavailability and bioactivity of protein hydrolysates and peptide fractions derived from AHCK, BSFL, and SWP before and after simulated gastrointestinal digestion (SGD). Hydrolysates were produced using 2SD Amano protease (3% w/w), and peptide fractions were obtained by ultrafiltration into four molecular weight ranges (<3, 3–5, 5–10, and >10 kDa). Bioactivities were assessed through angiotensin-converting enzyme (ACE), α-glucosidase, dipeptidyl peptidase IV (DPP-IV), and pancreatic lipase inhibition assays, alongside in vitro antioxidant assays (DPPH, metal chelating activity (MCA), and ABTS) and *Caenorhabditis elegans* in vivo antioxidant models. SGD significantly enhanced the bioactivities of most fractions, particularly peptides <3 kDa. The <3 kDa SWP peptides exhibited strong ACE inhibition (IC_{50} = 0.05 mg/mL), while <3 kDa AHCK peptides demonstrated potent α-glucosidase inhibition (IC_{50} = 0.07 mg/mL), comparable to acarbose (IC_{50} = 0.06 mg/mL). LC–MS/MS identified bioactive sequences such as GPAGPQGPR (DPP-IV inhibitor), LPLP (ACE inhibitor), and AGDDAPR (multifunctional peptide in AHCK). Peptide fractions were rich in glutamic acid, aspartic acid, and essential amino acids, which contributed to their biological potency. Antioxidant assays revealed that SGD peptides exhibited significantly greater ($p < 0.05$) MCA and ABTS activities than their pre-digested counterparts. In vivo, *C. elegans* fed SGD peptides showed extended lifespan under oxidative stress conditions.

In the third phase, antimicrobial properties of insect oils and <3 kDa peptide fractions were evaluated against five foodborne pathogens (*Escherichia coli* TISTR 527, *Staphylococcus aureus* TISTR 746, *Bacillus cereus* TISTR 2372, *Salmonella typhimurium* TISTR 2519, and *Pseudomonas*

aeruginosa TISTR 2370). BSFL oil, enriched with lauric acid, exhibited strong inhibitory effects against *E. coli*, *S. aureus*, *B. cereus*, and *S. typhimurium*, though in effective against *P. aeruginosa*. Lauric acid was identified as the key antimicrobial constituent.

Finally, a techno-economic evaluation of AHCK protein hydrolysate production was performed using SuperPro Designer v11 software. The overall findings demonstrate that AHCK, BSFL, and SWP oils, proteins, and peptides possess significant bioactive potential, supporting their applications as natural antimicrobial, antioxidant, and enzyme inhibitory agents. Edible insect-derived biomolecules offer valuable prospects for use in functional foods, nutraceuticals, and cosmetic formulations, as well as sustainable alternatives in feed and food industries.

LIST OF ABBREVIATIONS

ABTS	2,2'-Azino-Bis (3-Ethylbenzothiazoline-6-Sulfonic Acid)
ACE	Angiotensin-converting Enzyme
AHCK	Adult house cricket
AOAC	Association of Official Analytical Chemists
ANOVA	Analysis of variance
AV	Acid value
BSFL	Black soldier fly larvae
CFU	Colony-forming unit
CO_2	Carbon dioxide
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
DPP-IV	Dipeptidyl Peptidase-IV
ETA	Eicosatetraenoic acid
FFA	Free fatty acid
HPLC	High-performance liquid chromatography
HPP	High-pressure processing
IC_{50}	Half-maximal inhibitory concentration
KOH	Potassium hydroxide
MIC	Minimum inhibitory concentration
MCA	Metal chelating activity
MW	Molecular weight
MWCO	Molecular weight cut-off
NCDs	Non-communicable diseases
NIZ	No inhibition zone
PV	Peroxide value
qPCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species

SC-CO_2	Supercritical CO_2 extraction
SE	Solvent extraction
SEM	Scanning electron microscope
SGD	Simulated gastrointestinal digestion
SPSS	Statistics Package for Social Science
SWP	Silkworm pupae
tBOOH	Tertiary-butylhydroperoxide
TAG	Triglyceride
TFA	Trifluoroacetic acid
TPC	Total polyphenol content
TVC	Total viable count
US	Ultrasonication

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CHAPTER I

INTRODUCTION

1.1 Background and Rationale

Edible insects have emerged as a promising solution for sustainable nutrition, providing high-quality proteins, healthy fatty acids, and other essential nutrients through more environmentally friendly production processes compared to conventional protein sources (Kim & Oh, 2022). The two principal components of insects—proteins and fats—are widely utilized in the food and animal feed industries. Their protein content ranges from 37% to 76%, while fat content varies from 20% to 40% (SrarČeviĆ et al., 2016). Insect proteins are rich in bioactive peptides with significant potential; peptides identified from edible insects have demonstrated anti-inflammatory, antihypertensive, antidiabetic, and antimicrobial properties (Rivero-Pino et al., 2024).

Insect-derived oils are equally noteworthy for their favorable fatty acid profiles, comprising oleic, palmitic, linoleic, and linolenic acids, which contribute moisturizing and nourishing properties (Kolobe et al., 2023). These oils are an emerging source of beneficial omega-3, -6, and -9 fatty acids with potential applications in food, nutraceutical, and cosmetic formulations (Franco et al., 2022). Studies have also indicated that different insect species possess distinct protein and oil compositions, contributing to species-specific nutritional and functional characteristics (Li et al., 2023). Furthermore, insect-based foods have been reported to be acceptable to consumers with regard to sensory attributes such as flavor, aroma, and texture (Roma et al., 2020).

Despite their potential, extracting high-quality oils from insects presents several challenges. Conventional extraction techniques often involve harsh conditions that may reduce nutritional value, degrade thermolabile bioactive compounds, and negatively affect functional properties (Vieira et al., 2023). Consequently, research has increasingly focused on the development of alternative, mild, and sustainable extraction methods and pretreatment processes.

Supercritical carbon dioxide (SC-CO_2) extraction has emerged as an efficient and environmentally friendly technology for recovering oils from insect biomass. This green extraction method maximizes yield while preventing thermal degradation (Cheseto et al., 2020; Kim & Oh, 2022; Saviane et al., 2021). The moderate critical parameters of SC-CO_2 (31 °C, 7.38 MPa) and its nonpolar, diffusive characteristics facilitate effective lipid solubilization from complex insect

matrices with minimal oxidative or thermal damage (Femenia et al., 2001; Temelli et al., 2012). Moreover, the use of entrainers such as ethanol can enhance the recovery of polar bioactive compounds during SC-CO_2 extraction (Roff & Greenlief, 2017).

To further improve extraction efficiency and quality, pretreatment processes such as high-pressure processing (HPP) and ultrasonication (US) have been explored. HPP has proven effective as a non-thermal preservation technology that maintains the fresh flavor, texture, and nutritional integrity of edible insects without the need for chemical preservatives (Huang et al., 2017). Operating at pressures between 100 and 600 MPa, HPP inactivates microorganisms and enzymes by inducing protein denaturation, cell membrane disruption, and molecular reconfiguration (Tornberg, 2005; Sevenich et al., 2014). Ultrasonication, in contrast, enhances matrix disintegration, extraction efficiency, and component recovery by generating localized pressure gradients, shear forces, and cavitation effects (Bußler et al., 2016; Gallo et al., 2018). These mechanical phenomena promote cell wall rupture, thereby facilitating oil release (Yao et al., 2018; Naik et al., 2013).

Although previous studies have examined the effects of extraction techniques on insect lipid composition (Tzompa-Sosa et al., 2014), limited research has investigated the combined influence of pretreatments and extraction methods on the physicochemical properties of insect oils. Therefore, this study aims to address this knowledge gap by evaluating the effects of HPP and US pretreatments—individually and in combination—on the microbial reduction, proximate composition, and structural characteristics of three selected edible insect species: adult house cricket (*Acheta domesticus*, AHCK), black soldier fly larvae (*Hermetia illucens*, BSFL), and silkworm pupae (*Bombyx mori*, SWP). These species were selected for their commercial availability in Thailand and their distinctive nutritional profiles. For instance, BSFL oil is particularly rich in lauric acid (38.43%), known for its antimicrobial potential (Ushakova et al., 2016), while silkworm pupae contain essential long-chain fatty acids such as eicosatetraenoic acid (ETA) and docosahexaenoic acid (DHA), which play critical roles in fetal development and cognitive function (Swanson et al., 2012; Rowe, 2020; Zhou et al., 2022).

This research investigates the effects of HPP and US pretreatments on microbial load reduction, proximate composition, and structural modifications of pretreated insect samples. Additionally, the study evaluates the oil yield, physicochemical characteristics, and fatty acid

composition of oils extracted using SC-CO_2 and compares the results with those obtained through conventional solvent extraction. The defatted insect proteins were subsequently hydrolyzed using commercial enzymes and fractionated by membrane ultrafiltration. The resulting insect protein hydrolysates and peptide fractions were evaluated for their bioavailability and biological activities, including α-glucosidase inhibition, angiotensin-converting enzyme (ACE) inhibition, dipeptidyl peptidase IV (DPP-IV) inhibition, pancreatic lipase inhibition, antioxidant activity, antimicrobial potential, and identification of specific bioactive peptide sequences.

1.2 Research Objectives

- To investigate the effects of combined pretreatment processes—high-pressure processing (HPP) and ultrasonication (US)—coupled with supercritical carbon dioxide (SC-CO_2) extraction on the yield and physicochemical properties of edible insect oils.
- To evaluate the effects of simulated gastrointestinal digestion (SGD) on the bioavailability and biological activity of insect protein hydrolysates and peptide fractions.
- To examine the antimicrobial activities of edible insect oils and peptide fractions against selected foodborne pathogenic bacteria, including *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Salmonella typhimurium*, and *Pseudomonas aeruginosa*.
- To generate fundamental laboratory data to support the design and optimization of industrial-scale production processes for insect-derived proteins and oils.

1.3 Hypothesis of the Research Project

- Pretreatment processes applied prior to oil extraction, such as high-pressure processing (HPP) and ultrasonication (US), enhance the efficiency of supercritical carbon dioxide (SC-CO_2) extraction. Furthermore, variations in SC-CO_2 extraction pressure (25, 30, and 35 MPa) significantly influence the yield and physicochemical properties of edible insect oils.
- Insect-derived proteins and peptides possess bioactive potential, exhibiting inhibitory effects against key metabolic enzymes, including α-glucosidase, dipeptidyl peptidase IV (DPP-IV), angiotensin-converting enzyme (ACE), and pancreatic lipase.

- Extracts obtained from edible insects display antimicrobial properties and demonstrate strong potential as alternative natural ingredients for applications in the food, feed, and cosmetic industries.

1.4 Concepts, Scope of Research

The raw materials used in this research consist of 3 species of edible insects.

Adult house crickets; AHCK (*Acheta domesticus*), adult stage, 40 - 45 days old

Black soldier fly larvae; BSFL (*Hermetia illucens*), larval stage, 20 - 25 days old.

Silkworm pupae; SWP (*Bombyx mori*), pupal stage, 45 - 50 days old

All edible insects were kindly supported by Thai Ento Food Co., Ltd., Samutprakarn, Thailand. The step of this research is shown in Figure 1.

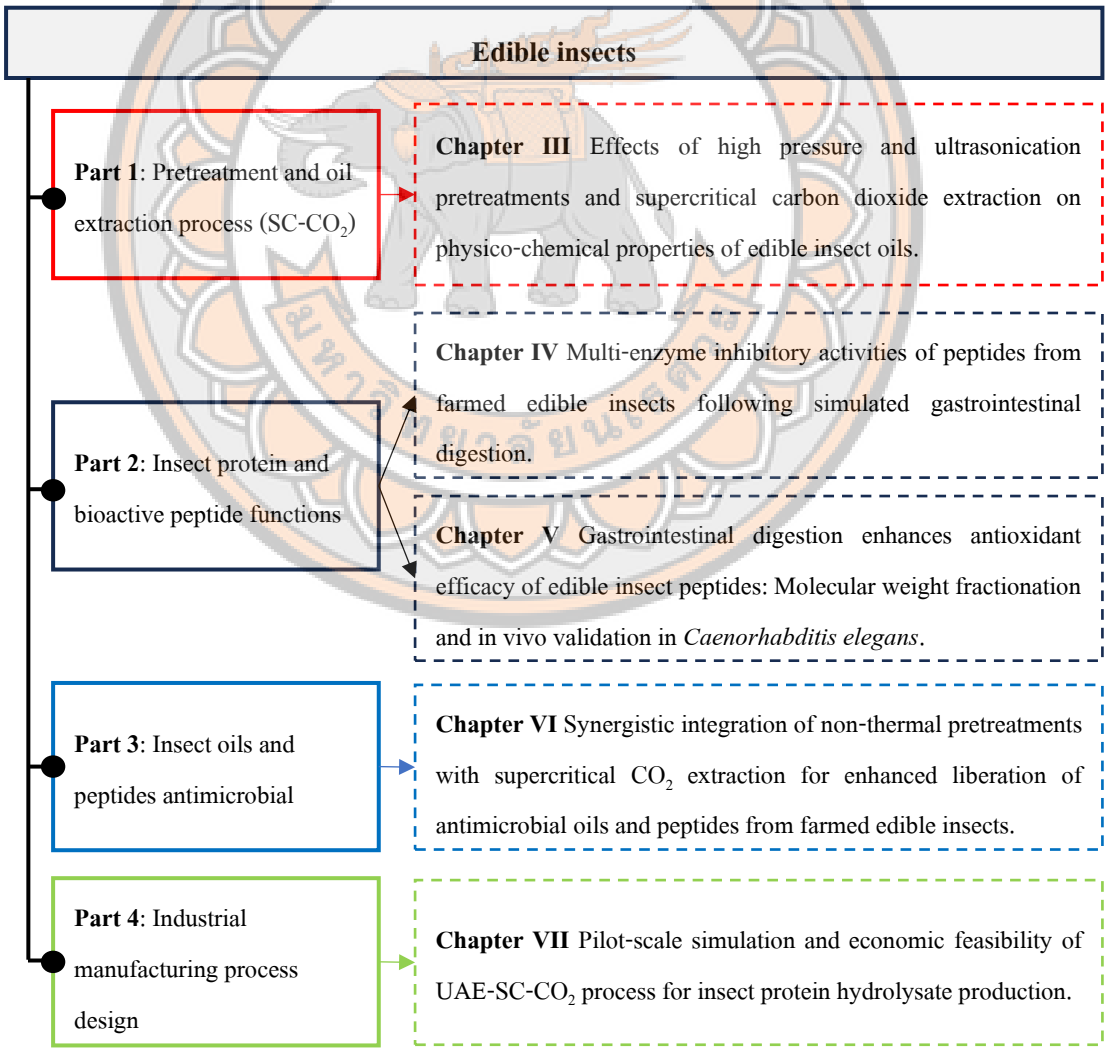


Fig. 1 Step of research

Insects were stunned using carbon dioxide and stored at -18 ºC before being used in the research process. Insect samples were transported frozen at -18 ºC and packed in 54 cm x 38 cm x 20 cm foam boxes to maintain the temperature during the transport process. This research was divided into 4 parts as follows:

Analysis of raw materials

After receiving the sample, before starting the research process, the AHCK, BSFL, and SWP samples were thawed by placing them in a refrigerator at 4 ± 1 ºC overnight. Then start by sorting out extraneous materials that may be contaminated with the raw materials during the raw material storage process, cleaned with distilled water, and heated at 100 ± 1 ºC for 5 min to reduce the initial bacteria. When the water temperature reaches 100 ºC, the heated time should be counted. Then, 100 kg of boiled insects were separated to 1 kg per bag and hot-filled in a 20 x 30-inch Polyethylene plastic bag. Store at -18 ºC before using in the research studies. In this part, the initial quality of the insects was analyzed, including moisture content, protein content, fat content, fiber, ash, carbohydrate content, and the initial microbial load, as total viable count and yeast & molds.

Part 1: Study of pretreatment processes and oil extraction processes

Boiled AHCK, BSFL, and SWP underwent pretreatment using HPP at 200, 400, and 600 MPa for 5 min or US at 37 kHz for 5, 10, and 15 min, followed by dehydration using hot air oven at 70 °C, for 10 - 12 h until the samples reached a target moisture content between 5 - 8 %, verified using a halogen moisture analyzer. The dried insect samples were then milled into a powder using a commercial blender (Vitamix) and sieved through an 18-mesh screen to standardize particle size distribution. The AHCK, BSFL, and SWP powdered samples were sealed in polyethylene bags and stored at 4 ± 1 °C until use for oil extraction with SC-CO_2. The effects of HPP or US pretreatments on bacterial reduction (whole insect samples) and structural changes (dried full fat insect milled) were investigated. Additionally, the yield, oil qualities, physicochemical properties, and fatty acid profiles of the oils obtained through SC-CO_2 extraction were evaluated. SE was used for reference.

The optimal condition of the oil extraction process was selected by considering the amount of oil extracted (% Yield), followed by consideration of the quality of the extracted oils, including peroxide value (PV), acid value (AV), and free fatty acid (FFA), respectively.

Part 2: Testing of bioavailability and biological properties of protein hydrolysate and peptide extracted from AHCK, BSFL, and SWP.

Part 2.1, insects were pretreated with US (37 kHz, for 10 min), partially defatted using SC-CO_2 (25 MPa at 37 ± 1 °C for 2 h and a CO_2 flow rate of 24 - 26 L/h), enzymatic hydrolysis, and peptide fractionation. Insect protein hydrolysates were fractionated by molecular weight (<3 kDa, 3-5 kDa, 5-10 kDa, >10 kDa) and evaluated for inhibitory activities against DPP-IV, α-glucosidase, ACE, and pancreatic lipase both before and after SGD.

Part 2.2, the samples were prepared following Part 2.1. Total polyphenol content, in *vitro* antioxidants (ABTS, DPPH, and MCA), and in *vivo* antioxidants (antioxidant activities under chronic and acute oxidative stress) in *C. elegans* model were evaluated.

Step 3: Testing of antimicrobial activities of oil and peptide extracted from insects.

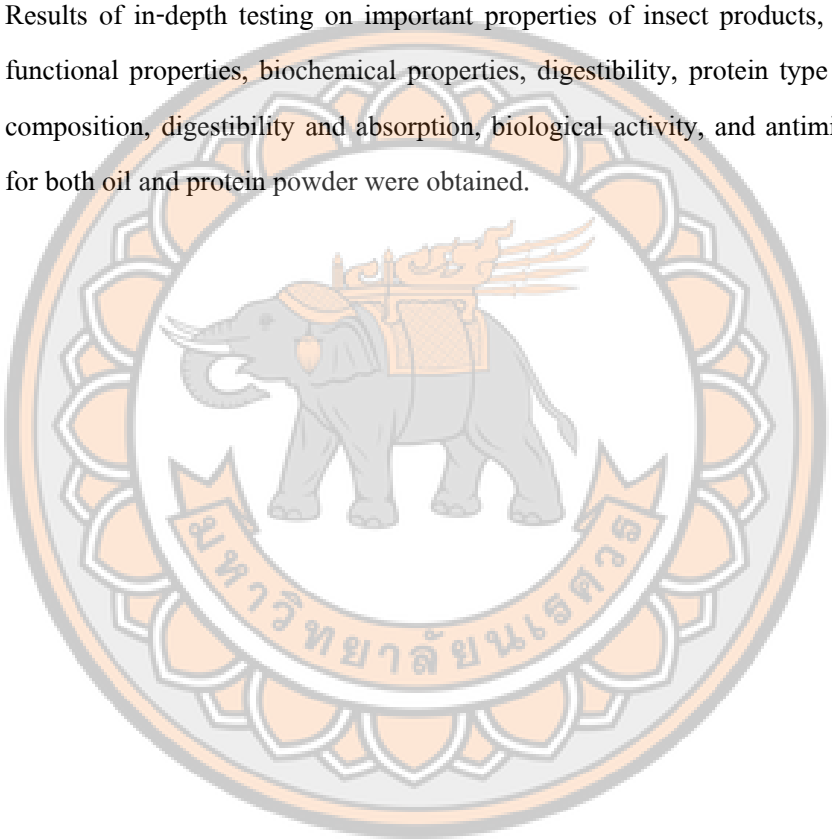
Five strains of foodborne pathogenic bacteria, consisting of *Escherichia coli* TISTR 527, *Staphylococcus aureus* TISTR 746, *Bacillus cereus* TISTR 2372, *Salmonella typhimurium* TISTR 2519, and *Pseudomonas aeruginosa* TISTR 2370, were used to evaluate the potential of insect oils and peptides for antimicrobial properties. Insect oils obtained from all oil extraction processes. In terms of insect peptide, only <3 kDa for each were used. Firstly, all insect oils and peptides were measured for antimicrobial properties using the agar diffusion method. After that, the potential samples (show an inhibition zone) were selected and used for the minimum inhibitory concentration (MIC) test.

Step 4: Industrial production process design

This research focuses on the industrial process design of AHCK protein hydrolysate production. Based on the selected hydrolysis method identified in Step 2, the study aims to develop an efficient and scalable production process suitable for industrial application. The scope includes designing the process flowsheet, establishing material and energy balances, and performing techno-economic analysis using SuperPro Designer v11 software. The simulation results will be used to estimate production costs, evaluate process feasibility, and identify key cost drivers.

1.5 Expected Benefits from the Research Project

- Obtain appropriate technology and sample preparation conditions before the extraction process of oil and protein from edible insects.
- Obtain suitable technology and process for extracting oil from insects using appropriate semi-industrial supercritical fluids.
- The process of extracting protein powder from insects using commercial enzymes and filtration through suitable membranes was developed.
- Results of in-depth testing on important properties of insect products, such as techno-functional properties, biochemical properties, digestibility, protein type and amino acid composition, digestibility and absorption, biological activity, and antimicrobial activity, for both oil and protein powder were obtained.



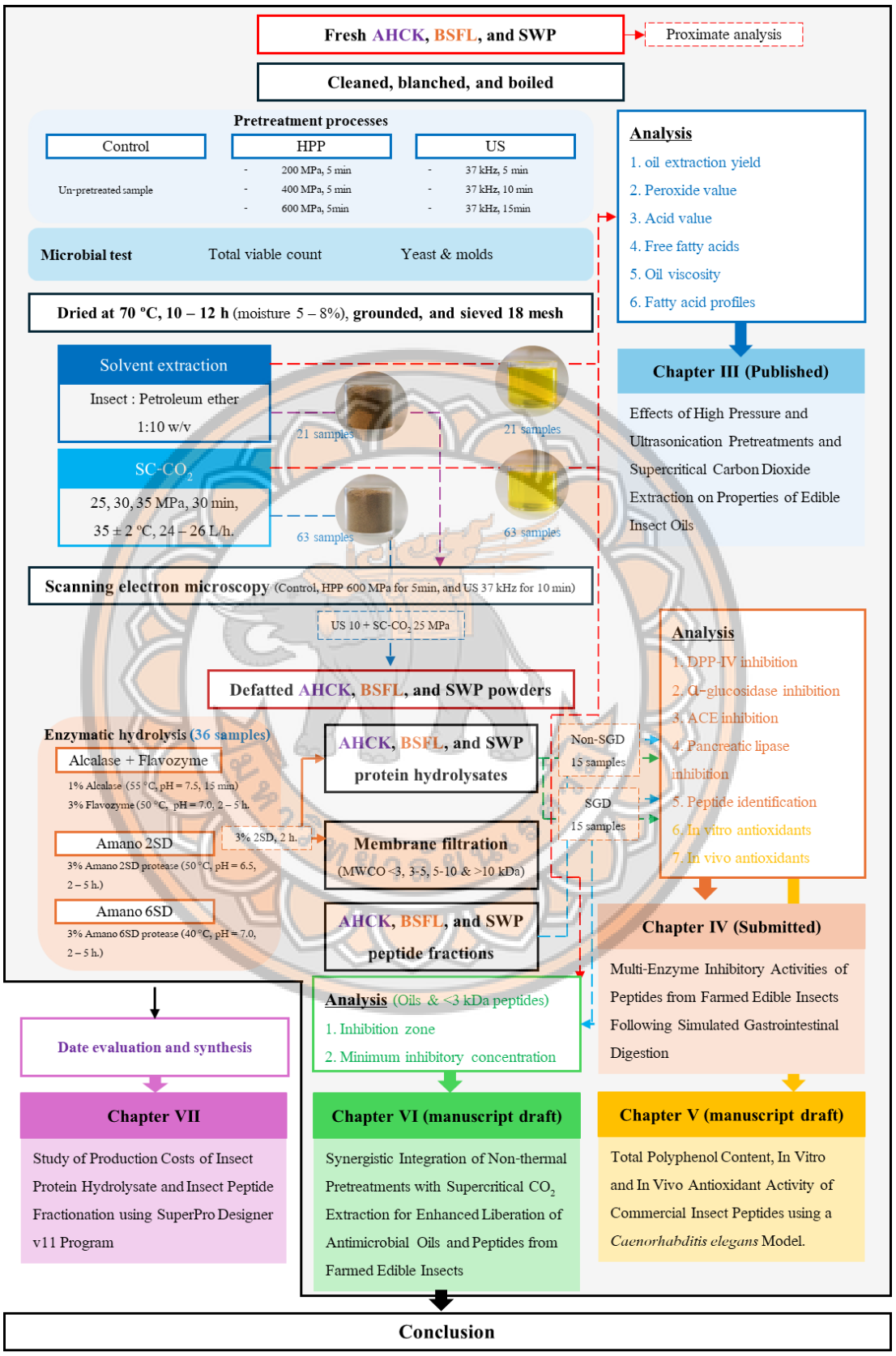


Fig. 2 Research flow chart

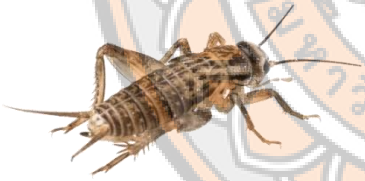
CHAPTER II

LITERATURE REVIEW

2.1 Edible Insects

Edible insects, also referred to as edible bugs or insects for food, are species that can be safely consumed by humans. They represent a nutritious and sustainable food source, being rich in proteins, lipids, vitamins, and minerals (Tang et al., 2019). Traditionally consumed across many cultures, edible insects are increasingly recognized worldwide as a promising component of future food systems. In light of global challenges such as population growth, water scarcity, limited arable land, and climate change, enhancing the sustainability of food production has become essential. Owing to their high feed conversion efficiency, low environmental impact, and favorable nutritional profile, edible insects hold significant potential as an alternative source of food. Our research group has investigated various aspects of insect incorporation into the human diet (Liu & Zhao, 2019).

2.1.1 House Cricket



Scientific classification

Scientific name: *Acheta domesticus*

Kingdom: *Animalia*

Phylum: *Arthropoda*

Class: *Insecta*

Order: *Orthoptea*

Family: *Gryllidae*

Fig. 3 House crickets

House crickets (Figure 3) are an edible insect that is gaining attention and has the potential to be used in the animal feed and food industries because they are an alternative source of protein and other nutrients, such as fat. They are an alternative source of protein and fat that is safe, environmentally sustainable, and has a high biological value (Pilco-Romero et al., 2023). On February 10, 2022, Commission Implementation Regulation (EU) 2022/188 was enacted, authorizing the marketing of *Acheta domesticus* and house crickets in frozen, dried, and powdered forms as new foods according to Regulation (EU) 2015/2283 of the European Parliament and the Council (Siddiqui et al., 2023).

2.1.2 Black Soldier Fly Larvae

Scientific classification

Scientific name: *Hermetia illucens*

Kingdom: *Animalia*

Phylum: *Arthropoda*

Class: *Insecta*

Order: *Diptera*

Family: *Stratiomyidae*

Fig. 4 Black soldier fly larvae

Black soldier fly larvae (Figure 4) are the larval stage of the black soldier fly, scientifically known as *Hermetia illucens*. They are a crucial part of the fly life cycle, lasting approximately 7-30 days depending on environmental conditions (Ferrarezi et al., 2016). During this stage, the larvae feed voraciously on organic materials and waste. It has been proven to convert organic waste into high-quality nutrients, such as proteins and oils, for pet foods and animal feeds (Siddiqui et al., 2022). Black soldier fly females can lay hundreds of eggs. The growth and reproduction of black soldier fly larvae are similar to those of other insects and depend on complex interactions, such as ambient temperature, humidity, lighting, nutritional value of the substrate consumed, and microbial colonization (Liu et al., 2022). The primary fatty acids in black soldier fly larvae are lauric acid, followed by palmitic, oleic, myristic, and linoleic acids. Lauric acid is an active saturated fatty acid that can be converted to monolaurin, which has potent antibacterial, antifungal, and antiviral properties (Phongpradist et al., 2023).

2.1.3 Silkworm Pupae

Fig. 5 Silkworm pupae

Scientific classification

Scientific name: *Bombyx mori*

Kingdom: *Animalia*

Phylum: *Arthropoda*

Class: *Insecta*

Order: *Lepidoptera*

Family: *Bombycidae*

Silkworm pupae (Figure 5) are the dormant, pre-moth stage of silkworms and are a good source of protein and other nutrients. They are often used as a food source for humans, pets, and animal farms. Silkworm pupae are a rich source of protein, lipids, minerals, and vitamins, and are considered a valuable source of nutrients for humans (Wu et al., 2021). In recent years, the biological functions of silkworm pupae have gradually been identified and confirmed. There were positive effects on liver protection, immune enhancement, antiapoptosis, antitumour, antibacterial, regulation of blood glucose and blood lipids, and lowering of blood pressure (Zhou et al., 2022).

2.1.4 Nutrition Composition of Edible Insects

Edible insects are a rich source of essential nutrients, including protein, healthy fats, vitamins, and minerals. They are also a good source of dietary fiber and can be a sustainable food source. The specific nutritional profile of insects can vary depending on the species, life stage, and diet (Figure 6) (Anankware et al., 2021; Shah et al., 2022; Kouřimská & Adámková, 2016). Many studies have shown that proteins, lipids, and other elements from edible insects can replace conventional sources of food and animal feed (Zhou et al., 2022; Sosa et al., 2017).

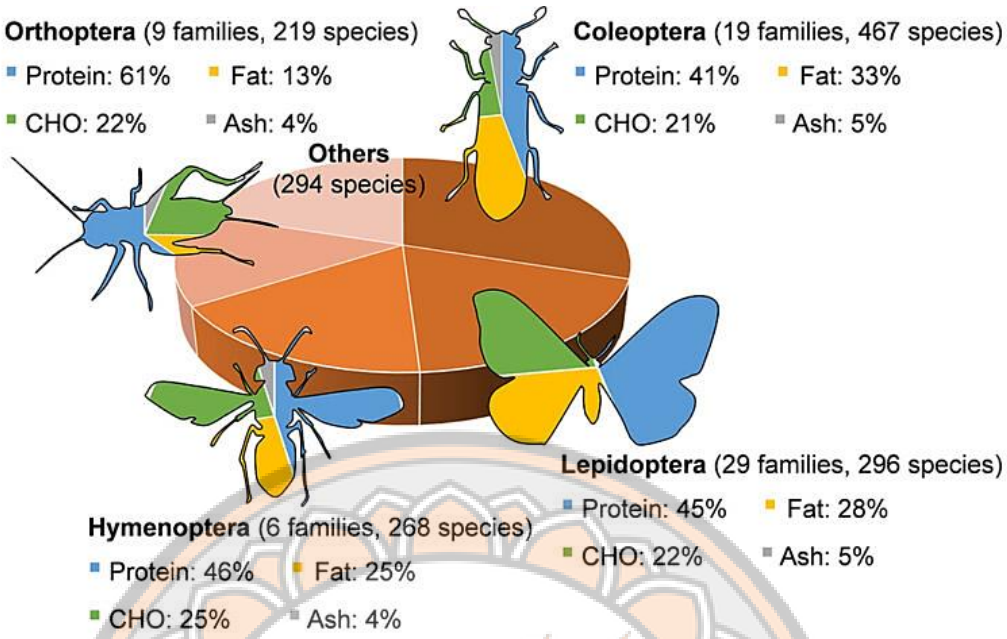


Fig. 6 The nutritional profiles of different orders of insects

Source: Liu & Zhao (2019)

Edible insects are used as a high-protein raw material and can be a part of animal meat in commercial baked and food products (Figure 7). Insect protein powders can increase the protein, dietary fiber, and essential amino acids content of bakery products and show similar content of protein, essential amino acids, and essential fatty acids of meat products (Yang et al., 2023).

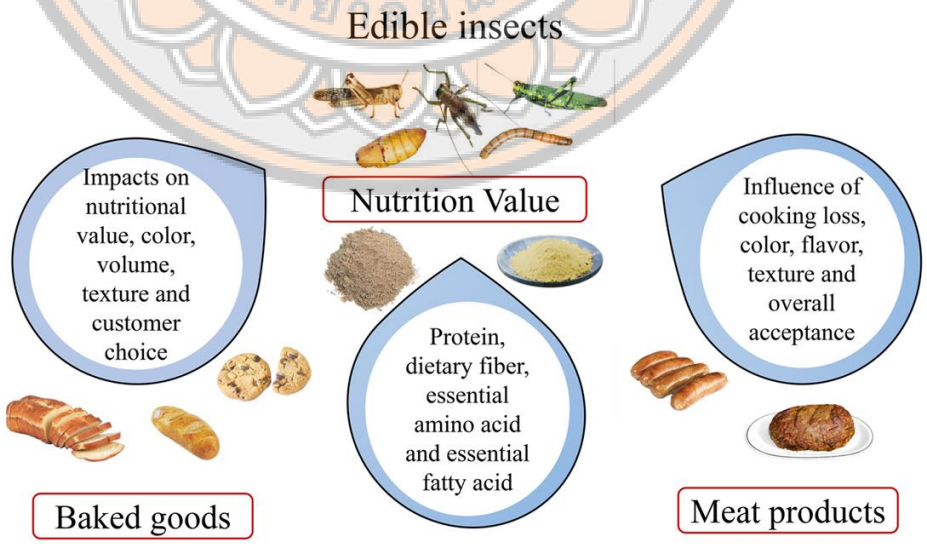


Fig. 7 The use of edible insects in bakery and meat products

Source: Yang et al. (2023)

The proximate compositions of edible insects, such as house crickets, black soldier fly larvae, silkworm pupae, mealworms, and locusts, are shown in Table 1. The nutritional value of edible insects varies widely and depends on the species, stage, origin, and diet (Kouřimská & Adámková, 2016). Edible insects generally contain high levels of protein (Yang et al., 2023). On a dry matter basis, the protein content of edible insects has been reported to ranges from 20 % to 70 % (Hasnan et al., 2023; Köhler et al., 2019). The lipid content of edible insects depends on several factors, such as the life stage and feed, and ranges from 10 % to 60 % on a dry matter basis (Perez-Santaescolastica et al., 2023).

Table 1. Proximate nutrient compositions of whole edible insects used as food and feed

Compositions	AHCK[a]	BSFL[b]	SWP[c]	Mealworm[d]	Locust[e]
Crude protein (%db)	51.12	43.10	49.19	49.08	46.27
Crude lipid (%db)	13.42	38.60	30.47	34.56	32.29
Ash (%db)	8.99	2.70	4.73	3.02	6.68
Crude fiber (%db)	8.14	4.10	5.46	4.84	4.83
Carbohydrate (%db)	18.33	11.50	10.15	No data	No data

Source: [a] Adesoye et al. (2023); [b] Lu et al. (2022); [c] Hirunyophat et al. (2021); [d] Ravzanaadii et al. (2012); [e] Kinyuru (2021).

Edible insects typically have higher levels of crude protein than conventional protein. Furthermore, based on the comparison conducted by Van Huis et al. (2013) and the literature reviewed by Churchward-Venne et al. (2017), the protein content of selected insect orders is comparable to that of eggs, soy, and milk. Remarkably, the protein content of grasshoppers (*Sphenarium purpurascens*) (35 - 48 %) is higher than that of conventional protein sources such as beef (17.17 - 32.27 %), pork (17.43 - 21.1 %), chicken (16.52 - 22.5 %), and fish (16.38 - 29.4 %) (Hasnan et al., 2023). Table 2 shows a comparison of the nutrient compositions of crickets and conventional protein products.

Table 2. Comparison of proximate nutrient compositions found on edible insects and conventional protein products used for human consumption

Compositions	Crickets[a]	Pork[b]	Chicken Breast[c]	Milk[d]	Soybean[e]	Egg york[f]
Protein (%db)	63.00	81.71	81.99	42.21	34.35	32.10
Lipid (%db)	20.40	17.82	11.23	15.65	17.97	50.90
Ash (%db)	8.30	4.61	5.80	5.31	1.37	3.30
Fiber (%db)	0.66	No data	No data	6.97	23.33	0.80
Carbohydrate (%db)	7.64	No data	No data	6.18	22.94	12.90

Source: [a] Egonyu et al. (2025); [b] Zduńczyk et al. (2024); [c] Fakolade (2015); [d] Hew et al. (2023); [e] Kowmudi et al. (2023); [f] Kruenti et al. (2022).

2.1.5 Bioactive Compound and Biological Activity of Edible Insects

Edible insects are rich in bioactive compounds such as chitin, peptides, and phenolic compounds, which exhibit antioxidant, antimicrobial, and disease-preventing properties (Figure 8). These compounds contribute to the potential health benefits associated with insect consumption, including anti-inflammatory, antidiabetic, and antihypertensive effects (Sánchez-Estrada et al., 2024).

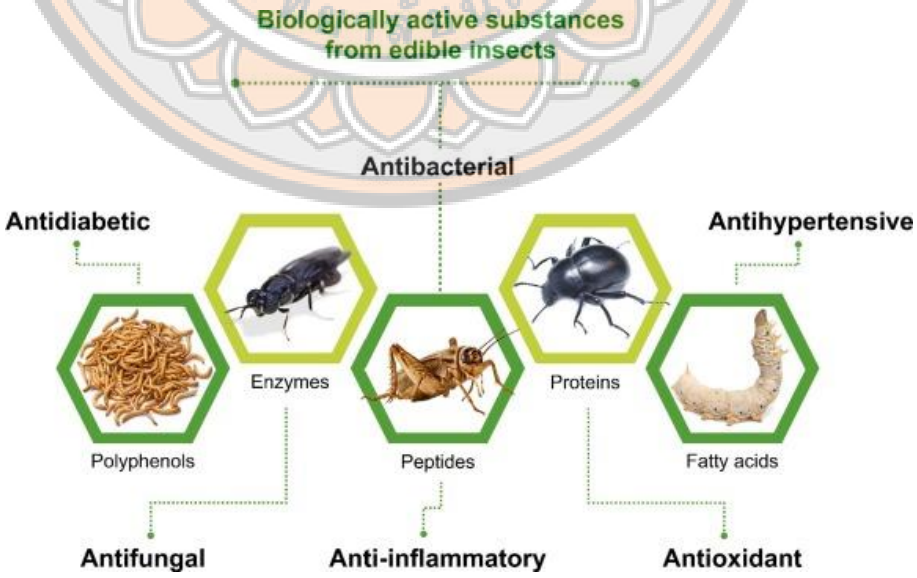


Fig. 8 Edible insects as a source of biologically active substances and properties

Source: Castro-López et al. (2020)

2.1.5.1 Insect oils

Edible insect oils are a sustainable and nutritious alternative to traditional oils, offering a rich source of healthy fatty acids, such as lauric acid, oleic acid, omega-3 and -6, vitamins, and other bioactive compounds. It can be extracted from various insects, such as crickets, silkworms, and beetles, and has potential applications in food, feed, biofuel, and cosmetics (Lumanlan et al., 2022).

The fatty acid composition and bioactive compounds in edible oils play an important role in determining their nutritional value and suitability for use in various food systems. As an important food ingredient, the physicochemical properties of oil also affect the quality and consumer acceptability of the final food product. Therefore, this review compiles information to provide knowledge and support for the future applications of insect-based oils in various food systems (Figure 9).

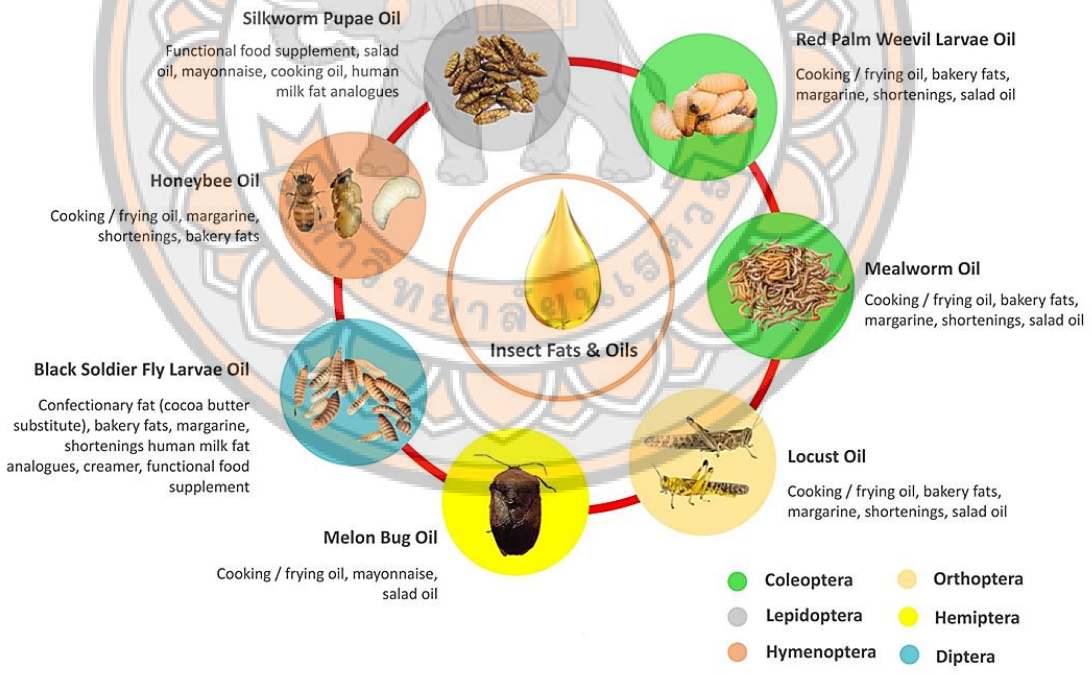


Fig. 9 Applications of insect-based oils and fats in various food systems

Source: Yap et al. (2021)

Insect oils are a rich source of fatty acids (Table 3), both saturated and unsaturated, with varying profiles depending on the insect species and diet. Common fatty acids found in insect oils include palmitic, oleic, myristic, and linoleic acids. Some insects also contain significant amounts of polyunsaturated fatty acids, such as linoleic and linolenic acids (Jayanegara et al., 2020; Kolobe et al., 2023).

Table 3. Fatty acid composition of selected edible insects

	Insect species				**FFA requirements**	
	Mealworm	Grasshoppers	Crickets	Black soldier fly	Humans	Poultry
Stage	*Larvae*	*Adult*	*Adult*	*Larvae*		
Fats (%)	24.7 - 43.1	30.5	12.2 – 23.0	18.0 – 32.6		
Compositions (g/ 100g)						
SFA	25.3 – 30.1	46.7 – 56.9	32.2 – 32.8	61.4 – 67.9	26.9	
C12:0	**0.14 – 0.29**		**0.02 – 0.10**	**43.1**		
C16:0	18.0 – 28.2	39.0 – 43.0	5.5 – 22.7	9.4 – 13.3		
UFA		43.2	64.4 – 67.4			
MUFA	43.3 – 66.8	25.7 – 28.0	21.7 – 33.5	17.4 – 26.1	30.9	
C18:1	40.9 – 60.6	22.9 – 26.2	20.2 – 31.1	2.6 – 12.8		
PUFA	3.1 – 31.4	17.5 – 25.2	33.9 – 42.6	9.2 – 14.7	16.9	
C18:2	2.8 – 29.7	3.4 – 9.3	32.2 – 41.3	6.4 – 13.9	15.0	
Omega 3	1.6	6.7	0.4	0.0		
Omega 6	29.7	10.7	42.6	8.2		0.8 – 2.0

Source: Kolobe et al. (2023); Suryati et al. (2023); Siddiqui et al. (2023)

Table 3 describes the fats and fatty acids composition of various commonly reared and harvested edible insects. All selected edible insects contain high fat content, ranging from 12.20 to 43.10 g/100g. The highest fat content is observed in mealworms. Similar results were reported by Hlongwane et al. (2020), who observed higher fat content in insect species from the Lepidopteran order (*caterpillars*) compared to other insect orders. The accumulation of lipid and fatty acid contents in different insect species depends on a combination of factors such as species type, sex, reproduction stage, developmental stage, living temperature, season, feed substrates, geographical origin, and processing methods, as well as biological factors including enzymatic activity (Akullo

et al., 2018; Kim et al., 2019; Kolobe et al., 2023; Mouter-Miranda et al., 2018; Mutungi et al., 2019)

Lauric acid, systematically dodecanoic acid, is a saturated fatty acid with a 12-carbon atom chain, thus having many properties of medium-chain fatty acids. It's found in many tropical oils, particularly coconut oil and palm kernel oil. The current study shows that BSFL fat (37.55 % lauric acid) is a comparable and alternative source to coconut oil (53.98 % lauric acid) to produce lauric acid-rich products (Vázquez et al., 2024; Kim et al., 2020). Lauric acid is also known to the pharmaceutical industry for its excellent antimicrobial properties (May & Cotton, 2012). In the body, lauric acid will change its form to monolaurin to make it more functional in maintaining health (Barlina et al., 2022).

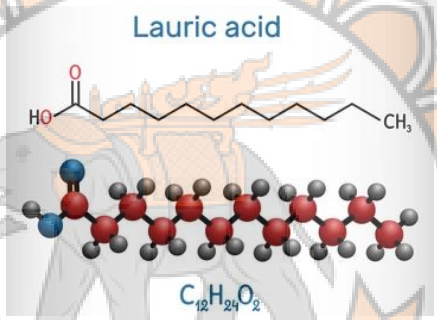


Fig. 10 Lauric acid

Source: Lauric acid, by Shutterstock, 2025, (https://www.shutterstock.com/image-vector/lauric-acid-dodecanoic-c12h24o2-molecule-saturated-1720399936)

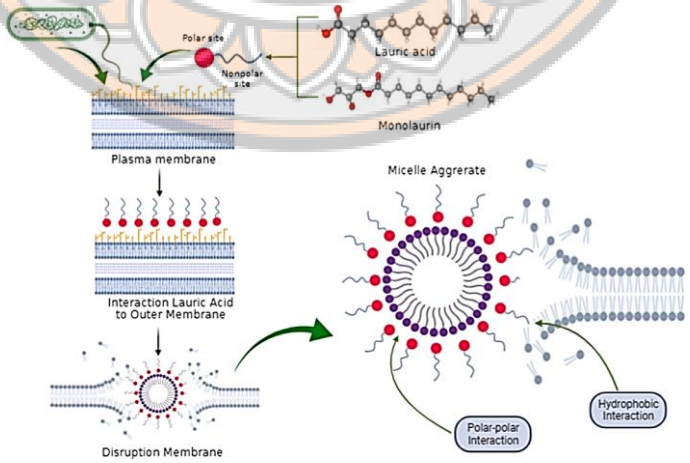


Fig. 11 Disruption membrane by lauric acid and monolaurin

Source: Nitbani et al. (2022)

Mechanism of lauric acid and monolaurin to antibacterial have been shown in Figure 11. The amphiphilic properties enable lauric acid and monolaurin to attack the structure of the cell membranes. Consequently, the damaged cell membranes are exposed to the surrounding environment, and the cytoplasmic fluid potentially leaks out. Meanwhile, the cytoplasmic fluid contains numerous organic compounds and enzymes that play a role in the cell metabolism of the bacteria. Therefore, leaking of the cytoplasmic fluid causes a decrease in the cell activity of the bacteria, leading to the death of the cells (Nitbani et al., 2022).

2.1.5.2 Insect proteins/ peptides

Edible insect proteins/ peptides are a source of high-quality protein derived from insects, offering a potentially sustainable and nutritious alternative to traditional protein sources. They are rich in essential amino acids, vitamins, and minerals. Insects can be a complete protein source, meaning they contain all nine essential amino acids, and their protein content can be comparable to or even higher than some plant-based proteins, like soybeans or lentils (Castro et al., 2018; Kim et al., 2019).

The amino acid composition of edible insects is comparable to that of traditional protein sources like meat and dairy. While the specific amounts of each amino acid can vary between insect species, some key essential amino acids found in high concentrations include lysine, leucine, and valine (Köhler et al., 2019; Oliveira et al., 2024). For example, based on data of five insects, including yellow mealworm, house cricket, super worm, lesser mealworm, and the roach, it was found that the amount of essential amino acids (EAA) was high and that the content of protein was similar to that of conventional meat products (Meyer-Rochow et al., 2021).

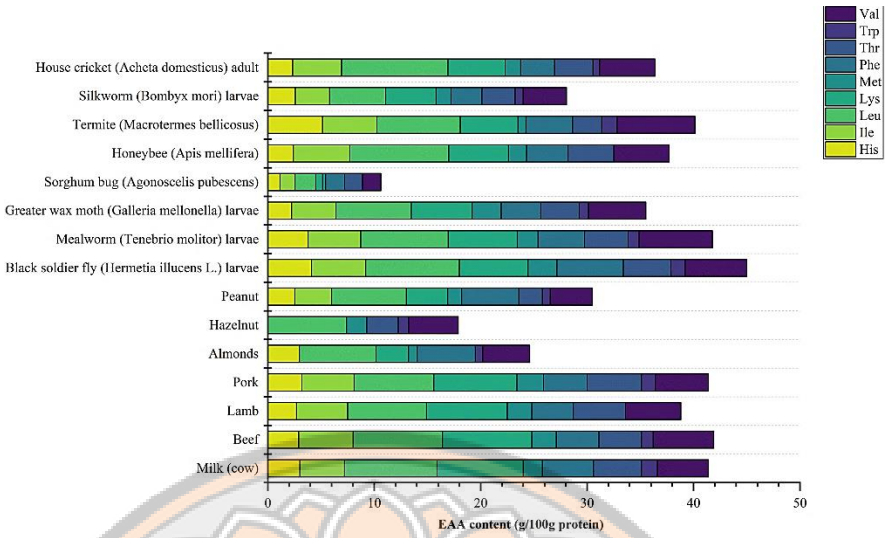


Fig. 12 Comparison of essential amino acid contents of insect and common food sources.

Source: Ma et al. (2023)

A comparison of essential amino acid contents of diverse insect sources and well-known protein sources is displayed in Figure 12. The essential amino acid levels of a diverse set of insects were comparable to soybean proteins, while being lower than casein. BSFL seems to be the insect containing the highest essential amino acid amounts, reaching 45.00 g/100 g protein, comparable to beef (41.90 g/100 g protein). Widely researched mealworm, AHCK, and honeybee also contain a high content of essential amino acids: 33.84 g/100 g protein, 36.39 g/100 g protein, and 37.70 g/100 g protein, respectively. Moreover, the protein content in common edible insects such as AHCK (72.45 %), mealworm (45.00 %) and muga SWP (38.05 %) are higher than that in common legumes such as lentils (26.70 %), beans (23.50 %) and soybean (41.10 %) (Ma et al., 2023).

Table 4. The essential amino acid profile of insect soluble protein (g/ 100g dry basis).

	Gryllus bimaculatus[a]	***Acheta domesticus***[a]	***Bombyx mori***[a]	***Hermetia illucens***[b]	**Human daily requirements FAO/WHO/UNU (1985)**
His	0.65	0.19	0.08	3.02	1.5
Ile	2.15	0.21	0.18	5.21	3.0
Leu	3.50	0.33	0.49	7.87	5.9
Val	1.85	0.65	0.20	5.74	3.9
Lys	2.39	0.31	0.27	8.19	4.5
Met	4.01	0.02	0.04	Met+Cys 3.41	2.2
Phe	1.62	0.10	0.08	Phe+Tyr 14.28	3.8
Thr	1.09	0.23	0.17	4.46	2.3
Trp	-	-	-	1.57	0.6
Total EAA	17.26	2.03	1.52	53.76	

Source: [a]Sornkhan et al. (2024); [b]Miron et al. (2023)

The amino acid content of four insect-soluble proteins is shown in Table 4, compared with the WHO daily requirements. The nine essential amino acids, including histidine (His), Isoleucine (Ile), Leucine (Leu), Valine (Val), Lysine (Lys), Methionine (Met), Phenylalanine (Phe), Threonine (Thr), and Tryptophan (Trp), were identified. As illustrated in Table 4, when comparing the essential amino acid profiles of the four insects with the WHO-recommended daily requirements for adults, it becomes evident that some essential amino acids in the *Gryllus bimaculatus* and *Hermetia illucens* were present at higher concentrations than in milk for several key amino acids, though at lower levels than in pork, showing quantitative differences as reported by Mazhangara et al. (2019) and Landi et al. (2021). This positioning between plant and animal protein sources reinforces the potential of insects as intermediate protein alternatives in sustainable food systems, as suggested by Van Huis & Oonincx (2017).

In general, insects exhibit a vast biodiversity, comprising 95% of the animal kingdom. It has been estimated that more than 2000 different edible species of insects are consumed by humans, including beetles, caterpillars, bees, ants, grasshoppers, locusts, worms, and crickets (Tarahi et al., 2025). However, only a few species have been studied for their potential bioactive peptides. Common sources of insect-derived bioactive peptides are presented in Figure 13.

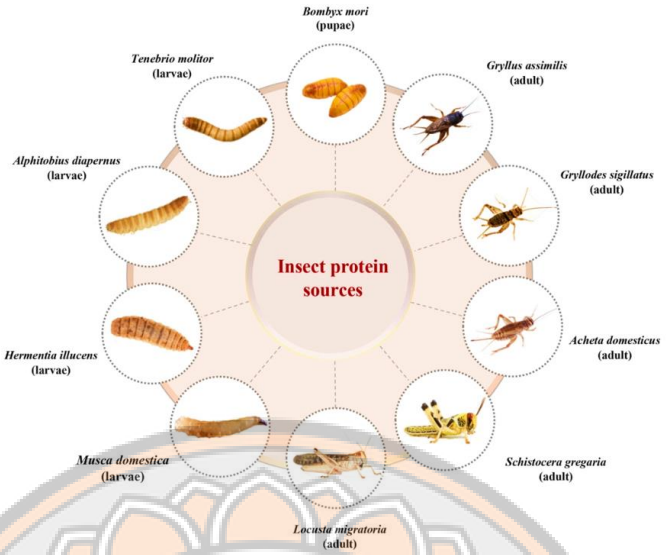


Fig. 13 Common sources of insect-derived bioactive peptides.

Source: Tarahi et al. (2025)

Bioactive peptides are polypeptides with a relatively low molecular weight, usually consisting of around 2 to 20 amino acid residues (Liu et al., 2023). These peptides have been shown to exhibit a diverse range of biological activities depending on their amino acid composition and sequence, including antioxidant, antihypertensive, anti-obesity, anti-cancer, antimicrobial, and immunomodulatory properties (Figure 14) (Singh et al., 2023).

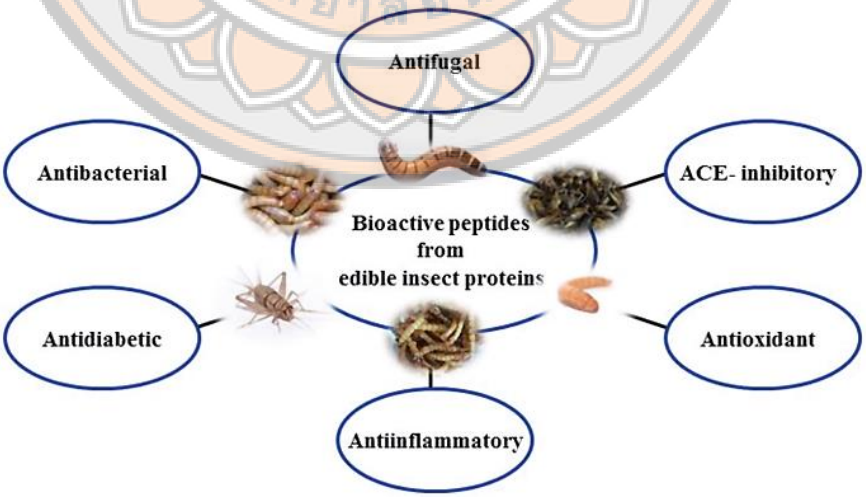


Fig. 14 Bioactive peptides from edible insects.

Source: Zielińska et al. (2019)

In general, bioactive peptides have a wide range of potential applications in food and non-food sectors due to their diverse health benefits and functional properties, as summarized in Table 5. These peptides can be used in developing nutraceuticals, functional foods, cosmetics, personal care products, and agricultural products to enhance human health, animal welfare, and environmental sustainability (Tarahi et al., 2025). Bioactive peptides derived from edible insects show promise as functional food components with potential health benefits. These peptides, which are short chains of amino acids, exhibit various bioactivities, including antioxidants, antihypertensive, anti-inflammatory, and antimicrobial effects. Edible insects provide a sustainable and nutritious source of these bioactive peptides, potentially aiding in the prevention and management of various health conditions (Ferrazzano et al., 2023; Psarianos et al., 2025).

Table 5. Summary of insect-derived bioactive peptides: bioactive activities, molecular characteristics, and key findings.

Insects	MW	Sequences	Bioactive peptides
SWP	<3 kDa	AAEYPA, AKPGVY	Antioxidant
	-	ASL	Anti-hypertensive
Mealworm	<1 kDa	FFGT, LSRVP, CTKKHKPNC	Antioxidant
	-	KVEGDLK, YETGNGIK	Antidiabetic
		AIGVGAIR, IIAPPER, FDPFPK	Anti-obesity
	90% less than 1 kDa	YKPRP, PHGAP, VGPPQ	Anti-hypertensive
AHCK	-	KDNEEAEAKPT, SLGGEMKQTAK	Antioxidant
	90% less than 1 kDa	YKPRP, PHGAP, VGPPQ	Antidiabetic Anti- hypertension
BSFL	955.4–1495.7 kDa	VEEPPKEE, VEEPPKEEKNPK, MAAGTNLLDTK, RPEELGPNK, FPGGETEALRR, AGGGGGGGGGGGKNL, IHKAGGGGGGGGGGK, HPERPIPEH, NWDLKEVGGGALP, SATTAIYMNALL,	Antioxidant

Source: Tarahi et al. (2025)

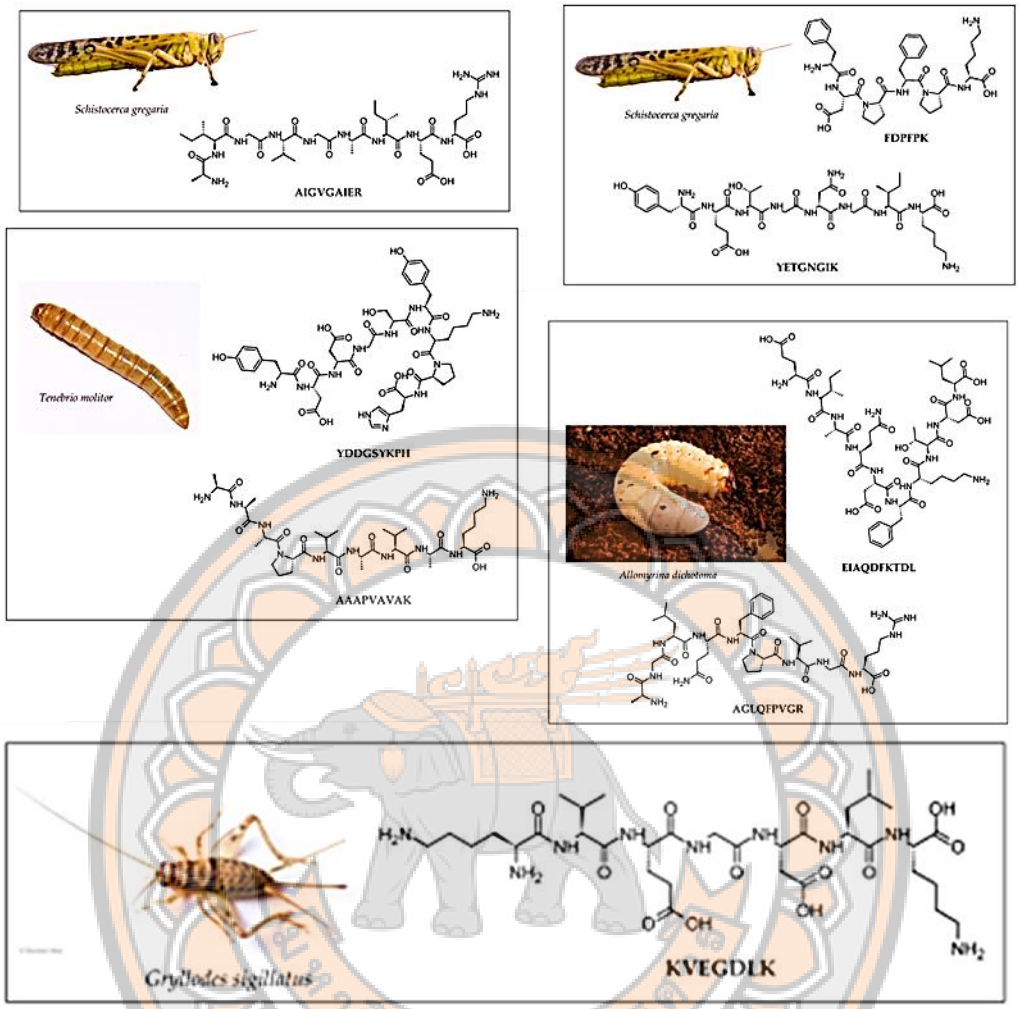


Fig. 15 The two-dimensional stuctures of insect bioactive peptides

Source: Quah et al. (2023)

The peptide FDPFPK (Figure 15) ranks among the most effective antioxidant peptides. Among the ACE inhibitory peptides derived from edible insects, YETGNGIK and KVEGDLK are noted for their strong properties. IIAPPER and AIGVGAIER are peptides that inhibit lipase, sourced from *Gryllodes sigillatus* and *Schistocerca gregaria*, respectively. The peptides AIGVGAIER, YDDGSYKPH, and AAAPVAVAK are identified as the most powerful Fe^{2+}chelating agents (Quah et al., 2023).

2.1.6 Edible Insects Market

The global edible insects market size was estimated at USD 1,350.0 million in 2024 and is projected to reach USD 4,383.0 million by 2030, growing at a CAGR of 25.1 % from 2025 to 2030 (Figure 16). The rising demand for high-protein, low-fat foods is a major driver as consumers increasingly seek healthier alternatives to traditional protein sources (Choudhury & Vidhate, 2025).

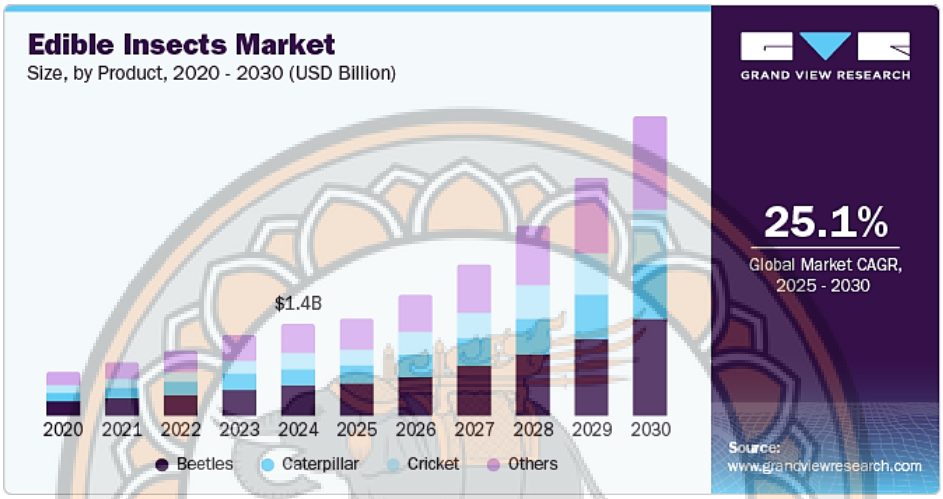


Fig. 16 Global edible insect market size

Source: Edible insect market 2020-2030, by Grand view research, 2025, (www.grandviewresearch.com)

The increasing demand for insects as a food source, driven by the rising cost of seafood or aquacultures, scarcity of conventional protein (Rumpold & Schlüter, 2013; Arru et al., 2019), and the low-cost, high-nutritional benefits of insect farming, is indeed expected to significantly impact the market positively. This trend is fueled by the need for sustainable and cost-effective food production, particularly as global populations grow (Boissat, 2025; Safavi et al., 2024).

Thailand's edible insect market is experiencing significant growth, driven by both domestic demand and export opportunities. The country is positioning itself to capitalize on the expanding global market for sustainable protein sources, with a focus on insect farming and growing domestic acceptance of edible insects. Popular insects consumed in Thailand include locusts, palm weevils, silkworm pupae, bamboo caterpillars, crickets, red ants, and giant water bugs (Krongdang et al., 2023).

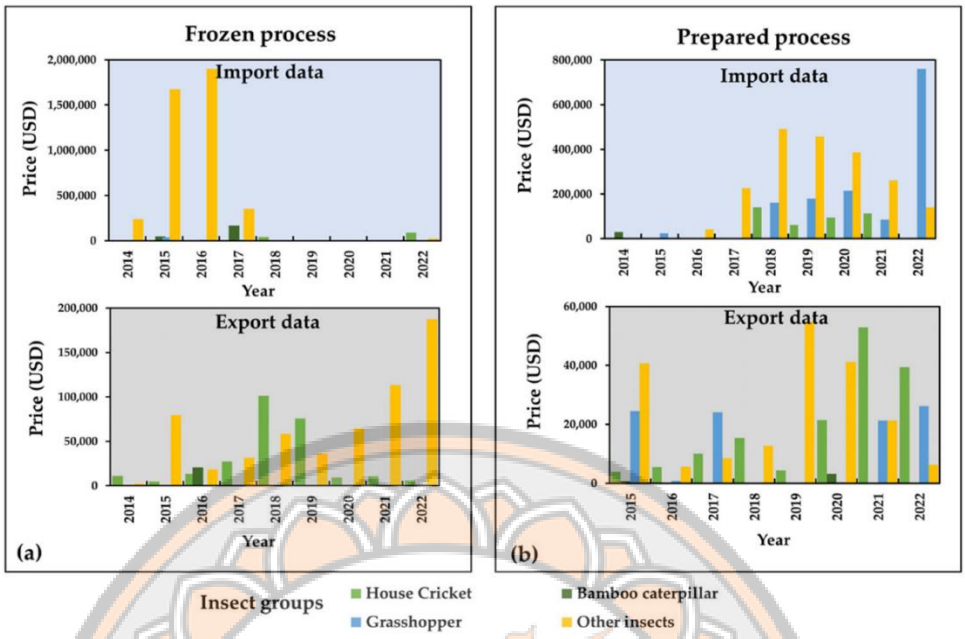


Fig. 17 Import and export data on edible insects in 2014–2022

(a) frozen edible insect products and (b) prepared edible insect products.

Source: Krongdang et al. (2023)

Thailand has the potential to become a leading producer of edible insect products for the world's population. Since 2004, Thailand has been a leading developer of insect-based products in the Asia-Pacific region, holding a 12% global market share for edible insect products or insect protein (Van Huis et al., 2023; Chantawannakul et al., 2020). Insect farming is widespread throughout Thailand, with the majority of farms located in the northeastern region of Thailand. It is estimated that 7,500 tons of insects, including species collected or farmed in neighboring countries such as Myanmar, Laos, and Cambodia, are consumed in Thailand annually. An overview of trade logistics and the supply chain of edible insects mainly originates from Southeast Asia, particularly Thailand, which has well-established farms and trade routes (Krongdang et al., 2023).

In Thailand, the edible insect market has experienced significant growth in recent years, focusing on both export and import flows of products, including frozen and processed goods (Figure 17). From 2014 to 2022, over five species of edible insects have been commercially imported and exported, including crickets, bamboo caterpillars, and grasshoppers, among others (Krongdang et al., 2023).

2.2 Pre-Treatment Process

2.2.1 High Pressure Processing

HPP is a non-thermal food preservation technique or food pasteurization method that uses high pressure to inactivate microorganisms, enzymes, and extend shelf life in food, while maintaining the food's natural qualities (Figure 18) (Balasubramaniam et al., 2015). This technique helps in achieving pasteurization with the aid of high pressure between 400 - 600 MPa under room temperature or slightly elevated temperature (<45 °C) (Gokul Nath et al., 2023).

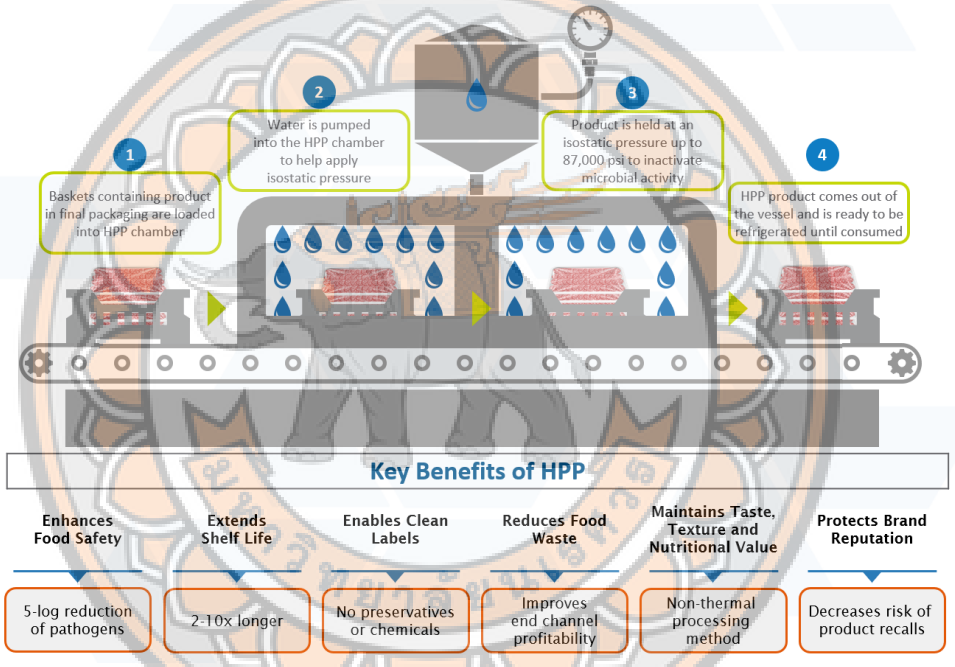


Fig. 18 High pressure processing: principle and key benefits

Note: From high pressure processing (HPP) for food safety and quality, by, Universalpure, 2025, (https://universalpure.com/high-pressure-processing/)

HPP can be used as a pretreatment before extraction to enhance the extraction of bioactive compounds. HPP disrupts cell walls, increasing cell permeability and facilitating solvent penetration, which leads to higher extraction yields, improved efficiency, and reduced extraction time (Zhao et al., 2024; Kotsoni et al., 2024). HPP can be utilized to enhance the extraction of bioactive components from animal, plant, and fungal sources (Tepsongkroh et al., 2023) and also improve the efficiency and yield of the oil extraction process (Zhang et al., 2021).

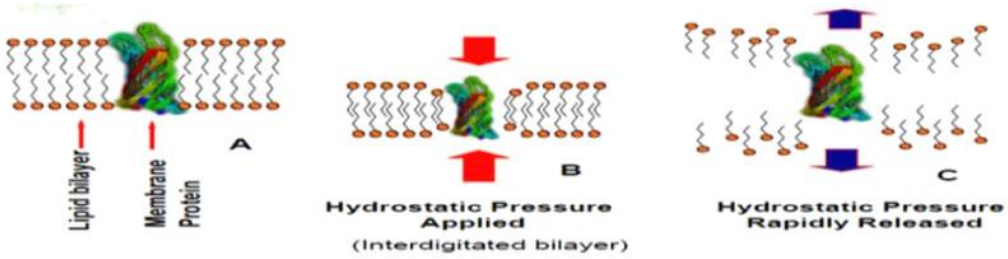


Fig. 19 Effect of high pressure on cell membrane materials

Source: Naik et al. (2013)

Figure 19 depicts the damage of cell membranes at high-pressure cycle; Figure 19A showed normal cell membrane, compression of cell membrane or cell wall material as and when pressure is applied (Figure 19B) and sudden expansion of lipid bilayer after pressure release (Figure 19C), leading to destruction and loses of cell membrane integrity (Naik et al., 2013).

HPP pretreatment can affect oil extraction yield, with the impact varying depending on the specific material, pressure, and duration of treatment. In some cases, HPP can increase oil yield, while in others, it may decrease it (Zhang et al., 2021; Kotsoni et al., 2024).

Extracts from the house cricket and mealworm were obtained by ultrasound-assisted extraction (UAE) and pressurized-liquid extraction (PLE) using ethanol (E) or ethanol: water (E: W). The highest extraction yield corresponded to PLE-T. *molitor* extracts. The highest extraction yield corresponded to PLE-mealworm extracts (Otero et al., 2020).

Zhang et al. (2021) reported that fish oil was extracted from yellowfin tuna (*Thunnus albacares*) heads using enzymatic hydrolysis extraction after single ultra-high-pressure pretreatment. The results showed that ultra-high pressure pretreatment of the tuna heads before enzymatic hydrolysis was suitable for extracting fish oil and effectively increased the oil yield.

The HPP pretreatments (50 - 250 MPa, 20 - 60 °C, 10 - 60 min) generally resulted in higher free oil recoveries from ground-sieved kernels than from the whole kernels. When the temperature and time were increased, the linear increment in free oil recovery also increased, but not the pressure level. Without the pre-boiling step in the aqueous enzymatic extraction process, the use of HPP pre-treatment at 50 MPa and 60 °C for 35 min resulted in approximately 73 % (w/w) free oil recovery (Yusoff et al., 2017).

2.2.2 Ultrasonication

US is a green pretreatment technique used to generate high shear stress for cell disruption (Priya et al., 2023; Li et al., 2022). It's a key technique in various scientific and industrial applications, is the process of applying high-frequency sound waves (above 20 kHz) to agitate particles in a liquid medium, using ultrasonic frequencies (Figure 20). This technique is widely used in various scientific and industrial applications due to its ability to induce cavitation bubbles that create intense localized heat and pressure, facilitating processes like mixing, homogenization, and extraction (Goutham, 2024).

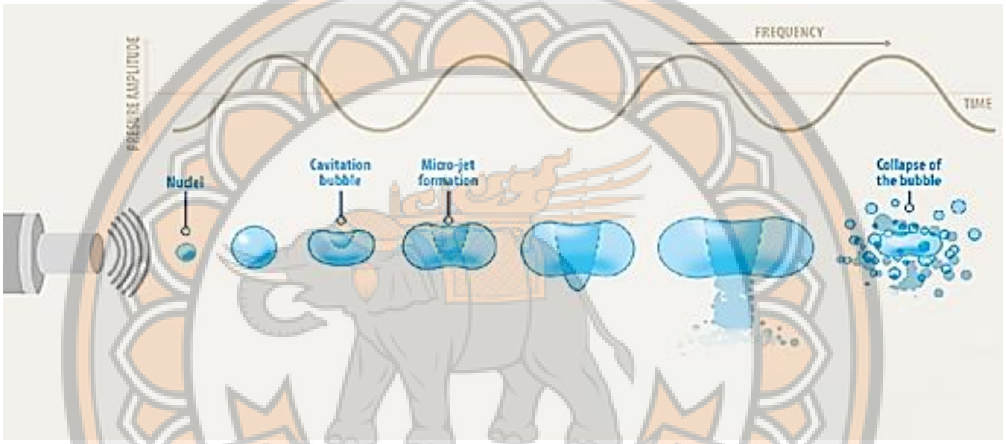


Fig. 20 Schematic representation of the ultrasonic cavitation phenomena

Source: Córdova et al. (2022)

US utilizes high-frequency sound waves to generate acoustic cavitation, which involves the formation and violent collapse of microscopic bubbles in a liquid. This process releases significant energy, disrupting molecular interactions and causing phenomena like dispersion, mixing, and sometimes even chemical reactions (Córdova et al., 2022; Vela et al., 2024). The physical effects of cavitation include intense microjets streaming with high velocity (hundreds of m/s) toward the surface of the treated particles in a very short time, shear forces, and shock waves generated by the collapsing bubbles. During collapse, the bubble becomes asymmetric, and its wall accelerates more on the side opposite to the solid surface, resulting in the formation of a strong microjet of water directed toward the particle's surface, which brings material fatigue followed by a gradual tearing off of microscopic particles capable of breaking polymeric chains (Figure 20) (Vela et al., 2024).

Ultrasonic pretreatment is a technique that uses high-frequency sound waves to disrupt materials, often to enhance subsequent processing. It works by generating cavitation bubbles that implode, creating localized heat and pressure that can break down structures and improve mass transfer. This method is applied in various fields like food processing, wastewater treatment, and biomass conversion to improve efficiency and product quality (Ramirez Cabrera et al., 2024)

Ultrasonic bath configurations are commonly used setups when conducting sonochemical reactions, particularly in the valorization of biomass. In an ultrasonic bath, a container is filled with a liquid medium, such as water or a solvent, and an ultrasonic transducer is immersed in the liquid (Lee et al., 2020). The transducer generates high-frequency sound waves above 20 kHz, which propagate through the liquid, creating cavitation bubbles. These bubbles implode, generating intense localized heat and pressure, which leads to the breakdown of biomass structures and facilitates chemical reactions (Sabaruddin et al., 2023). To use an ultrasonic bath for biomass valorization, the sample (biomass material) is typically placed in a suitable container or vessel that can be immersed in the liquid inside the ultrasonic bath (Figure 21).

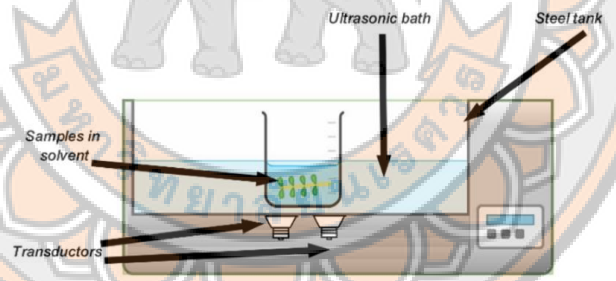


Fig. 21 Ultrasonic bath for biomass valorization

Source: Ramirez Cabrera et al. (2024)

The container holding the sample should be positioned in such a way that it is fully submerged in the liquid to ensure the efficient transmission of ultrasonic energy to the biomass material (Wu et al., 2019). The ultrasonic bath is then activated, and the high-frequency sound waves induce cavitation in the liquid, enhancing the breakdown of the biomass and promoting chemical reactions for valorization purposes. A transducer in an ultrasonic bath works by converting electrical energy into mechanical energy, which generates ultrasonic waves in the liquid medium. The transducer is typically attached to the bottom or sides of the bath, and it vibrates at a

high frequency, causing the liquid to vibrate as well. This vibration creates cavitation bubbles in the liquid, which implode and generate high temperatures and pressure, leading to the breakdown of the biomass and the promotion of chemical reactions for valorization purposes (Ramirez Cabrera et al., 2024; Sangave et al., 2004).

Factors affecting ultrasonic pretreatment:

- Frequency and power: different frequencies and power levels can be used depending on the application and material.
- Duration: longer sonication times can lead to greater disruption but may also cause undesired effects.
- Temperature: temperature control is important to manage the heat generated during cavitation.
- Biomass characteristics: the type and properties of the material being treated can influence the effectiveness of ultrasonic pretreatment.
- Ultrasonic extraction promotes the disintegration of plant inclusions through cavitation and mechanical vibration effects, which improves the extraction efficiency of essential oils (Ma et al., 2025).

The grounded mealworm larvae, cricket adults, and silkworm pupae powder were defatted with n-hexane for up to 48 h, sonication was applied for 1 - 20 min, and the protein yield was measured. All samples showed a total residual fat percentage below 1.36 %, and a 35 % to 94 % improvement in protein yield (%) (Choi et al., 2017).

The extraction of essential oil from *Pinus koraiensis* seed using ultrasonic pretreatment-microwave-assisted hydrodistillation. Under the optimization parameters of a liquid material ratio of 14 mL/g, power of 100 W, time of 15 min, temperature of 50 °C, and tea saponin dose of 0.4 % for the ultrasonication and then microwave at 458 W for 18 min, the actual yield was 8.02 ± 0.35 μL/g and was highly consistent with the theoretical yield of 8.21 μL/g (Li et al., 2024).

Sandhu et al. (2021) reported the effects of ultrasound pretreatment on the extraction of bioactive compounds and composition of essential oils extracted from citrus waste. The mass transfer rate of antioxidants from peel and pomace was 30% increased as a result of ultrasound-assisted treatment.

2.3 Oil Extraction Processes

Mechanical pressing and solvent extraction are conventional oil extraction processes. Extractions have moved from the conventional solvent and mechanical extractions to improved nonconventional techniques (Nde & Foncha, 2020). The major technologies being adopted in the recent scenario for the extraction of essential oil are microwave-assisted extraction, supercritical fluid extraction, pulsed electric field, ultrasound-assisted extraction, and subcritical extraction (Gaikwad et al., 2025).

2.3.1 Solvent Extraction

SE is the process of removal of a solute component from a solid using a liquid solvent and is one of the modern extraction processes. It is also called leaching or solid–liquid extraction (Babazadeh et al., 2022). Solvent extraction is based on the principle of differential solubility (González-Hernández et al., 2024).

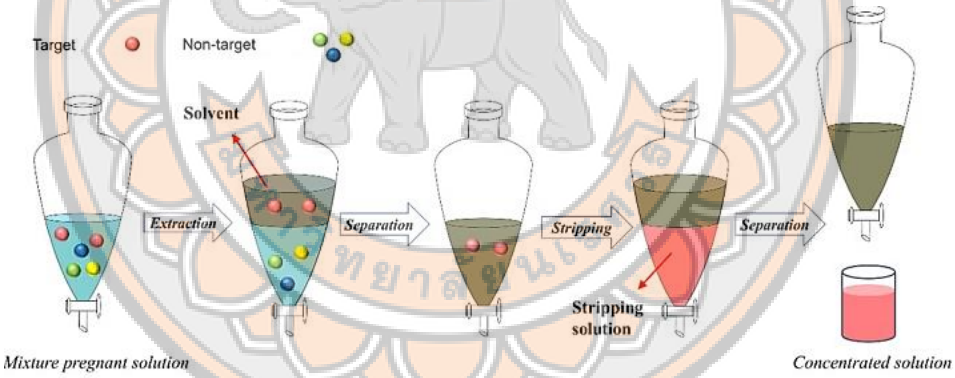


Fig. 22 The application of the solvent extraction method for solution purification

Source: Xia & Ghahreman (2023)

Figure 22 illustrates a typical solvent extraction process for target compound recovery, where the target compound is selectively recovered from the aqueous phase into the organic phase (or ionic liquid) and then stripped from the organic/ionic liquid phase to produce a concentrated aqueous solution. The advantages of solvent extraction include high recovery rates, good selectivity, and fast kinetics (Xia & Ghahreman, 2023).

2.3.2 Supercritical CO_2 Extraction

Fig. 23 SC-CO_2 extraction machine.

Source: From Co_2 Extraction Supercritical Co_2 Extraction Machine 5L, by Conversant, 2025, (https://www.conversant.co.th/co2extraction)

SC-CO_2 extraction (Figure 23) is a process that uses SC-CO_2 to extract compounds from a material. SC-CO_2, which exists at a specific temperature and pressure, exhibits properties of both a liquid and a gas, allowing it to effectively dissolve and separate desired substances. This method is favored for its ability to be both highly selective and easily separated, making it useful in various industries like food, pharmaceuticals, and cosmetics (Echim et al., 2011; Herzyk et al., 2024).

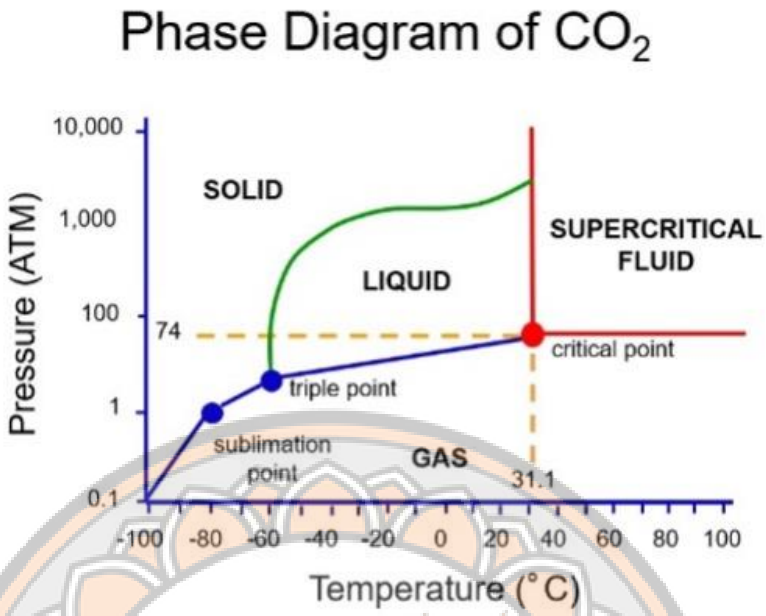


Fig. 24 Phase diagram of CO_2

Source: Ali (2018)

The CO_2 phase diagram (Figure 24) shows the conditions of temperature and pressure where solid, liquid, and gaseous CO_2 can exist. It features a triple point, where all three phases coexist, and a critical point, above which CO_2 becomes a supercritical fluid. Unlike water, CO_2's melting curve slopes upwards and to the right, and at standard atmospheric pressure, solid CO_2 (dry ice) sublimes directly to gas (Ramachandran et al., 2014).

SC-CO_2 extraction and solvent extraction are two different methods for separating desired compounds from a mixture. SC-CO_2 extraction utilizes carbon dioxide in its supercritical state, offering benefits like selectivity, reduced solvent usage, and environmentally friendly processing. Traditional solvent extraction relies on liquid solvents, which can be less efficient and potentially harmful due to solvent residues and environmental concerns (Yildırım et al., 2024; Olivira et al., 2025).

SC-CO_2 extraction generally yields lower oil extraction than traditional solvent extraction methods (Table 6), but it offers advantages in terms of purity and environmental impact (Xu et al., 2000). While solvent extraction, like using ethanol, can achieve higher oil yields (Laroche et al., 2019).

Table 6. Lipid extraction yield of house cricket and mealworm meals, according to the defatting method.

Insect meal	Defatting method	Extracted fat (% (w/w) of sample mass)
House cricket	Soxhlet (Hexane)	14.6 ± 0.1^{c}
	Soxhlet (Petroleum ether)	14.7 ± 0.2^{c}
	Soxhlet (Ethyl acetate)	15.1 ± 0.3^{b}
	Soxhlet (Ethanol)	22.7 ± 2.9^{a}
	Three-phase partitioning	19.3 ± 2.0^{ab}
	SC-CO_2	11.9 ± 1.4^{c}
Mealworm	Soxhlet (Hexane)	25.5 ± 0.1^{c}
	Soxhlet (Petroleum ether)	24.3 ± 1.2^{c}
	Soxhlet (Ethyl acetate)	25.7 ± 0.3^{b}
	Soxhlet (Ethanol)	28.8 ± 5.9^{a}
	Three-phase partitioning	23.7 ± 2.4^{ab}
	SC-CO_2	22.1 ± 0.6^{c}

Source: Laroche et al. (2019)

2.4 Protein Extraction and Purification

2.4.1 Protein Hydrolysis

Protein hydrolysis is a chemical reaction catalyzed by proteases that involves the cleavage of proteins by breaking the peptide bonds between two amino acids. This can be achieved through various methods, including enzymatic hydrolysis (using enzymes) and acid hydrolysis (using acids like hydrochloric acid) (Cruz-Casas et al., 2021).

Enzymatic hydrolysis has become the most used production method for bioactive peptides production. Different enzymes (Alcalase, Savinase, Flavourzyme, Neutrase, Trypsin, Pepsin, and Papain) have been employed to liberate bioactive peptides from food proteins (Fadimu et al., 2022). Enzymes used in the hydrolysis of food proteins can be classified according to their origin (plant, microbial, or animal), catalytic action (exopeptidase or endopeptidase) (Table 7) (Werb, 1981), and the nature of the catalytic site (e.g., serine proteases, aspartic proteases, metalloproteases, cysteine proteases, and threonine proteases) (Clemente, 2000; Hunsakul et al., 2022).

Table 7. Endopeptidase and exopeptidase enzymes

Feature	Endopeptidase	Exopeptidase
Cleavage site	Internal peptide bonds	Terminal peptide bonds
Protein breakdown	Breaks down proteins into smaller fragments	Releases individual amino acids from fragments
Enzymes	Pepsin, Trypsin, Chymotrypsin, Subtilisin	Aminopeptidases, Carboxypeptidases

Source: Nandan & Nampoothiri (2020)

Advantages of enzymatic hydrolysis

- Specificity: Enzymes are highly selective, meaning they only act on specific bonds, leading to controlled reactions.
- Mild conditions: Enzymatic reactions often occur under moderate temperatures and pH, minimizing damage to the substrate.
- Reduced byproducts: Compared to chemical hydrolysis, enzymatic hydrolysis often generates fewer unwanted byproducts (Chinnadurai et al., 2023).

Insect protein hydrolysate is a water-soluble form of insect protein, created by breaking down insect protein (typically from insects like black soldier flies, mealworms, or crickets) into smaller peptides and amino acids through enzymatic hydrolysis. This process improves solubility and makes it suitable for various food applications, particularly in liquid-based products (Tarahi et al., 2025; Lisboa et al., 2024).

Firmansyah and Abduh (2019) investigated the potential of protein hydrolysate from BSFL as a bioactive hydrolysate through enzymatic hydrolysis using bromelain. BSFL contains 25.6% protein and 35.5% lipids. The BSFL protein hydrolysate had a molecular weight in the range of 14–25 kDa based on Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis, with an antioxidant activity of 72.6 in terms of its ability to inhibit free radicals DPPH with an IC_{50} of 0.84%

Cricket protein hydrolysate with Alcalase (0.2 units/g dry sample) showed the highest ABTS radical-scavenging activity (19.53 ± 0.15 µmol TE/g dry sample) and FRAP (5.90 ± 0.10 µmol TE/g dry sample), compared to that prepared by Flavozyme hydrolysis ($p < 0.05$) (Chotphruethipong et al., 2024).

2.4.2 Bioactive Protein/ Peptide Purification

Bioactive protein and peptide purification involves isolating specific proteins or peptides with desired biological activity from complex mixtures. This often involves a multi-step process utilizing techniques like chromatography, membrane filtration (Table 8), and enzymatic hydrolysis to achieve the desired purity and yield (Tarahi et al., 2025; Kan et al., 2025).

Table 8. Protein purification method

Chromatography	Reversed-phase HPLC
	Ion-exchange chromatography
	Hydrophobic interaction chromatography
	Size exclusion chromatography
	Gel filtration
	Affinity chromatography
Membrane filtration	Ultrafiltration
	Nanofiltration

Source: Insuasty Cepeda et al. (2019)

2.4.2.1 Membrane filtration

Membrane filtration is a physical separation process that uses a selective barrier to separate biomolecules and particles based on their size (Barman et al., 2021). It's a pressure-driven process where a feed stream is passed through the membrane, and components smaller than the membrane's pore size pass through as permeate, while larger components are retained as retentate (Figure 25 and 26). This method is widely used for various applications, including water purification, wastewater treatment, and in the food and pharmaceutical industries (Hube et al., 2020).

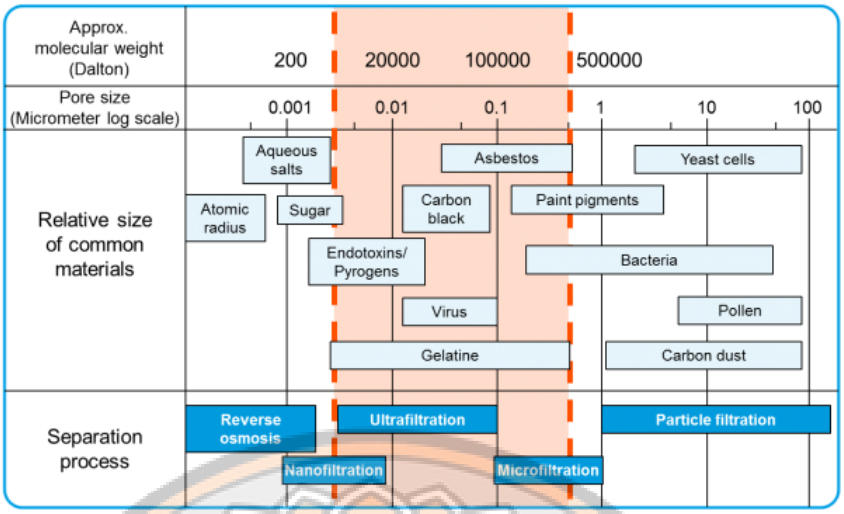


Fig. 25 Overview of common pore sizes and their suitable separation processes

Source: From Technology, by Alpha plan, 2025, (https://www.alpha-plan.de/en/filter/membrane-filter-technology/)

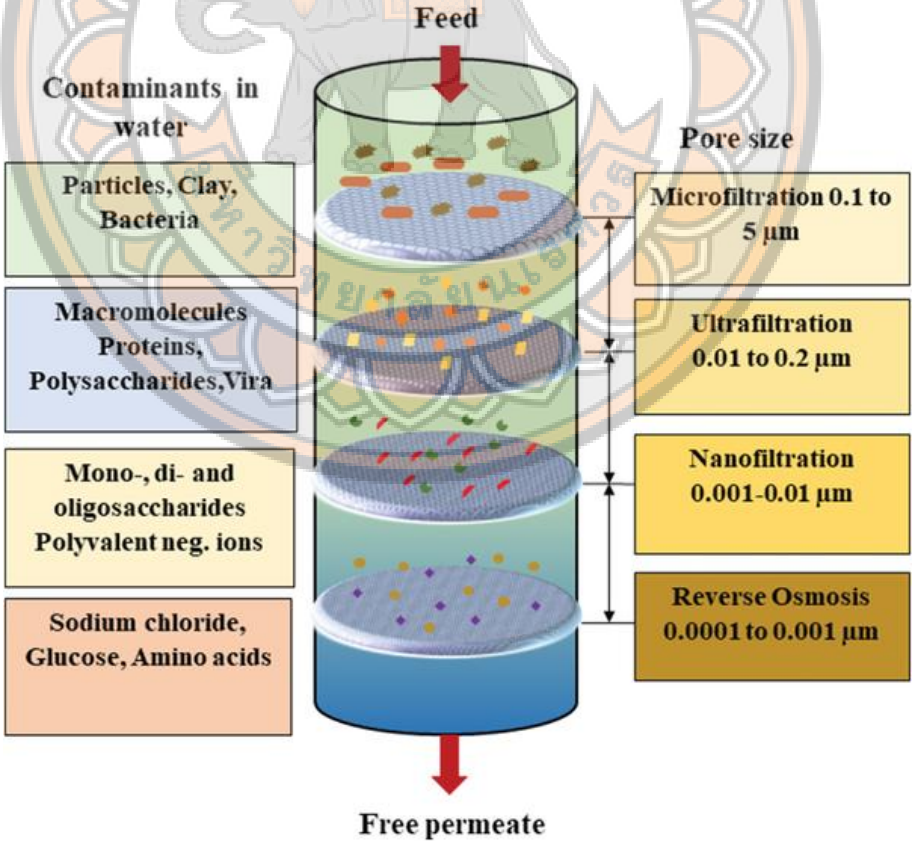


Fig. 26 Schematic illustration of pressure-driven membrane filtration processes

Source: Mousa et al. (2022)

- Microfiltration with a pore size of 0.1 to 5 µm
- Ultrafiltration with a pore size of 0.01 – 0.2 µm
- Nanofiltration with a pore size of 0.001 – 0.01 µm
- Reverse osmosis uses dense membranes without pores to desalinate seawater and produce potable water. In gas separation, membranes are used to separate oxygen from nitrogen or to improve natural gas quality by removing carbon dioxide.

Membrane filtration, particularly ultrafiltration and nanofiltration, is effective for purifying peptides based on molecular weight. These methods have yield, purification, and product quality that are comparable to chromatography processes, but they are more affordable and productive (Kalita et al., 2025).

Table 9. Summary of studies on insect-derived bioactive peptides: molecular characteristics and key bioactivity findings

Insects	**Molecular weight**	**Sequences**	**Bioactive properties**
Cricket	90% < 1 kDa	YKPRP, PHGAP, VGPPQ	Antidiabetic Anti-hypertension
Larva of the Japanese rhinoceros's beetle	0.943 kDa	AGLQFPVGR	Amti-obesity
lesser mealworm	< 1 kDa	-	α-glucosidase inhibition ACE inhibition
Larva of the white-spotted flower chafer	0.262 – 0.441 kDa	ST, PF, YPY, WI	Anti-hypertensive
Mealworm	< 1 kDa	FFGT, LSRVP, CTKKHKPNC	Anti-hypertension
Silkworm	-	WW, ASL, GNPWM	Anti-hypertensive

Source: Tarahi et al. (2025)

2.5 Industrial Production Process Design

SuperPro Designer is a software tool for process simulation, evaluation, and optimization, specifically designed for batch and continuous chemical and bioprocesses. It helps engineers and scientists model processes, perform material and energy balances, analyze costs (including capital and operating costs), and identify bottlenecks to optimize production and debottleneck processes across various industries like pharmaceuticals, biotechnology, and food processing (Hengevoss, 2023; Canizales et al., 2020)

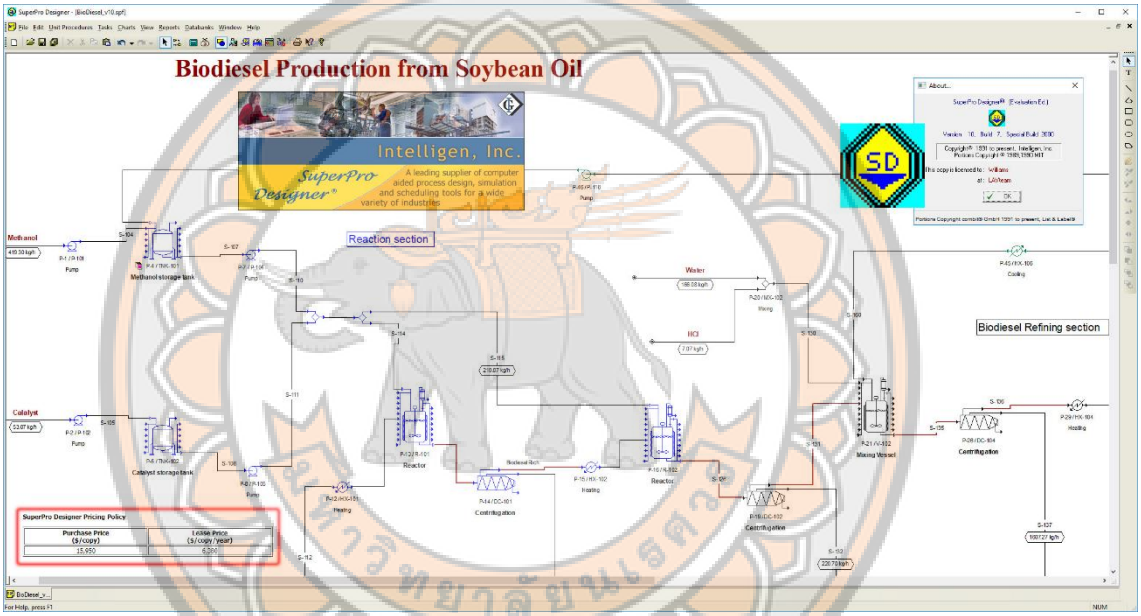


Fig. 27 Example of production process simulated by SuperPro Designer program

Source: From Intelligen SuperPro Designer v10 build 7, by Usoftly, 2025, (https://usoftly.ir/software/intelligen-superpro-designer/)

2.5.1 Key features and capabilities (Canizales et al., 2020)

- Process Modeling: Create detailed models of integrated batch and continuous processes with a user-friendly interface and large databases for chemicals, equipment, and resources.
- Material and Energy Balances: Calculate comprehensive material and energy balances for all streams within the process, including waste streams and emissions.

- Cost Analysis & Economics: Perform detailed techno-economic analyses, including equipment sizing and costing, capital investment (CAPEX), and operating costs (OPEX), providing a platform for cost-effective process design.
- Debottlenecking & Scheduling: Generate Gantt and Equipment Occupancy Charts (EOC) to visualize process scheduling, identify cycle time bottlenecks, and optimize throughput.
- Waste Stream Characterization: Calculate the amounts and compositions of all waste streams.
- Resource Tracking: Track demand for resources such as raw materials, labor, and utilities.

2.5.2 Industries and Applications (Carnegie Mellon University, 2025)

- Biotechnology
- Pharmaceuticals
- Specialty Chemicals
- Food Processing
- Water and Wastewater Treatment
- Air Pollution Control
- Metallurgical and Materials Processing

2.5.3 Benefits for Engineers and Scientists (Canizales et al., 2020)

- Technology Transfer: Facilitates the transfer of technology from development to manufacturing by providing detailed process models and reports.
- Process Optimization: Identifies cost-sensible factors and operational bottlenecks to increase efficiency and throughput.
- Decision Support: Provides data and tools to support decision-making throughout the entire process development lifecycle, from conceptual design to process operation.

2.6 Related Research

2.6.1 Extraction of Nutritional and Functional Components from Edible Insects

Table 10. Extraction methods for insect lipids and their characteristics

Insects	Extraction process	Characteristics	Source
House cricket	SC-CO_2 (32.5 MPa, 55 °C, 75 min)	Higher monounsaturated fatty acid contents than Soxhlet extraction	Laroche et al. (2019)
House cricket	SC-CO_2 (30-45 MPa, 30-80 °C)	Defatting efficiency = 85 %	Davalos-Vazquez et al. (2024)
Moth	Ultrasound assisted extraction (20 kHz, 650 W, 15 min)	Lower acid and peroxide levels and higher polyunsaturated fatty acid contents and thermal stability than Soxhlet extraction	Sun et al. (2018)
Mealworm	SC-CO_2 (40/25 MPa, 45 °C, 105 min)	Defatting efficiency = 95 %	Purschke et al., (2017)
Black soldier fly	SC-CO_2 (25-30 MPa, 60 °C) + EtOH (10% cosolvent)	The yields of the extracts were different by 32.5 to 53.9%.	Cruz et al., (2024)
Silkworm	SC-CO_2 (20.3 MPa, 50 °C, 145 min)	At this optimized condition, the highest oil yield was found to be 30.10 g/100 g.	Srinivas et al., (2019)

Table 11. Extraction methods for insect proteins

Insects	Extraction process	Characteristics	Source
Black soldier fly	Defatting and alkaline (alkaline solution to sample ratio of 15:1 (v/w))	Improvement of water and oil absorption properties	Mintah et al. (2020)
Yellow mealworm	Hexane defatting and sonication (20 kHz with a 75% AMP, 1-20 min)	Improvement of the protein extraction yield. 35% (protein extraction yield)	Choi et al. (2017)
Field crickets	Hexane defatting and sonication (20 kHz with a 75% AMP, 1-20 min)	Improvement of the protein extraction yield. 37% (protein extraction yield)	Choi et al. (2017)
Silkworm pupae	Hexane defatting and sonication (20 kHz with a 75% AMP, 1-20 min)	Improvement of the protein extraction yield. 94% (protein extraction yield)	Choi et al. (2017)
House crickets	SC-CO_2 (32.5 MPa, 55 °C, 75 min)	The protein extraction yield ranged from 12.4 to 38.9%	Laroche et al. (2019)
Mealworm	SC-CO_2 (32.5 MPa, 55 °C, 75 min)	The protein extraction yield ranged from 11.9 to 39.3%	Laroche et al. (2019)

Table 12. Bioactive peptides of edible insects and their potential

Insects	Peptides	Bioactivity	References
Crickets	IIAPPER	ACE inhibition: IC_{50}, 6.93 µg/mL	Zielińska et al., (2020); Zielińska et al., (2018)
		Lipase inhibition: IC_{50} 49.44 µg/mL	
		α-Glucosidase inhibition: IC_{50}, 22.86 µg/mL	
		Radical scavenging activity (ABTS assay): IC_{50}, 15.62 mg/mL	
		Antioxidant activity (DPPH assay): IC_{50}, 1.01 mg/mL	
		Fe^{2+} chelating activity: IC_{50}, 0.14 mg/mL	
	KVEGDLK	ACE inhibition: IC_{50}, 3.67 µg/mL	
		Lipase inhibition: IC_{50} 115.44 µg/mL	
		α-Glucosidase inhibition: IC_{50}, 18.37 µg/mL	
		Radical scavenging activity (ABTS assay): IC_{50}, 2.88 mg/mL	
		Antioxidant activity (DPPH assay): IC_{50}, 8.73 mg/mL	
		Fe^{2+} chelating activity: IC_{50}, 0.122 mg/mL	
	LAPSTIK	ACE inhibition: IC_{50}, 11.14 µg/mL	
		Lipase inhibition: IC_{50} 104.95 µg/mL	
		α-Glucosidase inhibition: IC_{50}, 45.60 µg/mL	
		Radical scavenging activity (ABTS assay): IC_{50}, 15.69 mg/mL	
		Antioxidant activity (DPPH assay): IC_{50}, 0.66 mg/mL	

	Fe^{2+} chelating activity: IC_{50}, 0.456 mg/mL	

Table 12. *Cont.*

Insects	Peptides	Bioactivity	References
Crickets	VAPEEHPV	ACE inhibition: IC_{50}, 18.85 µg/mL Lipase inhibition: IC_{50} 100.13 µg/mL Radical scavenging activity (ABTS assay): IC_{50}, 3.49 mg/mL Antioxidant activity (DPPH assay): IC_{50}, 0.29 mg/mL Fe^{2+} chelating activity: IC_{50}, 0.155 mg/mL	Zieliński et al., (2020); Zieliński et al., (2018)
Silkworm pupa	AAEYPA	Radical scavenging activity (ABTS assay): IC_{50}, 70.32 µg/mL Antioxidant activity (DPPH assay): IC_{50}, 70.83 µg/mL	Khammuang et al., (2022)
	AKPGVY	Radical scavenging activity (ABTS assay): IC_{50}, 34.32 µg/mL Antioxidant activity (DPPH assay): IC_{50}, 58.50 µg/mL	
	FKGPACA SVLGTGC	Radical scavenging activity (ABTS assay): IC_{50}, 0.312 mM Radical scavenging activity (ABTS assay): IC_{50}, 0.181 mM	Zhang et al., (2021)
	ASL	ACE inhibition IC_{50}, 102.15 µM	Wu et al., (2015)
	GNPWM WW	ACE inhibition: IC_{50}, 21.70 µM ACE inhibition: IC_{50}, 10.76 µM	Tao et al., (2017)
Mealworm	LPDQWDWR APPDGGFWEWGD	DPP-IV inhibition: $IC_{50,}$ 0.15 mg/mL DPP-IV inhibition: $IC_{50,}$ 1.03 mg/mL	Tan et al., (2022)

Table 12. *Cont.*

Insects	Peptides	Bioactivity	References
Egyptian cotton leafworm	SGD hydrolysate	In vivo model: *Caenorhabditis elegans* ORAC: IC_{50}, 0.052 mg/mL Radical scavenging activity (ABTS assay): IC_{50}, 0.24 mg/mL Cellular antioxidant activity was similar to ascorbic acid (positive control) Protective effect in vivo against acute oxidative stress	Mudd et al., (2022)
Cricket	Cationic peptide fraction from sequential alcalase and SGD hydrolysates	ACE inhibition: IC_{50}, 1.922 µg/mL α-amylase inhibition: IC_{50}, 96.75 µg/mL α-Glucosidase inhibition: IC_{50}, 13.902 µg/mL	Hall et al., (2020)
Mexican katydid	SGD hydrolysate	ACE inhibition: IC_{50}, 0.49 mg/mL	Montiel-Aguilar et al., (2020)
	<3 kDa fraction of SGD hydrolysate	ACE inhibition: IC_{50}, 1.44 mg/mL α-amylase inhibition: IC_{50}, 0.68 mg/mL	

CHAPTER III

EFFECTS OF HIGH PRESSURE AND ULTRASONICATION PRETREATMENT AND SUPERCRITICAL CARBON DIOXIDE EXTRACTION ON PROPERTIES OF EDIBLE INSECT OILS*

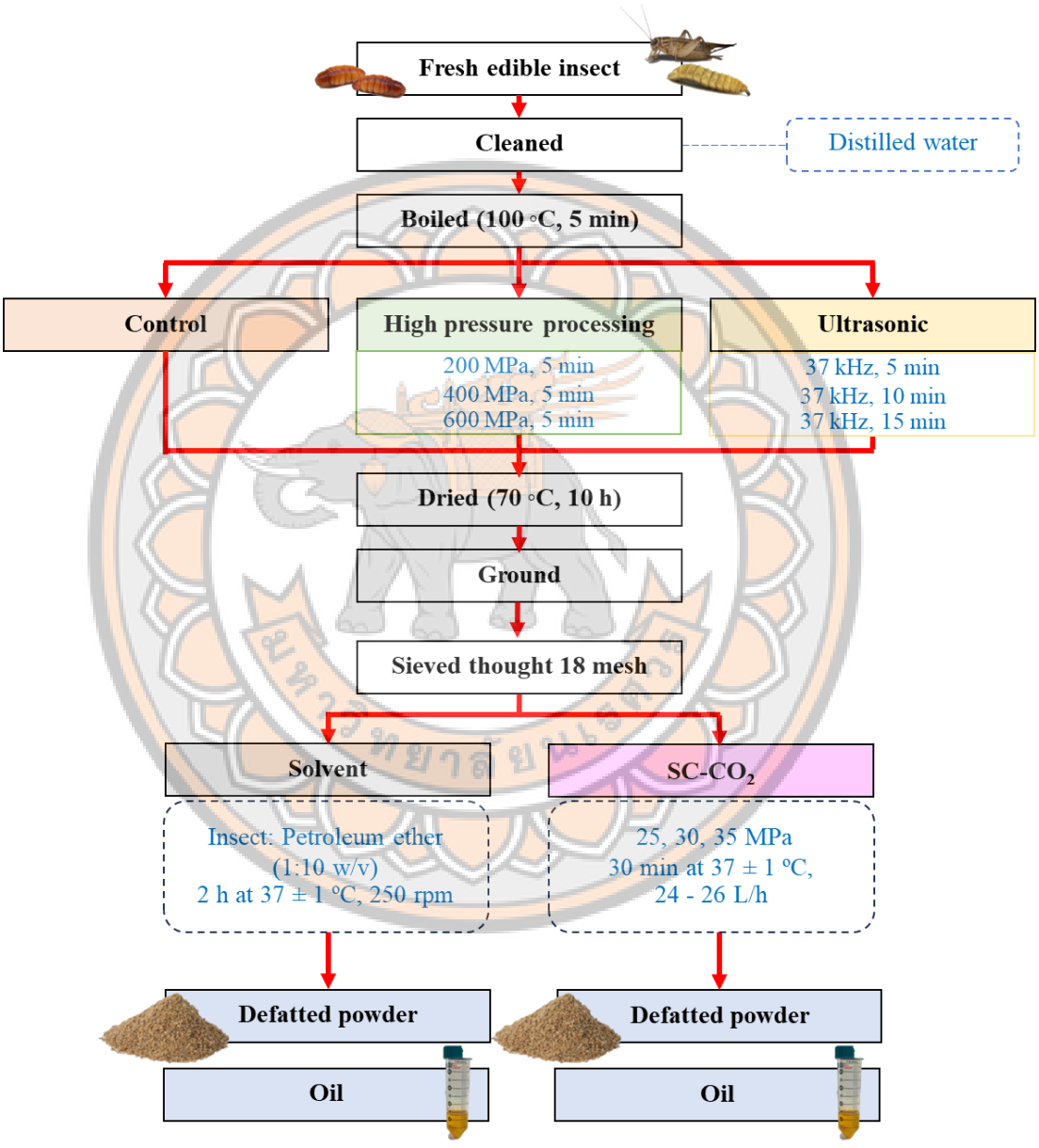


Fig. 28 Flow chart diagram of pretreatment processes

3.1 Abstract

Edible insects have gained increased attention as the important and sustainable source of nutrients for both human and animal consumption due to their rich content of protein, oil, fiber (as chitin), vitamins, and minerals. This study focuses on examining the effects of advanced pretreatments and oil extraction methods from prominent edible insect species commercially cultivated in Thailand, namely the AHCK, BSFL, and SWP. The raw insect samples underwent pretreatment using HPP at 200 - 600 MPa for 5 min or US at 37 kHz for 5 - 15 min, followed by dehydration and extraction using SC-CO_2 or solvent extraction. The study revealed that HPP significantly reduced the initial microbial load of all edible insects by 6 - 8 log cycles, while US showed a slight effect on microbial reduction. Additionally, pretreatments notably improved the oil extraction yield of most samples by disrupting the integrity of the cell membrane, facilitating the release of oils. The extracted edible insect oils were rich in unsaturated fatty acids, omega -3, -6, and -9, making them suitable for various applications such as cosmetics, food, and feed additives. Furthermore, the protein meals obtained as a residue after oil removal were identified as potential meat alternatives or replacements in food or feed formulations. This study provides valuable insights into the potential of edible insects as a sustainable source of oil and protein, highlighting the significance of pretreatments and extraction methods in maximizing their utility for various applications in the food, cosmetic, and feed industries.

Keywords: edible insect oil, high pressure processing, physico-chemical properties, SC-CO_2 extraction, ultrasonication.

*Based on the published paper: Boonmee, N., Chittrakorn, S., Detyothin, S., Tochampa, W., Sriphannam, C. & Ruttarattanamongkol, K. (2024). Effects of High Pressure and Ultrasonication Pretreatments and Supercritical Carbon Dioxide Extraction on Physico-Chemical Properties of Edible Insect Oils. *Journal of Food Process Engineering*, 47(11), e14781. https://doi.org/10.1111/jfpe.14781

3.2 Introduction

Edible insects have emerged as a promising solution, offering high-quality protein, healthy fatty acids, and other essential nutrients with a more sustainable production process compared to conventional protein sources (Kim & Oh, 2022). They offer impressive protein content ranging from 37 to 76 % and fat content from 20 to 40 % (StarČeviĆ et al., 2017). Insect-derived oils are particularly noteworthy for their rich composition of beneficial fatty acids, including oleic, palmitic,

linoleic, and linolenic acids, which confer moisturizing and nourishing properties (Kolobe et al., 2023). These oils present a promising new source of healthy omega-3, -6, and -9 fatty acids, with potential applications in both food and skincare products (Franco et al., 2022). Some studies indicate that various insect species possess distinct protein and oil profiles, contributing to their unique nutritional characteristics (Li et al., 2023) and many consumers find insect-based foods acceptable in terms of sensory attributes such as flavor, aroma, and texture (Roma et al., 2020).

However, the extraction of these valuable oils from insects presents several challenges. Traditional extraction methods often involve extreme conditions that can potentially reduce the nutritive values of oils, degrade bioactive compounds, and promote undesirable functional properties (Vieira et al., 2023). For these reasons, alternative extraction methods and pretreatment processes have been explored. SC-CO_2 extraction has emerged as an efficient green technology for obtaining oils from insect biomass. This method maximizes yields while avoiding thermal degradation (Cheseto et al., 2020; Kim & Oh, 2022; Saviane et al., 2021). The moderate critical conditions (31°C, 7.38 MPa) and nonpolar, diffusive properties of SC-CO_2 allow effective solubilization of lipids from insect matrices with minimal thermal and oxidative damage (Femenia et al., 2001; Temelli et al., 2012). Furthermore, coupling SC-CO_2 extraction with entrainers like ethanol can enhance the recovery of polar bioactive compounds from edible insects (Roff & Greenlief, 2017).

To further improve extraction efficiency, various pretreatment processes have been investigated. HPP and US have shown particular promise. HPP has demonstrated effectiveness as a nonthermal preservation method for edible insect products, maintaining fresh flavor, texture, and nutritional quality without added preservatives (Huang et al., 2017). Through pressures of 100 - 600 MPa, HPP inactivates microbes and enzymes in insects by inducing protein denaturation, cell membrane damage, and altered molecular structures (Tornberg, 2005; Sevenich et al., 2014). US, on the other hand, has been explored as a processing aid to enhance insect disintegration, oil extraction, and component recovery (Bußler et al., 2016). High-power ultrasound creates localized pressure changes, shear forces, and cavitation effects in liquid food systems that can facilitate homogenization, mixing, filtration, and extraction (Gallo et al., 2018). Both HPP and US have been found to cause the rupture of cell walls, allowing for easy release of oil (Yao et al., 2018; Naik et al., 2013).

While previous studies have explored the effects of various extraction techniques on insect lipid composition (Tzompa-Sosa et al., 2014), there is a need to further investigate the impacts of combined pretreatments and extraction methods on the physicochemical properties of edible insect oils. This study aims to address this gap by evaluating the effects of HPP and US pretreatments, both individually and in combination, on the physicochemical properties of oils extracted from selected edible insect species using SC-CO_2. The insects selected for this study include AHCK, BSFL, and SWP. These species were chosen due to their commercial availability in Thailand and their unique nutritional profiles. For instance, BSFLs contain high levels of lauric acid (38.43%), which has potential antiviral properties (Ushakova et al., 2016). SWPs are rich in essential fatty acids such as ETA and DHA (Zhou et al., 2017), which play crucial roles in fetal development and cognitive function (Swanson et al., 2012; Rowe, 2020).

This research investigated the effects of HPP and US pretreatments on microbial load reduction, proximate composition, and structural changes of the insect samples. Additionally, the extracted oil yield, physicochemical properties, and fatty acid profiles of the oils obtained through SC-CO_2 extraction were evaluated. By comparing these results with those from conventional organic solvent extraction, this study aimed to provide valuable insights into the potential of combined pretreatment and green extraction methods for producing high-quality edible insect oils.

3.3 Materials and Methods

3.3.1 Materials

The edible insect samples utilized in this study consisted of cooked and frozen AHCK, BSFL, and SWP, obtained from Thai Ento Food Company Limited, Thailand. The AHCK were 40 - 45 days old upon harvesting, the BSFL were 22 - 25 days old, and the SWP were harvested at the pupal stage. After harvesting, the raw insect samples were visually inspected and physically screened to remove any contaminants or filth. The samples were then washed three times with RO water to eliminate remaining debris and surface microorganisms. The washed insects were then blanched by boiling in water for 5 min, followed by packing in polyethylene bags (4 × 6 in.), sealing, and freezing storage at -18°C until experimental use. Petroleum ether 40 - 60 Ar. Grade (Density 0.645 - 0.665) and sulfuric acid 98% Ar. Grade (Density 1.84) were purchased from RCI Labscan, Ireland. All other chemical reagents used for analysis were analytical grade and supplied by Sigma Aldrich (St. Louis, MO, USA). Microbiological culture media used for microbial evaluation were purchased from Merck (Darmstadt, Germany).

3.3.2 Pretreatment Processes

3.3.2.1 High Pressure Processing (HPP)

To prepare samples for HPP treatment, 100-gram portions of each thawed insect sample with initial temperature of 10 ± 1 °C were weighed and sealed in polyethylene bags without water and sealed under vacuum, allowing them to equilibrate to ambient temperature. The HPP treatments were conducted using a 5L capacity pressure chamber equipped with a maximum pressure rating of 600 MPa (model HPP600 MPa/3-5L Pilot Scale, Baotou Kefa Inc., Baotou City, China). The insect sample bags were placed in the HPP chamber, surrounded by distilled water as the pressure-transmitting fluid as illustrated in Figure 29. The treatments applied discrete pressure levels of 200, 400, and 600 MPa sustained for a 5-minute hold time at ambient temperature. The initial water temperature in the quasi-adiabatic insulated vessel ranged from ambient temperature of 35 - 37 °C, and the rate of temperature rise during compression was 3°C per 100 MPa (Yordanov & Angelova, 2010). The HPP equipment uses an insulated pressure vessel. The temperature increase occurs due to adiabatic compression. The chamber's temperature was evaluated using the approach outlined by Balasubramaniam et al. (2004). During the HPP, the chamber temperature varied between 41 °C and 55 °C as the pressure escalated from 200 to 600 MPa. For HPP pressurization rate, it was based on the use of high isostatic pressure transmitted by water of up to 600 MPa, held for a few minutes. HPP pressurization rate was 5 - 10 s/100 MPa. The compression and decompression time ranged from 60 to 90 s and 10 to 15 s respectively. These pressure-time combinations were selected based on preliminary experiments and previous literature. Each insect type was subjected in triplicate to the three pressure levels along with an untreated control. All HPP conditions were monitored in real-time and recorded using the chamber instrumentation to ensure consistency between replicates. Immediately after depressurization, the treated insect samples were removed and kept refrigerated (4 ± 1°C) until analysis.

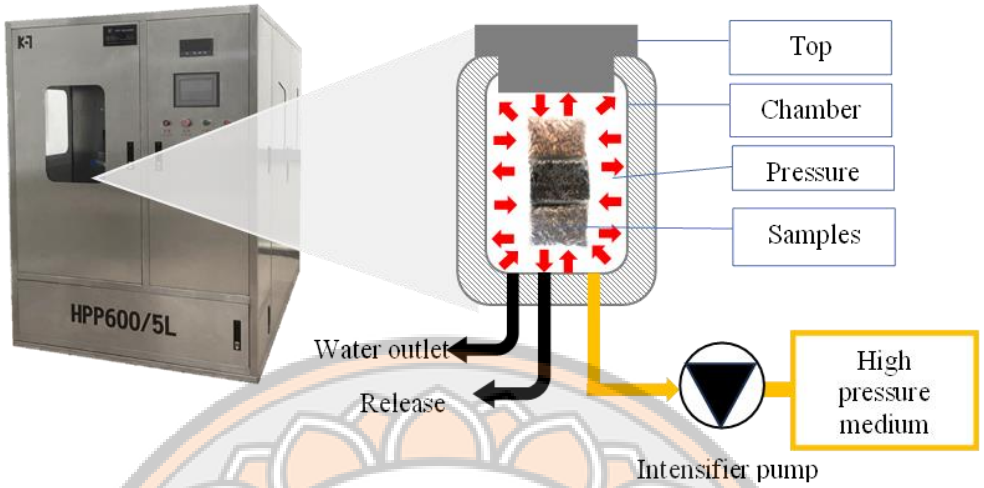


Fig. 29 Schematic diagram of HPP treatments of edible insects

3.3.2.2 Ultrasonication

To prepare insect samples for US pretreatment, 100 g of each insect species were weighed into polyethylene bags which were then heat-sealed. The sealed bags were then fully submerged in an ultrasonic bath (E100H, Elma, Germany) filled with distilled water acting as the coupling medium. US was conducted at a fixed frequency of 37 kHz for treatment times of 5, 10, and 15 min, which were chosen based on preliminary experiments. The temperature of the sample during US treatment remained ambient (35 ± 2 °C) and was controlled using a circulating water bath. The ultrasonic power input was calibrated prior to experiments using a standard calorimetric method. Triplicate samples of each insect type were subjected to the three US times along with an untreated control sample. There was no difference in the physical appearance of all insect powder samples of control and pretreatment.

After US or HPP pretreatment, the insect samples underwent controlled drying to reduce moisture content. Drying was conducted at 70 °C in a convection oven with continuous air circulation for approximately 10 h until the samples reached a target moisture content between 5 - 8%, verified using a halogen moisture analyzer (HR83, Mettler Toledo). The dried insect samples were then milled into a powder using a commercial blender (Vitamix) and sieved through an 18-mesh screen to standardize particle size distribution. The powdered samples were sealed in polyethylene bags and stored at 4 °C until further analysis.

3.3.3 Microbial Analysis

Microbiological quality of the raw and processed insect samples was assessed by determining TVC as well as yeast and mold levels. Serial decimal dilutions of each sample were prepared in peptone water to obtain suitable dilution levels for microbial enumeration. The diluted samples were inoculated using the pour plate technique with 1 mL aliquots dispersed onto 90 mm Petri dishes containing appropriate growth media. Total aerobic mesophiles were enumerated using Plate Count Agar incubated at 37 ± 1°C for 24 h as per FDA BAM Chapter 3 guidelines. Yeast and mold counts were obtained using Rose Bengal Agar with plates incubated at 25°C for 5 days as specified in BAM Chapter 18. All microbiological analyses were performed in a biosafety cabinet under aseptic conditions. Duplicate platings were conducted for each dilution and counts were reported as colony-forming units per gram (CFU/g) on a dry weight basis. Proper dilutions resulting in 30 - 300 colonies were selected for microbial enumeration and standard plate count protocols were followed to quantify microbial load reductions resulting from the insect pretreatments.

3.3.4 Scanning Electron Microscope

SEM provided insights into the effects of HPP and US on the solid matrix and protein structures within the dried insect powders. The microstructure and morphology of selected dried insect powder samples were visualized using SEM. Samples were sputter-coated with an 11.34 nm layer of gold using a mini sputter coater (SC7620, Quorum Technologies) at 18 mA for 90 s with a 1 kV target voltage. This conductive coating prevents charging and allows surface visualization. SEM imaging was performed using a Quanta 200 microscope (FEI Oregon, USA) under a high vacuum with an acceleration voltage of 12.5 kV. Secondary electron images were collected at various magnifications up to 12,000× using a large field detector with a spot size of 4.5. Representative regions of interest were imaged for each sample to qualitatively assess microstructural changes induced by the pretreatment processes compared to untreated insect powder.

3.3.5 Oil Extraction

3.3.5.1 Solvent Extraction

Approximately 100 g (particle size less than 18 mesh) of pretreated insect powder samples including control (non-pretreated sample); HPP 200, HPP 400, and HPP 600 (HPP pretreated samples at 200 MPa for 5 min, 400 MPa for 5 min, and 600 MPa for 5 min, respectively); US 5, US 10, and US 15 (US pretreated samples at 37 kHz for 5 min, 37 kHz for 10 min, and 37 kHz for 15 min, respectively) was mixed with 1000 mL of petroleum ether. The extraction process was carried out using a sample-to-solvent ratio of 1:10 (w/v) in an extraction beaker. The samples were stirred for 2 h at 37 °C ± 1°C, and the Petroleum Ether was subsequently separated through centrifugation at 4830× g for 15 min. Following this, the samples were left overnight in a fume hood to allow the solvent to evaporate. The resulting oil was then stored in a sealed container at –18 °C until analysis.

3.3.5.2 Supercritical CO_2 Extraction

The oil extraction from pretreated insect powder samples (control, HPP 200, HPP 400, HPP 600, US 5, US 10, and US 15) was carried out using a pilot-scale SC-CO_2 extraction machine (SFE-01 L, CAREDDI SCF, China) with a 1 L extraction tank, as illustrated in Figure 30. Initially, approximately 300 g of the pretreated insect powder was placed in the SC-CO_2 chamber and tightly sealed in an extraction tank. Subsequently, the extraction process was conducted at a pressure ranging from 25 to 35 MPa, with a fixed extraction temperature of 37 °C, a time of 30 min, and a CO_2 flow rate of 24 - 26 L/h. The resulting extract was obtained from the separator and then subjected to centrifugation at 4830 x g for 15 min to separate the oil phase from other components. The liquid oil was then collected and stored in a sealed container at -18 °C until analysis.

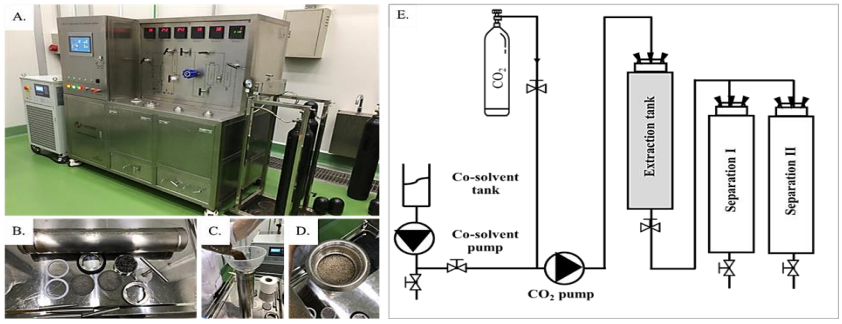


Fig. 30 Pilot-scale SC-CO_2 extraction unit A) SC-CO_2 extraction machine 1L, B) SC-CO_2 chamber, C) samples were placed in SC-CO_2 chamber, D) defatted samples and E) SC-CO_2 extraction diagram.

3.3.6 Proximate Analysis

Moisture content, crude protein, crude fat, total ash, and crude fiber contents of each sample were determined according to the standard methods of the Association of Official Analytical Chemists (AOAC 2012). Each analysis was carried out in triplicate.

3.3.7 Oil Extraction Yield

Oil yield (%) obtained by solvent and SC-CO_2 extraction techniques was calculated as shown in Equation (1).

$$\% \text{ Oils extraction yield} = \frac{Extracted\ oil\ amount}{Initial\ oil\ amount} \times 100 \quad (1)$$

where the initial oil amount is the total quantity of oil originally presented in the sample prior to extraction.

3.3.8 Oil Properties

AV, FFA, and PV were evaluated according to the methods outlined in AOAC (1990). To determine the AV, 5 mL of the oil samples were dissolved in 50 mL of ethanol. A few drops of phenolphthalein indicator were added, and the solution was titrated with 0.5 M KOH until a persistent pink color appeared. This titration process was repeated three times, and an average titer was calculated. The AV was then converted to FFA content using a factor obtained by dividing the molecular weight of the fatty acid concerned (MW = 282.4) by 10 times the molecular weight of KOH (56.1). For the PV determination, 5 g of the edible insect oil was added to 30 mL of a solvent mixture (acetic acid and chloroform in a 3:2 ratio). Subsequently, 0.5 mL of saturated potassium iodide was added, and the mixture was shaken and kept in a dark place for 1 min. A 1% starch solution in 30 mL of distilled water was then titrated with 0.1 N sodium thiosulfate. All analyses were performed in triplicate.

3.3.9 Viscosity

The viscosity of insect oil samples was determined using the Rheometer (Anton Paar, Austria). A concentric cylinder CC27-SS and CC27 probe were used to evaluate the oil viscosity at a constant temperature of 37 °C and performed under the low-viscosity liquid mode. The shear rate was performed in a range of 0.1 – 100 [1/s] under a shear stress of 0.5 – 5 [Pa] and a torque of 0.025 – 0.25 [mN.m], respectively.

3.3.10 Fatty Acid Profiles

Fatty acid profiles of selected edible insect oil samples obtained SC-CO_2 extraction were performed according to the procedure of AOAC (2012) 996.06 using the GC–MS technique. Identification of peaks was done by comparison with relative retention times for the standard fatty acid methyl esters (FAMEs). Concentrations of each fatty acid were reported as g/100 g sample.

3.3.11 Statistical Analysis

Statistical analyses were performed using the Statistics Package for Social Sciences (SPSS) IBM SPSS Statistics version 26. The statistical model for this test used ANOVA for a completely randomized design experiment (CRD experiment). Each experiment was performed in triplicate and results were reported as mean ± standard deviation (SD). Duncan tests ($\alpha = 0.05$) were used as multiple comparison tests.

3.4 Results and Discussion

3.4.1 Raw Material Composition

The proximate analysis results of the edible insect species utilized as raw materials in this study have been comprehensively documented in Table 13. Notably, the protein and fat contents of the studied edible insects spanned from 49 to 65 % and 18 to 34 %, respectively. Of significance, the AHCK demonstrated the highest levels of protein and fiber, while the SWP exhibited the highest fat content. In comparison to prior findings by Amarender et al. (2020), the protein and fat contents of the AHCK and SWP were found to be consistent with the previously reported values, providing additional validation to our study's results. However, it is noteworthy that the BSFL displayed discrepancies in protein and fat contents compared to previous reports, potentially attributable to variations in rearing conditions, climate, and feed, as elucidated by Smets et al. (2020). These findings not only contribute to the understanding of the nutritional composition of the studied edible insects but also underscore the influence of environmental and dietary factors on their nutritional profile, thereby enriching the existing body of literature in this field.

Table 13. Compositions of studied edible insects as the raw material.

Insects	% w/w on dry matter basis				
	Crude protein	Crude oil	Fiber	Ash	Carbohydrate
AHCK	64.69 ± 0.35^{a}	21.96 ± 0.10^{b}	13.77 ± 0.01^{a}	5.17 ± 0.03^{c}	8.18 ± 0.22^{c}
BSFL	58.86 ± 0.66^{b}	18.37 ± 0.60^{c}	10.68 ± 0.17^{b}	8.00 ± 0.17^{a}	14.77 ± 0.28^{a}
SWP	49.15 ± 0.35^{c}	34.15 ± 0.24^{a}	4.17 ± 0.12^{c}	6.73 ± 0.00^{b}	9.97 ± 0.59^{b}

Note: Values in the same column followed by different letters (a, b, c) are significantly different ($p < 0.05$).

3.4.2 Microbial Reduction

Based on the data presented in Table 14, the control (non-pretreated) samples of all three edible insects studied had very high total viable counts, indicating a high microbial load. This is expected for untreated insect samples. HPP at all pressures (200, 400, and 600 MPa) dramatically decreased the total viable counts for all three insects by 6 - 8 log units compared to the control. This significant microbial reduction by HPP is consistent with other studies showing HPP can effectively pasteurize and reduce microbial loads in foods (Rastogi et al., 2007). It shows that the higher the pressure, the greater the log reduction. Govaris & Pexara (2021) reported that HPP has the potential to enhance food safety by eliminating microorganisms responsible for foodborne illnesses and spoilage. The application of high pressure induces conformational changes in cell membranes and cell morphology, affecting biochemical reactions and the genetic mechanisms of microorganisms. Consequently, this process ensures a reduction in microbial counts. At lower pressures ($\leq$ 100 MPa), HPP significantly impacts cellular functions such as DNA replication, transcription, and translation, leading to impaired bacterial growth. Conversely, at higher pressures (> 300–600 MPa), microorganisms experience lethal injuries due to the loss of cell membrane integrity and protein functionality (Sehrawat et al., 2021; Salvador-Castell et al., 2020). In our study, we applied intermediate (200 MPa) to high pressure levels (400 - 600 MPa). Notably, the highest efficiency in reducing microbial load in insect samples was achieved with HPP at 600 MPa. US also decreased total viable counts but was not as effective as HPP, especially at lower treatment times. It was reported that the combination of critical temperature (50 - 60 °C) with US can inactivate microbes (BaŞlar et al., 2016). Yeasts and molds were present in both the control and US treated samples across all insect species, but their levels fell below detectable limits after HPP pretreatments (Table 14). The US treatments proved ineffective in controlling yeast and mold growth due to the brief exposure time and lack of elevated temperatures during the process. In contrast, HPP methods successfully inhibited the proliferation of yeasts and molds.

Table 14. Total viable count and yeast & molds of edible insect samples after HPP or US pretreatments

Edible insects	Pretreated samples	Colony forming unit (CFU/g)	
		Total viable count	Yeast & Molds
AHCK	Control	4.17×10^{11}	Spr
	HPP 200	2.72×10^{3}	<25
	HPP 400	1.10×10^{3}	<25
	HPP 600	6.83×10^{2}	<25
	US 5	3.38×10^{10}	Spr
	US 10	2.05×10^{10}	Spr
	US 15	6.85×10^{8}	Spr
BSFL	Control	1.03×10^{10}	Spr
	HPP 200	5.07×10^{3}	<25
	HPP 400	3.22×10^{3}	<25
	HPP 600	1.91×10^{3}	<25
	US 5	1.40×10^{9}	Spr
	US 10	2.75×10^{8}	Spr
	US 15	2.95×10^{8}	Spr
SWP	Control	2.90×10^{8}	Spr
	HPP 200	3.38×10^{2}	<25
	HPP 400	1.05×10^{2}	<25
	HPP 600	1.00×10^{2}	<25
	US 5	1.00×10^{8}	Spr
	US 10	1.00×10^{8}	Spr
	US 15	5.50×10^{7}	Spr

Note: Control = non-pretreated sample; HPP 200, 400, and 600 = High pressure processing pretreated samples at 200, 400, and 600 MPa for 5 min; US 5, 10, and 15 = Ultrasonication pretreated samples at 37 kHz for 5, 10, and 15 min. Spr = Spreaders (> 25% colony forming unit on agar plate).

Overall, HPP significantly reduced microbial loads in edible insects before further processing or consumption. HPP achieved greater microbial reductions likely due to the higher pressures rupturing cell membranes. US is a simpler process that may be more accessible and can achieve reasonable reductions, especially with extended treatment times or when combined with other methods like thermal treatments. This approach aims to ensure the food safety of edible insects for both human and animal consumption.

3.4.3 Microstructure by SEM

Microscopy serves as a useful tool for directly observing structural changes induced by food processing techniques. Based on the scanning electron micrographs in Figure 31, we can see visual evidence of the microstructural changes to the edible insect samples induced by HPP versus US pretreatments. For all three insect types, the control samples show intact cell structures with clear cell walls (Figure 31A, D, G). This is the expected morphology for untreated insect powders. However, after HPP at 600 MPa, major disruption of the cell structure was visible in all samples (Figure 31B, E, H). Cell walls appeared ruptured and porous, with a loss of structural integrity. The cell wall destruction was especially evident in BSFL, with larger pore formations visible at 1000× magnification (Figure 31E). This could correlate with increased solvent penetration and oil extraction post-HPP. These structural changes are attributed to the sudden expansion of lipid bilayers caused by HPP, which can destroy cell membrane integrity (Naik et al. 2013). By modifying the physical and biochemical structure, HPP likely contributed to the increased oil extraction yields, beyond just reducing microbial load. This matches previous discussions on how HPP can damage cell membranes through bilayer expansion and loss of membrane integrity at pressures over 100 MPa (Patterson et al., 1995). Therefore, high pressure treatment did not only influence the microbial load reduction of edible insect samples but also increased the oil extraction efficiency by causing a change in the physical biochemical environment of the sample (Farkas & Hoover, 2000).

In contrast, US pretreatment shows more subtle changes to cell morphology (Figure 31C, F, I). The cell walls retained shape and definition, with minimal pore formation visible. This agrees with the understanding that US causes minor membrane displacement and oscillation rather than wholesale destruction of cell membranes (Man et al., 2019). Overall, these micrographs provide visual confirmation on how HPP altered insect cell microstructure to a greater extent than US, corresponding to the differences shown in microbial reduction and oil extraction yields.

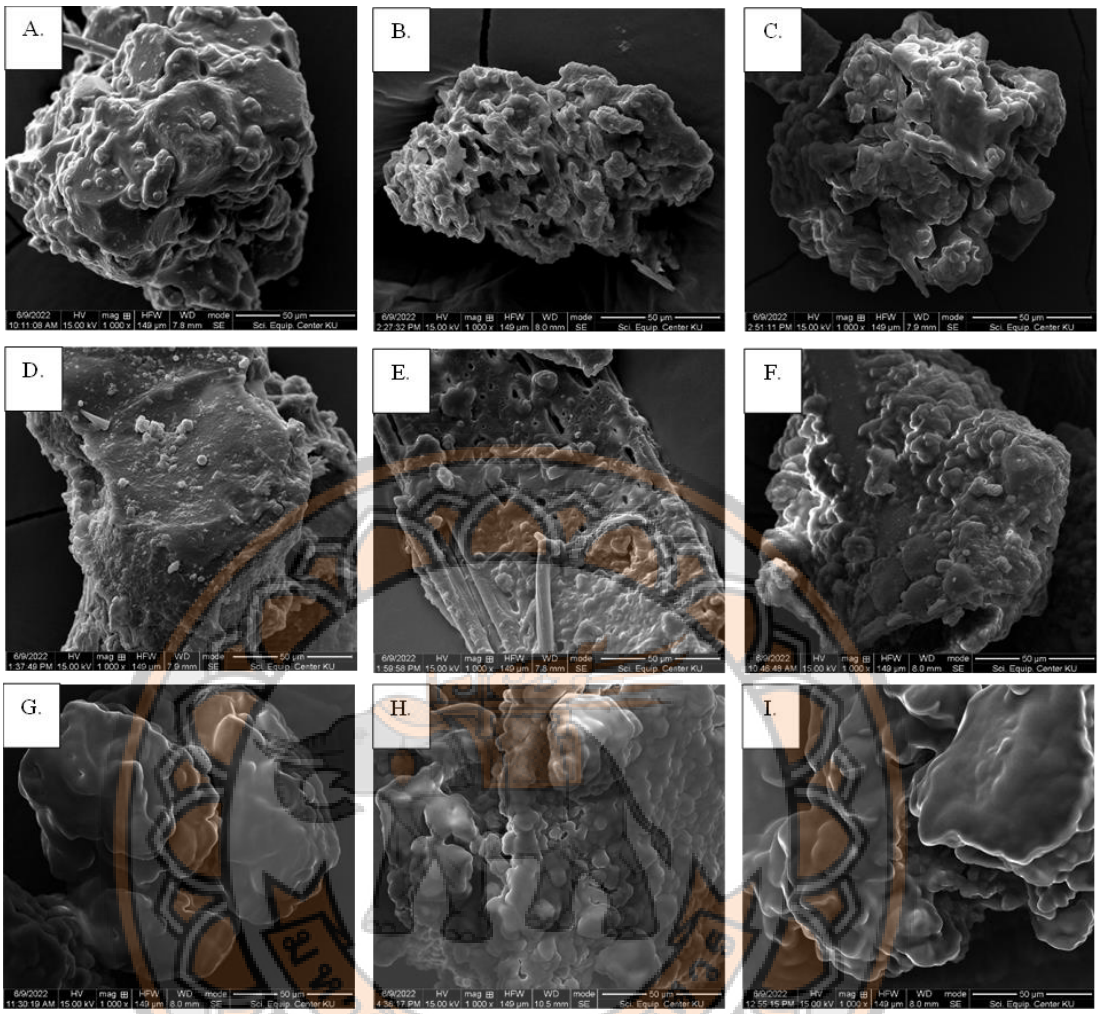


Fig. 31 Scanning electron micrographs of selected edible insect powder after HPP or US treatments

A - C for AHCK samples (A = control /non-pretreated sample, B = treated with HPP at 600 MPa 5 min, C = treated with US at 37 kHz 10 min) ; D - F for BSFL samples (D = control /non-pretreated sample, E = treated with HPP at 600 MPa 5 min, F = treated with US at 37 kHz 10 min); G - H for SWP (G = control /non-pretreated sample, H = treated with HPP at 600 MPa 5 min, I = treated with US at 37 kHz 10 min)

3.4.4 Oil Extraction

3.4.4.1 Solvent Extraction

The results of oil extraction using organic solvent are presented in Figure 32. The color values of oils from all pretreatments showed slight differences (Appendix B). It shows improved oil extraction yields from high HPP and US pretreatments of edible insects and could be explained through the cell disintegration theory. Applying external forces like pressure, ultrasonics, and other

methods could cause intracellular disruption and breakdown of cell walls and membranes. Pretreatments likely caused mechanical cell disintegration of the insect samples, rupturing the cell walls, and facilitating the release of intracellular oil during solvent extraction. HPP has been shown to cause cell disintegration through compression and shear forces as pressure transmitted through the intracellular liquid leads to cell wall fracture. This aligns with the significant improvement in oil yield seen with 600 MPa HPP pretreated AHCK. Likewise, US can induce acoustic cavitation, generating shockwaves and liquid jets that create pores and alter cell membranes (Brnčić et al., 2010). This matches prior results showing increased extraction efficiency of insect protein and chitin with US pretreatment (Wu et al., 2015). The higher yields from US-pretreated BSFL can be attributed to such cell disintegration. Cell disintegration theory provides a mechanistic explanation for the efficacy of HPP and US pretreatments in enabling intracellular oil recovery from insects. By causing physical disruption of cell integrity, these pretreatments likely permitted increased solvent access and extraction of the oil components.

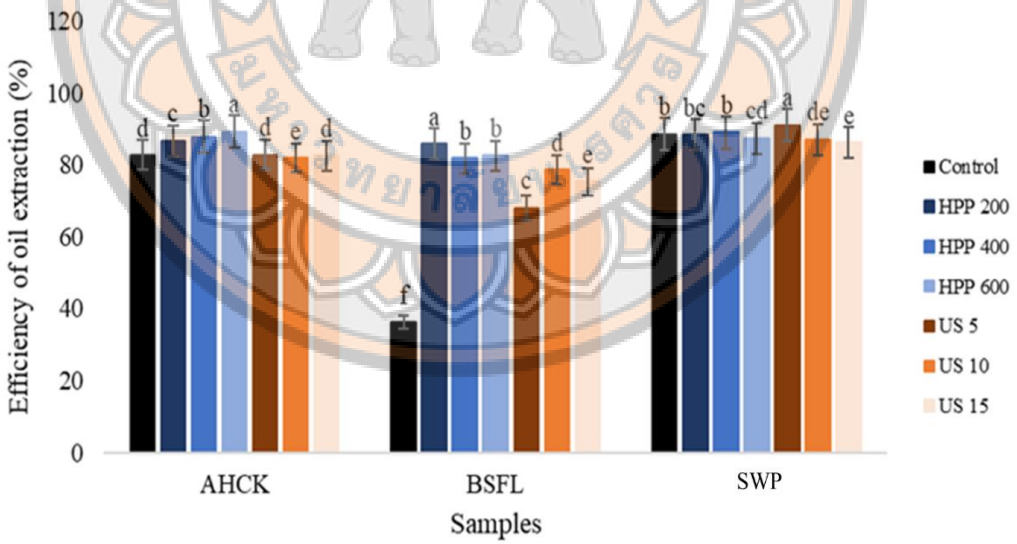


Fig. 32 Oil extraction yields from edible insects

Comparison of HPP and US pretreatments with SE versus non-pretreated control samples. Different superscript letters indicate significant difference ($p < 0.05$).

3.4.4.2 Supercritical CO_2 Extraction

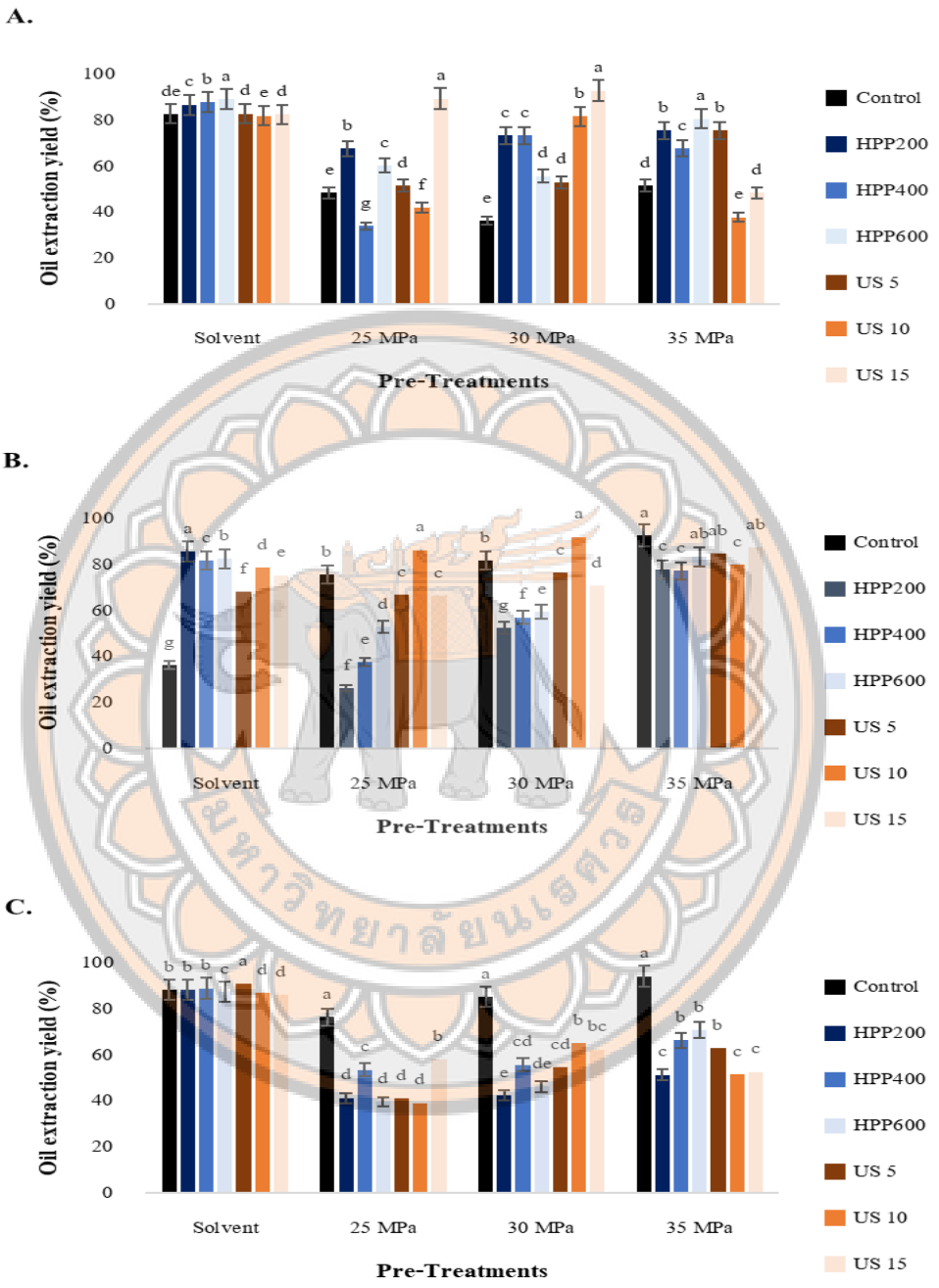


Fig. 33 Oil extraction yields from edible insects: Comparison of HPP and US pretreatments with SC-CO_2 extraction

Non-pretreated control samples, (A. = AHCK, B. = BSFL and C. = SWP). Different superscript letters indicate significant difference ($p < 0.05$).

The increased oil extraction yields from HPP and US pretreated insects using SC-CO_2 extraction are presented in Figure 33A – C, for AHCK, BSFL, and SWP, respectively. This finding could be related to the mass transfer theory as the rate of extraction is dependent on both the solubility of the solutes in the solvent as well as the mass transfer rate of the solutes through the solid matrix (Esquível et al., 1999). HPP and US pretreatments likely enhanced both solubility and mass transfer kinetics. HPP has been shown to increase cell membrane permeability, allowing greater penetration of solvents into the cell cytoplasm (Balasubramaniam et al., 2004). The pressure-induced disruption of cell walls also increases the available surface area for solute-solvent contact. This matches the higher extraction yields of oil from HPP-pretreated AHCK using SC-CO_2. US induces cavitation and shear forces that create pores in cell walls, which could explain the maximum oil yields from US-pretreated BSFL. Kim et al. (2019) reported the effect of SC-CO_2 at 15, 25, and 35 MPa on oil extraction from BSFL. They showed that the extraction yield increased with pressure, from 16% at 15 MPa to 24.5% at 35 MPa. As the pressure increased, the amount of fat in the flies gradually decreased. The results reported in this study are similar to Kim et al.'s findings, showing that as the pressure increased in the BSFL samples, the amount of oil extracted also increased.

The pores and damage allow increased diffusion and mass transfer of intracellular oil components. However, for silkworms, the pretreatments did not improve oil extraction. This may be because silkworms have more resilient cell walls that were not sufficiently altered by HPP or US to change mass transfer kinetics based on the parameters tested.

Our results show that SC-CO_2 extraction produced lighter-colored insect oils compared to solvent extraction (data not shown). This is likely due to the selective extraction of lipid components by the non-polar supercritical fluid (Taniguchi et al., 1985). SC-CO_2 extraction also reduced the oil's phosphorus content, which is beneficial for further refining and use. Previous studies have demonstrated that SC-CO_2-extracted oils generally have higher quality in terms of color, phosphorus content, and tocopherol retention compared to solvent-extracted oils (Piras et al., 2009; Taniguchi et al., 1985). Our SC-CO_2-extracted oils displayed a more solid-like texture than solvent-extracted oils, probably due to differences in the selective extraction of specific lipid components (Temelli et al., 2012).

3.4.5 Oil Properties

The lipid oxidation induced by HPP and US highlights the need to balance cell disruption to increase extraction yields with minimizing the degradation of unstable insect oils. A milder HPP below 300 MPa may be warranted over higher pressures that rapidly accelerate oxidation. Similarly, short US times and lower intensities may extract oil while better-preserving quality (Sethi et al., 2017; Moghimi et al., 2018). This provides an advantage over solvent extraction in mitigating pretreatment-induced oxidation. The higher oxidative stability of SWP oil aligns with the literature on its composition and suggests some insect oils may be less susceptible to degradation during processing. Further research could explore the impacts of oil composition on pretreatment effects. Overall, these findings indicate insect oil extraction requires tailored pretreatment and separation methods to balance yields and quality. Milder cell disruption, coupled with oxygen-free techniques like SC-CO_2 extraction, shows promise for stabilizing insect oils. Further process optimization and comparative studies are warranted. The findings that HPP and US pretreatments increased FFAs, AVs, and PVs of extracted insect oils have important implications for developing optimal insect oil extraction processes. The results presented in Figures 34A–C, 35A–C, and 36A–C illustrate the effects of different pretreatment methods on the quality parameters of oils extracted from AHCK, BSFL, and SWP. Overall, both HPP and US pretreatments were observed to increase the levels of FFAs, AVs, and PVs in oils extracted from AHCK and BSFL. The low FFA content observed in SWP oil (Figure 34) correlates with its low AV shown in Figure 34. This relationship is consistent with Tangsanthatkun et al. (2022), who reported that AV is indicative of FFA release. Both PV and AV are crucial quality parameters, reflecting the extent of oil oxidation and hydrolysis, respectively. The low FFA and AV levels in SWP oil may be attributed to the silk boiling process during silk reeling. As noted by Rashid et al. (2022), heat treatment can inhibit enzymatic oxidation, potentially preserving oil quality. In contrast, AHCK and BSFL samples were subjected to prolonged freezing before processing. Wereńska et al. (2022) found that frozen storage of meat can lead to hydrolysis of ester bonds between glycerol and fatty acids, as well as fatty acid oxidation. This storage-induced oxidation may explain the higher FFA values observed for these insects in Figure 34.

It was observed that the AV of AHCK oil pretreated using US was increased when the pressure of subsequent SC-CO_2 extraction was increased. However, the AV decreased with longer ultrasonic pretreatment times prior to SC-CO_2 extraction (Figure 35). This inverse relationship between US duration and AV aligns with the recent findings of Kerras et al., (2023), who reported decreased AVs resulting from longer ultrasonic pretreatments of the biomass. For BSF samples, only the combination of solvent extraction with US and SC-CO_2 decreased the AV, but it increased in AHCK. However, the potential mechanisms responsible for these trends require further elucidation. Nonetheless, our results further support ultrasonic pretreatment as a means of lowering crude oil acidity, thereby improving quality.

The lipid oxidation induced by HPP and US highlights the need to balance cell disruption to increase extraction yields while minimizing the degradation of unstable insect oils. It was noted that HPP at pressures below 300 MPa had a less severe impact on lipid oxidation compared to higher pressure levels. This observation agrees with previous findings by Sethi et al. (2017), who suggested that lipid assemblies are more susceptible to hydrophobic interactions at elevated pressure levels. HPP treatment of whole fresh insect samples results in a temperature increase of approximately 3°C per 100 MPa applied. This temperature rise, though modest, can impact oil quality. Mohammadian Fazli et al. (2022) observed that heating edible oils typically leads to an increase in AV due to the hydrolysis of triacylglycerols. This process releases free fatty acids, potentially affecting the oil's quality and stability. Similarly, short US times and lower intensities may extract oil while better-preserving quality. This phenomenon could be attributed to the potential oxidation reactions induced by ultrasonic waves, with the extent of oxidation potentially increasing with higher power and longer treatment times, as suggested by Moghimi et al. (2018). Furthermore, as the pressure of HPP increased, the lipid oxidation levels of the extracted oils also increased, as evidenced by the rise in PVs (Figure 36). Notably, PVs ranged from 1.11 to 21.18 mEqv.O_2/ kg for AHCK, 0.99 to 64.60 mEqv.O_2/kg for BSFL, and 1.40 to 33.51 mEqv.O_2/kg for SWP oils, respectively. PV of oils exceeding 10–20 mEqv.O_2/kg fat indicate the onset of lipid oxidation. The PV of SWP oil extracted with solvents was higher than that obtained using SC-CO_2 extraction. This difference can be attributed to the longer duration of the solvent extraction process compared to SC-CO_2 extraction. Additionally, samples subjected to pretreatments exhibited higher PV than control samples. This increase in PV is likely due to the extended processing time required for HPP and US treatments, which may promote oil oxidation.

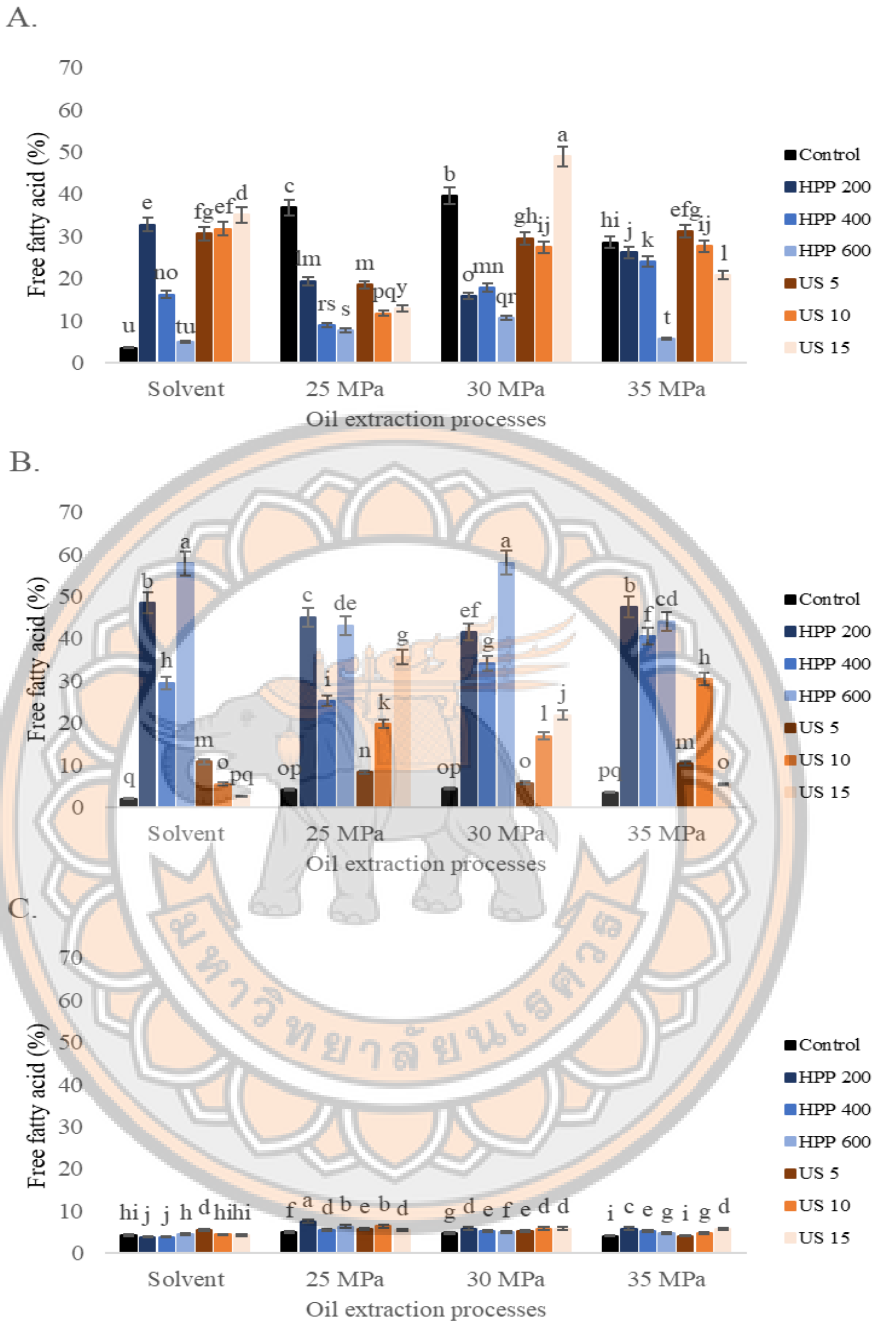


Fig. 34 Free fatty acid content in edible insect oils

Effects of HPP and US pretreatments combined with SE or SC-CO_2 extraction, compared to non-pretreated controls. (A) AHCK, (B) BSFL, (C) SWP. Different superscript letters indicate significant difference ($p < 0.05$).

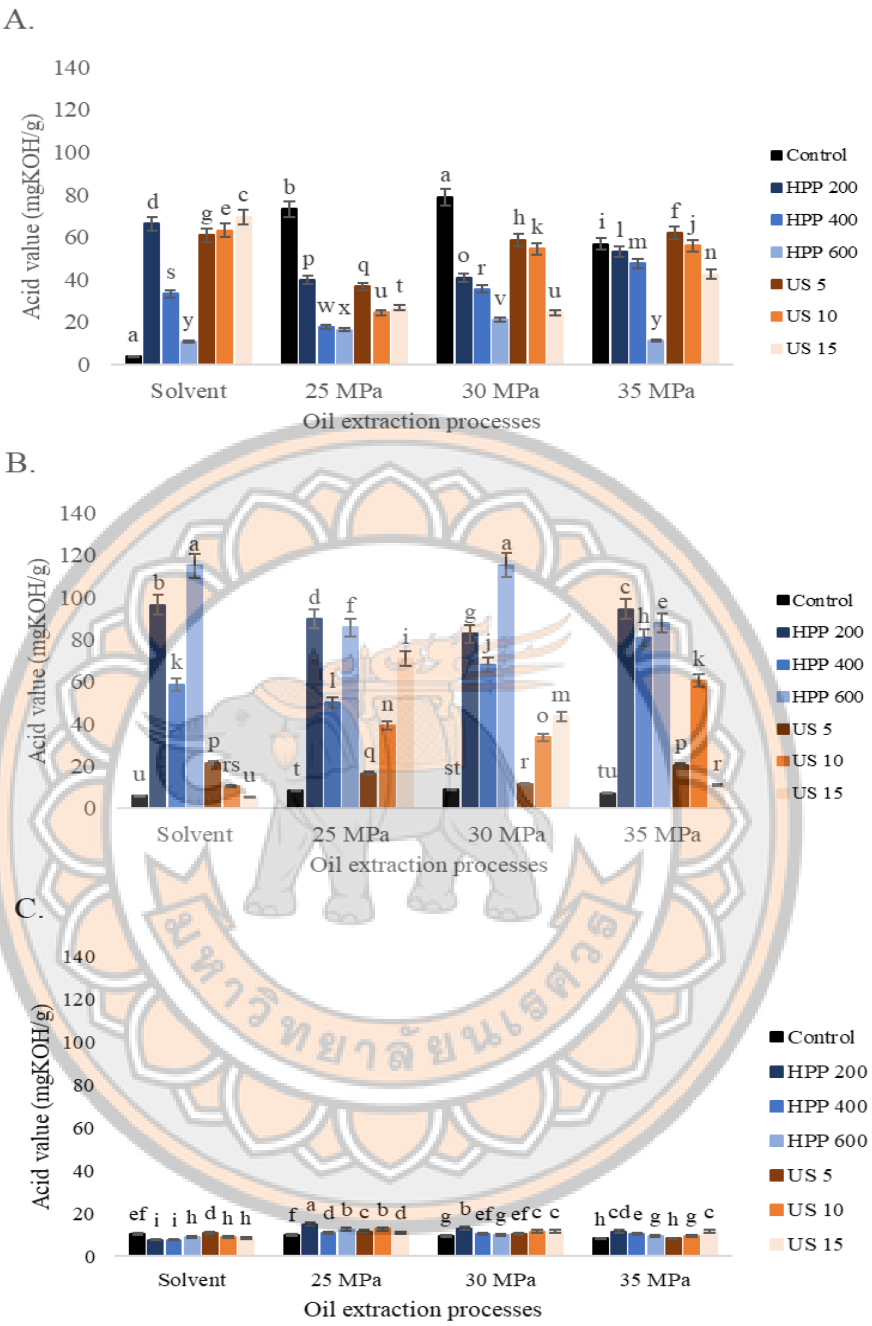


Fig. 35 Acid value in edible insect oils

Effects of HPP and US pretreatments combined with SE or SC-CO_2 extraction, compared to non-pretreated controls. (A) AHCK, (B) BSFL, (C) SWP. Different superscript letters indicate significant difference ($p < 0.05$).

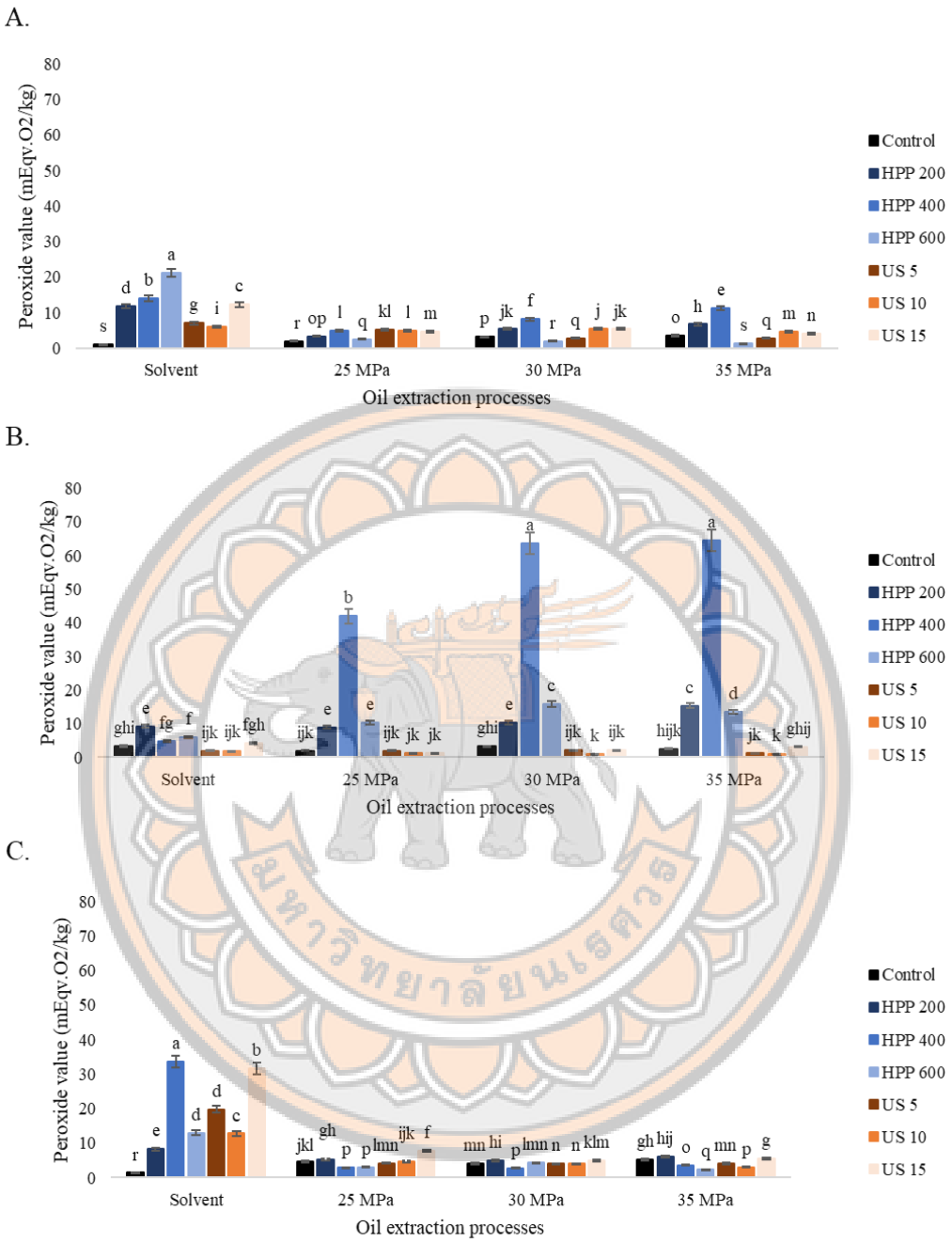


Fig. 36 Peroxide value in edible insect oils

Effects of HPP and US pretreatments combined with SE or SC-CO_2 extraction, compared to non-pretreated controls. (A) AHCK, (B) BSFL, (C) SWP. Different superscript letters indicate significant difference ($p < 0.05$).

Interestingly, oils extracted from US-pretreated samples, particularly using SC-CO_2, consistently exhibited PVs below the standard, suggesting better oxidative stability compared to HPP-pretreated samples. The increase in PV observed in US-pretreated samples could be attributed to the release of free radicals induced by ultrasonic waves, as suggested by Chemat et al. (2004). The lower PVs of US-pretreated oils using SC-CO_2 extraction point to this technique's potential antioxidant effects during extraction, likely due to eliminated oxygen exposure. This provides an advantage over solvent extraction in mitigating pretreatment-induced oxidation. The complex effects of pressure on lipid oxidation across different food systems have been previously mentioned. As stated by Wang et al. (2022), pressure can influence enzyme activities that in turn impact oxidative stability. Additionally, Ma et al. (2020) noted unsaturated fatty acids become more sensitive to oxidation at pressures exceeding 400 MPa, likely tied to structural changes in lipid membranes. Regarding AHCK oils specifically, Ugur et al. (2021) demonstrated a negative relationship between pressure under 300 MPa and oxidation, which aligns with our observations of increased oxidation at 400–600 MPa compared to 200 MPa. The enhanced solvent power of SC-CO_2 at higher pressures, as described by Mathew et al. (2023), may enable greater penetration and extraction of antioxidants that partially counteract oxidation. Hence, the balance of multiple chemical phenomena could determine overall effects on stability. However, further elucidation of specific mechanisms linking pressure changes to lipid oxidation kinetics is needed. Evaluating combinations of CO_2 processing with techniques like HPP, as noted from Li et al. (2023), represents another worthwhile direction for optimizing quality.

However, it is noteworthy that among the insect species studied, oils extracted from silkworms exhibited the least susceptibility to rancidity and oxidation, irrespective of the pretreatment and extraction methods employed. This suggests that silkworm oils may possess inherent qualities that confer greater resistance to lipid oxidation. The higher oxidative stability of silkworm oil aligns with the literature on its composition and suggests some insect oils may be less susceptible to degradation during processing. Further research could explore the impacts of oil composition on pretreatment effects.

3.4.6 Oil Viscosity

Figure 37 indicates that HPP and US pretreatments variably affected the viscosity of extracted insect oils. The higher viscosity of solvent extracted cricket oils versus SC-CO_2 extracted oils as shown in Figure 37A suggests solvent extraction may better preserve oil viscosity, while SC-CO_2 conditions may reduce viscosity. This points to tailoring extraction methods to target different oil viscosity requirements for products (Zulkurnain et al., 2016). The higher viscosity of solvent extracted cricket oil can be explained by the selectivity, mass transfer characteristics, and the mild condition of SC-CO_2 at the extraction pressure of 25 MPa, which minimizes extraction of very viscous components and effects such as emulsion formation and molecular aggregation. The SC-CO_2 acts as a co-solvent and can dissolve into the oil during the extraction process, thereby disrupting molecular interactions that contribute to viscosity. This plasticizing effect of dissolved supercritical CO_2 lowers viscosity. In addition, the SC-CO_2 extraction is able to selectively extract lower molecular weight, less viscous components compared to solvent extraction. The mild conditions of supercritical CO_2 extraction prevent these effects. Heat and mass transfer limitations during solvent extraction can preferentially concentrate more viscous, higher molecular weight components like wax esters and long chain triglycerides. The excellent mass transfer achieved with SC-CO_2 minimizes this effect.

However, for BSFL, the highest oil viscosity resulted from 600 MPa HPP pretreatment with solvent extraction in Figure 37B. This highlights the complex effects of pretreatments on oil properties based on insect species. HPP may increase viscosity in some cases through changes in fatty acid profiles (Yordanov & Angelova, 2010). The lack of significant viscosity changes from HPP and US pretreatments for SWP oil in Figure 37C indicates viscosity may be more stable for some insect oils. Further research on relating chemical composition to the rheological impacts of processing is needed. Overall, these findings reveal insect oil viscosity depends on the type of pretreatment, extraction method, and original insect species. To optimize the functional properties of insect oils, the interactions between processing parameters must be elucidated through further comparative studies.

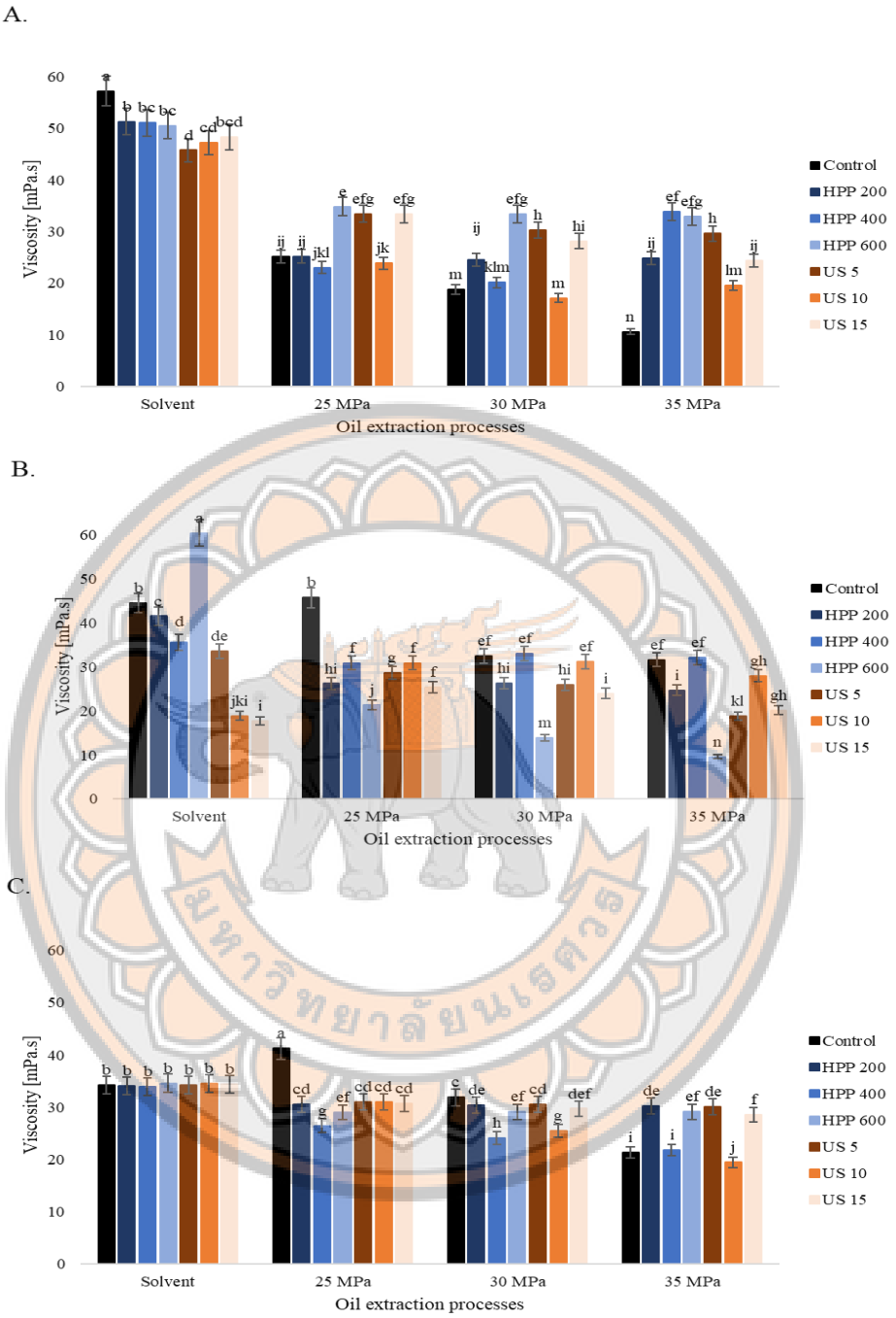


Fig. 37 Viscosity pretreated edible insect oils obtained by SE and SC-CO_2 extraction processes

Effects of HPP and US pretreatments combined with SE or SC-CO_2 extraction, compared to non-pretreated controls. (A) AHCK, (B) BSFL, (C) SWP. Different superscript letters indicate significant difference ($p < 0.05$).

3.4.7 Fatty acid profiles

Preliminary tests comparing the fatty acid profiles of oils obtained through conventional extraction methods versus SC-CO_2 extraction showed no significant differences between the two techniques. However, further investigation of the fatty acid compositions is needed, but these initial results suggest that SC-CO_2 extraction could produce an insect oil product with a similar nutritional quality to those derived using standard practices. This potential equivalency in terms of oil quality supports supercritical fluid extraction as a viable alternative to established approaches from a compositional standpoint, though additional study is required for confirmation.

The fatty acid compositions of various edible insect oils, subjected to US pretreatment at 37 kHz for 15 min before oil extraction by SC-CO_2 at 35 MPa pressure, were analyzed and presented in Table 15. It is noted that edible insects are recognized as a source of beneficial fats (Tang et al., 2019). Across all edible insect oils analyzed, high levels of omega-6 and omega-9 fatty acids were observed, along with minor but potentially nutritionally meaningful amounts of omega-3 fatty acids. Notably, the oil derived from BSFL exhibited the highest concentration of lauric acid (24.11 g/100 g). Lauric acid, abundant in coconut oil and human milk, is renowned for its antimicrobial properties (Sandhya et al., 2016). However, it is noteworthy that the lauric acid content in black soldier fly larvae oil in this study was lower than previously reported findings by Almeida et al. (2022), suggesting potential variations due to factors such as rearing conditions, feed, and climate.

The fatty acid profiles of the three edible insect oils also revealed significant amounts of C16:0, C18:1c9, and C18:2, which are known for their health benefits. This highlights the potential of insect oils as nutraceuticals. Additionally, all insect oils were found to contain high levels of omega-6 and omega-9 fatty acids, which play crucial roles in blood clotting, wound healing, immune system support, and cholesterol regulation (Mariamenatu & Abdu, 2021; Farag & Gad, 2022). Comparisons with previous studies indicate variability in omega-6 content, potentially attributable to differences in AHCK feed, rearing methods, and environments (Kipkoech et al., 2017). Furthermore, the omega-9 content observed in the oils from AHCK, BSFL, and SWP aligns with previous findings highlighting these insects as good sources of omega-9 fatty acids (Jayanegara et al., 2020). Overall, the fatty acid compositions of these edible insect oils demonstrate their potential as sources of beneficial fats with various health-promoting properties.

Table 15. Fatty acid profile of AHCK, BSFL and SWP oils extracted with SC- CO_2

Fatty acid	**AHCK (g/100g)**	**BSFL (g/100g)**	**SWP (g/100g)**
Caprylic acid (C8:0)	ND	0.02	ND
Capric acid (C10:0)	0.02	0.95	ND
Lauric acid (C12:0)	0.15	24.11	0.07
Myristic acid (C14:0)	1.01	6.41	0.23
Myristoleic acid C14:1	0.04	0.09	ND
Palmitic acid (C16:0)	27.15	14.65	23.44
Palmitoleic acid (C16:1n7)	0.65	1.53	0.74
Heptadecanoic acid (C17:0)	0.24	0.24	0.13
Stearic acid (C18:0)	6.58	3.37	7.11
Trans-9-Elaidic acid (C18:1n9t)	0.10	0.19	ND
Cis-9-Oleic acid (C18:1n9c)	31.44	17.08	30.42
Cis-9,12-Linoleic acid (C18:2n6)	30.83	27.32	10.25
Alpha-Linolenic acid (C18:3n3)	0.84	3.59	27.10
Arachidic acid (C20:0)	0.22	0.09	0.29
Saturated fat	35.83	50.10	31.40
Unsaturated fat	64.18	49.90	68.62
Monounsaturated fat	32.39	18.96	31.16
Polyunsaturated fat	31.79	30.94	37.46
Tran fat	0.10	0.19	ND
Omega 3 (mg/100g)	782.29	3566.41	27199.59
Omega 6 (mg/100g)	31011.82	27381.67	10254.79
Omega 9 (mg/100g)	31601.27	17145.68	30420.45

Note: ND = not detected.

3.5 Conclusion

In conclusion, this study demonstrates the potential of HPP and US pretreatments to enhance oil extraction yields from commercially produced insects, specifically AHCK, BSFL, and SWP. Our findings reveal that optimal extraction conditions are species-specific, underscoring the importance of tailored approaches in insect oil production. For AHCK, US15 pretreatment combined with SC-CO_2 extraction at 30 MPa yielded the highest oil extraction. BSFL showed two optimal methods: non-pretreated samples with SC-CO_2 at 35 MPa and US10 pretreatment with SC-CO_2 at 30 MPa. SWP oil yield was maximized using non-pretreated samples extracted with SC-CO_2 at 35 MPa. However, our results highlight a critical trade-off between extraction yield and oil quality. Non-pretreated samples extracted with conventional solvents consistently showed the lowest values for quality parameters (FFA, AV, and PV) across all insect species. This observation suggests that pretreatments, while enhancing yield, may accelerate lipid oxidation. Future research should focus on optimizing milder processing conditions that balance maximized yields with preserved oil quality. Notably, BSFL oil contained high levels of lauric acid, suggesting its potential use as an antimicrobial additive in animal feed or other applications. This finding opens new avenues for the utilization of insect-derived oils in various industries. Our study also revealed that the necessity for pre-treatment varies among insect species. BSFL, in particular, yielded satisfactory oil without pre-treatment, indicating potential for cost reduction and process simplification in oil production from this species. While SC-CO_2 extraction generally produced higher quality oils, its higher cost compared to conventional solvent extraction necessitates careful cost–benefit analysis. The choice of extraction method should consider factors such as the target insect species, desired oil quality, and economic constraints.

CHAPTER IV

MULTI-ENZYME INHIBITORY ACTIVITIES OF PEPTIDES FROM FARMED EDIBLES FOLLOWING SIMULATED GASTROINTESTINAL DIGESTION

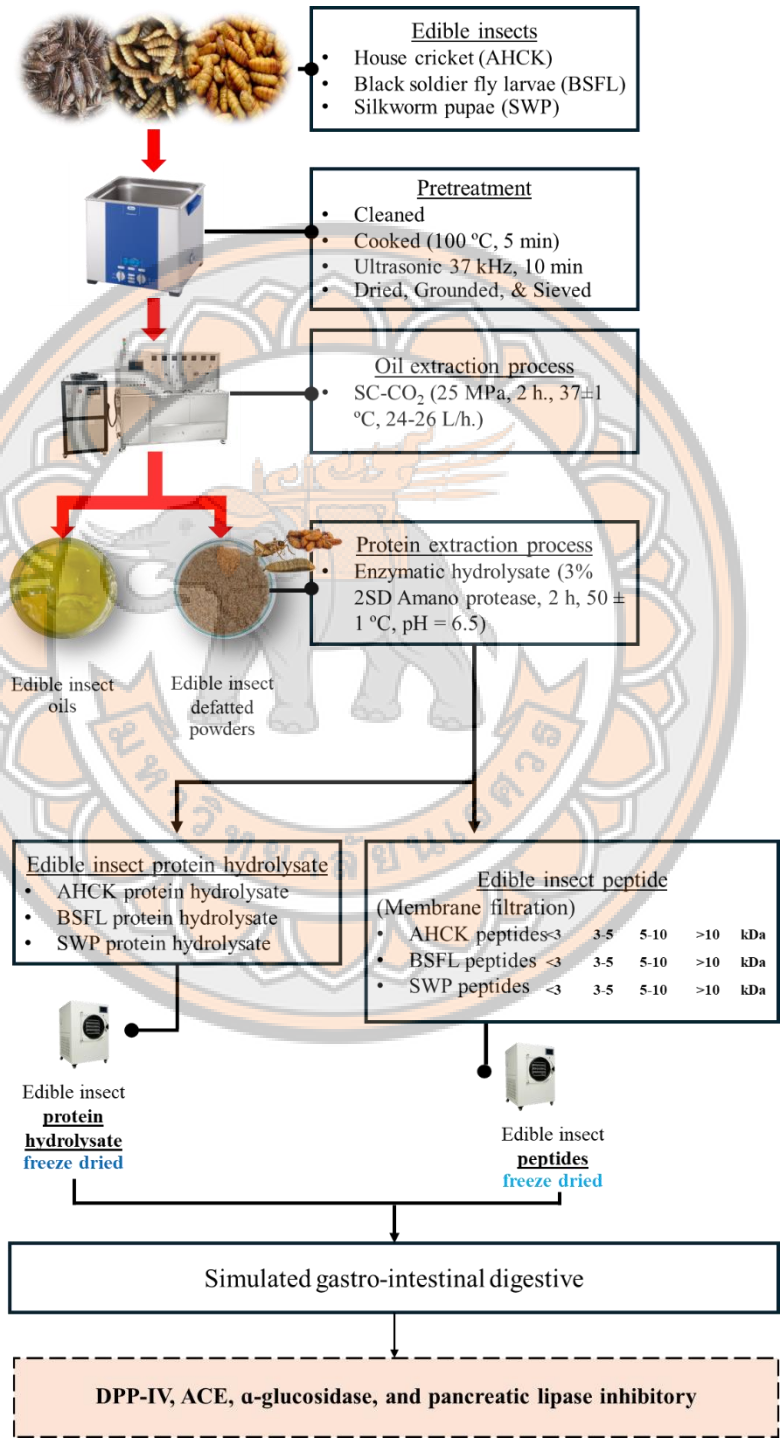


Fig. 38 Flow chart diagram of protein and bioactive peptide production

4.1 Abstract

Edible insects represent a sustainable protein source with potential health benefits. This study investigated enzyme inhibitory activities of protein hydrolysates and peptide fractions from AHCK, BSFL, and SWP. Insects were ultrasonicated, partially defatted using SC-CO_2, and hydrolyzed. The resulting fractions were evaluated for inhibitory activities against DPP-IV, α-glucosidase, ACE, and pancreatic lipase before and after SGD. SGD significantly enhanced bioactivities, particularly in <3 kDa fractions. The <3 kDa SWP peptides showed exceptional ACE inhibition (IC_{50} 0.05 mg/mL), while AHCK peptides exhibited potent α-glucosidase inhibition (IC_{50} 0.07 mg/mL) comparable to acarbose. LC-MS/MS analysis identified several bioactive peptide sequences, including GPAGPQGPR as a common DPP-IV inhibitor across all species. These findings highlight edible insect-derived peptides' potential as natural enzyme inhibitors for non-communicable disease management.

Keywords: Farmed edible insects; Bioactive peptides; Enzyme inhibition; Simulated gastrointestinal digestion; DPP-IV; ACE; Non-communicable diseases

4.2 Introduction

Chronic non-communicable diseases (NCDs) represent major global health challenges, affected by both external lifestyle factors and internal enzymatic activities (Rahman et al., 2024; Tulchinsky et al., 2023). Key enzymes, including DPP-IV, α-glucosidase, ACE, and pancreatic lipase, play significant roles in the pathogenesis of diabetes, hypertension, and obesity (Abbas et al., 2019; Liu et al., 2021; Urbizo-Reyes et al., 2022; Wu et al., 2009).

DPP-IV rapidly degrades incretin hormones essential for insulin secretion, contributing to type II diabetes and atherosclerotic diseases (Flatt et al., 2008; Röhrborn et al., 2015). Similarly, α-glucosidase, which hydrolyzes carbohydrates to glucose in the intestine, represents an important target for diabetes management through prevention of postprandial hyperglycemia (Bakhshi et al., 2018). ACE catalyzes the conversion of angiotensin I to angiotensin II, increasing blood pressure through vasoconstriction and contributing to cardiovascular disease (Fuchs & Whelton, 2020; Riordan, 2003). Pancreatic lipase breaks down dietary fats, and its unchecked activity contributes to obesity and related disorders (Mu & Høy, 2004; Shinde et al., 2024).

The increasing global demand for sustainable protein sources has directed significant attention toward edible insects, characterized by high protein content (50 - 70% dry weight) and a lower environmental footprint than conventional protein sources (Makkar et al., 2014; Shah et al., 2022). Approximately 2 billion people globally already incorporate insects into their diets, with the FAO advocating their use for enhanced food security (Attia et al., 2023; Khan, 2018). Among commercially farmed species, AHCK, BSFL, and SWP have emerged as promising candidates for large-scale production due to their efficient feed conversion ratios and favorable nutritional compositions (Apri & Komalassari, 2019; Hawkey et al., 2020). Their amino acid profiles, rich in glutamic acid, aspartic acid, and essential amino acids, make them suitable sources for bioactive peptides with enzyme inhibitory activities (Köhler et al., 2019; Udomsil et al., 2019).

Previous research has demonstrated that AHCK peptides exhibit ACE inhibition, while BSFL and SWP contain peptides with antioxidant, ACE inhibitory, and DPP-IV inhibitory properties (Ma et al., 2023; Wong et al., 2023; Zieliński et al., 2020). Processing methods, including enzymatic hydrolysis and SGD, significantly influence bioactivities (Ferrazzano et al., 2023; Mudd et al., 2022). Novel preprocessing techniques such as US and SC-CO_2 extraction have shown promise for enhancing protein recovery and bioactive peptide release while minimizing solvent use (Boonmee et al., 2024; Tang et al., 2018). Additionally, lower molecular weight (MW) peptides often exhibit enhanced enzyme inhibitory properties (Lee & Hur, 2019; Nongonierma et al., 2018; Wang et al., 2021).

While individual aspects of insect-derived bioactive peptides have been explored, a comprehensive comparative analysis across multiple commercially viable species, enzyme inhibition activities, and MW fractions remains lacking. This study investigated the amino acid composition of partially defatted AHCK, BSFL, and SWP, evaluating their potential for DPP-IV, α-glucosidase, ACE, and pancreatic lipase inhibitory activities across different protein fractions (<3 kDa, 3-5 kDa, 5-10 kDa, and >10 kDa) before and after SGD. Bioactive peptides were identified to elucidate the specific sequences responsible for these effects, providing insights into potential applications in functional foods and nutraceuticals targeting NCDs.

4.3 Materials and Methods

4.3.1 Materials

4.3.1.1 Raw Materials and Chemicals

The insect samples utilized in this study consisted of AHCK, BSFL, and SWP, obtained from Thai Ento Food Company Limited, Samutprakarn, Thailand. The AHCKs were 40 - 45 days old upon harvesting, the BSFLs were 22 - 25 days old, and the SWPs were harvested at the pupal stage. After harvesting, the raw insect samples were visually inspected and physically screened to remove any contaminants or filth. The samples were then washed 3 times with RO water to eliminate remaining debris and surface microorganisms. The washed insects were blanched by boiling in water for 5 min, followed by packing in polyethylene bags, sealing, and freezing storage at -18°C until further processing (Boonmee et al., 2024).

The protease (Protease A“Amano”2SD, 100,000 U/g) with both endo- and exopeptidase activity was procured from Amano Enzyme Inc (Naka-ku, Nagoya, Japan). For the SGD and enzymatic assays, pepsin from porcine gastric mucosa (EC 232-629-2, ≥2,500 units/mg protein), pancreatin from porcine pancreas (EC 323-619-9, Type II, ≥125 units/mg protein), bile salt, dipeptidyl peptidase-IV (EC 3.4.14.5, ≥10 units/mg) and its substrate Gly-Pro p-nitroanilide hydrochloride, angiotensin converting enzyme (EC 3.4.15.1, ≥2.0 units/mg protein) and its substrate Hippuryl-L-Histidyl-L-Leucine were obtained from Millipore Sigma (St. Louis, MO, USA).

Additional enzymes and reagents including α-glucosidase from Saccharomyces cerevisiae (EC 3.2.1.20, Type I, lyophilized powder, ≥10 units/mg protein), 4-Nitrophenyl α-D-Glucopyranoside, acarbose, lipase from porcine pancreas (EC 3.1.1.3, Type VI-S, ≥20,000 units/mg protein), 4-Methylumbelliferyl oleate, and orlistat were supplied by AT Life & Scientific Co., Ltd. (Chiang Mai, Thailand). All other chemical reagents used for analysis were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA).

4.3.2 Methods

4.3.2.1 Ultrasonication Pretreatment and SC-CO_2 Oil Extraction

Workflow of insect protein and peptide preparations is demonstrated in Figure 38. To prepare insect meal for proteolysis and peptide fractionation, frozen samples (20 kg each) were thawed, rinsed with deionized water, and ultrasonicated at 37 kHz for 10 min (37 ± 1 °C). Samples were then dried (70 °C, 10h), ground, and sieved (18-mesh) prior to oil extraction. Five kilograms of processed meal were subjected to the SC-CO_2 extraction using a pilot-scale system (50 L/batch, Shanghai Better Industry Co., Ltd.) for partial defatting. Extraction parameters were: 25 MPa, 37 ± 1 °C, 120 min, and CO_2 flow rate of 24 - 26 L/h. The resulting defatted powders (all <10 % residual fat content) were vacuum sealed in aluminum foil bags and stored at -18 °C until protein hydrolysate preparation and peptide fractionation.

Three insect species underwent pretreatment (US), defatting (SC-CO_2 extraction), proteolysis, and either direct freeze-drying or membrane fractionation (<3 to >10 kDa). All samples were subjected to SGD before analyzing enzyme inhibition activities and peptide identification.

Amino acid composition of insect meals was determined according to the Official Journal of the European Communities (L257/16) method using HPLC. The analysis was performed by Central Lab Thailand (Chiang Mai, Thailand). Briefly, the partially defatted insect meal samples (20 mg) underwent acid hydrolysis with 6 M HCl in vacuum-sealed tubes at 110 °C for 24 h. After cooling, hydrolysates were filtered (0.45 µm membrane), neutralized, and analyzed using an amino acid analyzer with cation-exchange chromatography and ninhydrin derivatization. Results were expressed as g/100 g of defatted insect powder.

4.3.2.2 Protein Proteolysis

Protein hydrolysates were prepared from the SC-CO_2 defatted insect meal using enzymatic hydrolysis. First, each insect meal was dispersed in distilled water at a ratio of 1:10 (w/v). The mixture was thoroughly homogenized using a hot plate stirrer (IKA C-MAG HS 7, Germany) at 300 rpm for 10 min to ensure uniform suspension. The pH of each suspension was then adjusted to 6.5 (optimal for protease activity) using 2M NaOH, and the temperature was raised to 50 ± 2 °C.

Enzymatic hydrolysis was initiated by adding the Protease A “Amano” 2SD at a concentration of 3 % (w/w of protein content). The hydrolysis reaction was conducted under constant stirring (200 rpm) for 120 min at 50 ± 2 °C to maintain optimal enzyme activity. Following

the hydrolysis period, the enzyme was inactivated by heat treatment in a water bath at 100 ± 1 °C for 10 min. The mixtures were then cooled to room temperature (25 ± 1 °C) in an ice bath to prevent further protein degradation. The cooled hydrolysates were centrifuged at 15,652 × g for 15 min (Sorvall Legend X1R, Thermo Fisher Scientific, USA) to separate insoluble residues from the soluble peptide fractions.

The supernatants containing the soluble peptides were carefully collected and dried using a freeze dryer (FreeZone® Console Freeze Dryer with Stoppering (12L capacity, Labconco, USA). The samples were initially frozen at -40 °C for 12 h to ensure complete crystallization, followed by primary drying at -20 °C under vacuum (0.05 mbar) for 24 h. Secondary drying was performed by gradually increasing the temperature to 20 °C over 12 h to remove bound moisture. The resulting dried products (moisture content < 5 %) were collected, sealed in airtight containers, and stored at -18 °C.

4.3.2.3 Peptide Fractionation by Ultrafiltration

The supernatants collected from AHCK, BSFL, and SWP protein hydrolysates from part *4.3.2.2* were subjected to tangential flow filtration using a Minimate™ Tangential Flow Filtration System (Pall Corporation, USA). The fractionation was performed as a cascade process using membranes with decreasing molecular weight cut-offs (MWCO). First, the hydrolysate was passed through a 10 kDa MWCO membrane. The retentate, containing peptides and proteins larger than 10 kDa, was collected as the >10 kDa fraction. The permeate from the first filtration step was then processed through a 5 kDa MWCO membrane. The retentate from this step, containing peptides between 5 and 10 kDa, was collected as the 5-10 kDa fraction. Next, the permeate from the 5 kDa filtration was passed through a 3 kDa MWCO membrane. The retentate, containing peptides between 3 and 5 kDa, was collected as the 3-5 kDa fraction. Finally, the permeate from the 3 kDa membrane, containing peptides smaller than 3 kDa, was collected as the <3 kDa fraction.

All MWCO fractions (<3 kDa, 3-5 kDa, 5-10 kDa, and >10 kDa) from each insect species were subsequently lyophilized using the same freeze-drying protocol described in section *4.3.2.2*. The lyophilization process was conducted until the moisture content of all fractions was below 5%. The dried peptide fractions were then weighed and sealed in airtight containers and stored at -18°C until further analysis.

4.3.2.4 Simulated Gastro-Intestinal Digestion (SGD)

SGD was performed to mimic the physiological digestion process that would occur in vivo, following the methodology described by Mudd et al. (2022). Insect protein hydrolysates or peptide fractions (1 g each) were combined with 10 mL of tris-buffer (pH = 8.0) and stirred at room temperature (25 ± 1 ºC) for 30 min. The mixture was adjusted to pH 2.0 with 6M HCl and shaken in a water bath at 37 °C. Then, 4 % (w/w of protein) pepsin was added and incubated for 2 h. After, the mixture was removed and placed on ice. The mixture was then adjusted to pH 6.0 using 1M $NaHCO_3$ and further adjusted to pH 7.0 using 6M NaOH. When the temperature in the mixture reached 37 °C, 4 % (w/w of protein) pancreatin and 10 mg/mL bile salts were added and incubated for 2 h. To terminate the enzymatic activity, the digests were heated at 90 °C for 10 min in a water bath. The samples were then cooled to room temperature and adjusted to a final pH of 7.0. The digesta was centrifuged at 12,000 × g for 30 min at 4 °C to separate the insoluble material from the soluble peptides. The supernatants, containing the bioaccessible peptides, were carefully collected, transferred to appropriate containers, and immediately subjected to freeze-drying. The freeze-dried post-SGD samples were weighed to calculate yield, sealed in airtight containers, and stored at -18 °C until further analysis of their bioactive properties.

4.3.2.5 DPP-IV Inhibition

The DPP-IV inhibitory activity of all insect protein hydrolysates and peptides was determined according to the method proposed by Urbizo-Reyes et al. (2021). Both before and after SGD, the edible insect peptide samples were dissolved in 100 mM Tris-HCl buffer (pH 8.0) to a final concentration of 0.5 mg/mL. In a 96-well microplate, 25 µL of sample solution was pre-incubated with 25 µL of substrate (6 mM Gly-Pro p-nitroanilide hydrochloride) at 37 °C for 10 min. Following pre-incubation, 50 µL of human DPP-IV enzyme solution (4.5 units/mL) was added to each well, and the plate was incubated at 37 °C for 1 h. The enzymatic reaction was then terminated by adding 100 µL of 1 M sodium acetate buffer (pH 4.0). The absorbance of released p-nitroanilide, which indicates DPP-IV activity, was measured at 405 nm using a Multiskan™ FC Microplate Photometer (Thermo Scientific, Waltham, MA, USA). For validation purposes, a commercial DPP-IV inhibitor was used as a reference standard, following the same procedure. A positive control was prepared by replacing the sample with buffer (representing 100 % enzyme activity), while a negative control was prepared by replacing the enzyme with buffer (representing 0 % enzyme activity). Sample blanks containing all components except the enzyme were also

included to account for any background absorbance. All measurements were performed in triplicate, and the percentage of DPP-IV inhibition was calculated using equation (2):

$$\text{DPP-IV inhibition (\%)} = 1 - \left[\frac{Abs\ sample - Abs\ sample\ blank}{Abs\ positive\ control - Abs\ negative\ control}\right] \quad (2)$$

4.3.2.6 α-Glucosidase Inhibition

The α-glucosidase inhibitory activity of protein hydrolysates and peptide fractions from the three edible insect species was evaluated following the method described by Urbizo-Reyes et al. (2022) with slight modifications. The enzyme α-glucosidase used in this assay was obtained from AT Life & Scientific Co. Ltd. (Chiang Mai, Thailand). Sample solutions were prepared by dissolving 1 g of each sample (both before and after SGD) in 10 mL of 0.1 M sodium phosphate buffer (pH 6.9) to achieve a final concentration of 0.5 mg/mL. The assay was conducted in 96-well microplates according to the following procedure. First, 50 µL of sample solution was added to designated wells, followed by 50 µL of α-glucosidase enzyme solution. The mixture was pre-incubated at 37 ± 1 °C for 10 min to allow potential inhibitors to interact with the enzyme. After pre-incubation, 100 µL of 5 mM 4-nitrophenyl α-D-glucopyranoside (substrate) was added to each well, and the reaction was allowed to proceed for 10 min at 37 ± 1 °C. The enzymatic reaction was terminated by adding 50 µL of 1 M sodium carbonate solution, which also enhances the color of the released 4-nitrophenol. The absorbance was measured at 405 nm using a Multiskan™ FC Microplate Photometer (Thermo Scientific, Waltham, MA, USA). Acarbose, a known α-glucosidase inhibitor used in the treatment of type 2 diabetes, served as a positive control. Sodium phosphate buffer was used as a control in place of inhibitor samples to represent 100 % enzyme activity. All measurements were performed in triplicate, and mean values were used for calculations. The percentage inhibition was calculated using equation (3).

$$\text{α-glucosidase inhibition (\%)} = 100 \times \left[\frac{Abs\ control\ (no\ inhibitor) - Abs\ sample}{Abs\ control\ (no\ inhibitor)}\right] \quad (3)$$

4.3.2.7 ACE II Inhibition

ACE inhibitory activity of all insect protein hydrolysates and peptides before and after SGD was measured according to the method described by Hall et al. (2020). Sample preparation involved dissolving edible insect peptides in 100 mM sodium phosphate buffer (pH 8.3) containing 300 mM NaCl to a final concentration of 0.5 mg/mL. The substrate Hippuryl-His-Leu (HHL) and

ACE were dissolved in the same buffer solution. The assay procedure consisted of pre-incubating 25 µL of sample with 25 µL of HHL substrate at 37 ± 1 °C for 4 min. Next, 80 µL of ACE enzyme solution (5 mU) was added to initiate the reaction, followed by incubation at 37 ± 1 °C for 2 h in a water bath with constant shaking. The enzymatic reaction was terminated by adding 50 µL of 1 M HCl. The resulting solution was filtered through a 0.22 µm nylon filter to remove particulates. Prior to HPLC analysis, 100 µL of a solution containing equal parts of acetonitrile with 1% (v/v) trifluoroacetic acid (TFA) and distilled water with 1% TFA was added to each sample. During the reaction, ACE hydrolyzes HHL to produce hippuric acid and histidyl-leucine. The amount of HA produced was quantified using HPLC (Model 2690, Waters Corporation, Milford, MA, USA) with an XBridge™ BEH130 C18 analytical column.

A control reaction was prepared by replacing the sample with buffer, representing 100 % ACE activity. All measurements were performed in triplicate, and the percentage of ACE inhibition was calculated using equation (4):

$$\text{ACE inhibition (\%)} = \left[1 - \frac{Abs\ inhibitor}{Abs\ control}\right] \times 100 \qquad (4)$$

4.3.2.8 Pancreatic Lipase Inhibition

Pancreatic lipase inhibitory activity of all insect protein hydrolysates and peptides before and after SGD was evaluated according to the method described by Urbizo-Reyes et al. (2022) with modifications. This fluorometric assay uses 4-methylumbelliferyl oleate (1 mM) as a substrate, which releases the fluorescent compound 4-methylumbelliferone upon hydrolysis by pancreatic lipase. All samples (both before and after simulated gastro-intestinal digestion) were dissolved in 0.1 M Citrate-Na_2HPO_4 buffer (pH 7.4) to a final concentration of 0.5 mg/mL. The pancreatic lipase enzyme and substrate were also prepared in the same buffer.

The assay was performed in black 96-well microplates to minimize light interference during fluorescence measurements. Initially, 25 µL of sample solution was pre-incubated with 50 µL of 4-methylumbelliferyl oleate substrate (1 mM) for 10 min at 37 ± 1 °C. Following pre-incubation, 25 µL of pancreatic lipase type-VI (200 U/mL) was added to each well to initiate the enzymatic reaction. The microplate was incubated at 37 ± 1 °C for 1 h, during which the enzyme hydrolyzes the substrate, releasing the fluorescent 4-methylumbelliferone.

Fluorescence was measured using a Fluoroskan Ascent FL Microplate Fluorometer and Luminometer (ThermoFisher Scientific, Waltham, MA, USA) with an excitation wavelength of

355 nm and an emission wavelength of 460 nm. Greater fluorescence indicates higher enzyme activity, while reduced fluorescence in the presence of samples suggests inhibition of pancreatic lipase. Buffer in place of the sample (representing 100 % enzyme activity) was used as a negative control, while Orlistat, a known pancreatic lipase inhibitor, was used as a positive control, respectively. For the sample blank, buffer in place of the enzyme (to account for background fluorescence from samples) was used, and the buffer in place of the sample and substrate was used as the enzyme blank. Sample readings were corrected by subtracting the corresponding sample blank values. All measurements were performed in triplicate, and the percentage of pancreatic lipase inhibition was calculated using equation (5):

$$\text{Pancreatic lipase inhibition (\%)} = \left[1 - \frac{(Abs\ sample - Abs\ sample\ blank)}{(Abs\ positive\ control - Abs\ negative\ control)}\ x\ 100\right] \quad (5)$$

4.3.2.9 IC_{50} Determination

The IC_{50} values, defined as the protein hydrolysate or peptide concentration required to inhibit 50 % of the enzyme activities, were calculated from a linear regression plot of each enzyme inhibition (%) and protein hydrolysate or peptide concentration (mg/mL).

4.3.2.10 Identification of Bioactive Peptides

This analysis focused specifically on the <3 kDa fractions obtained after SGD from each insect sample. The peptide identification was conducted at the Genomics Facility at Purdue University following the methodology described by Hall et al. (2020). The selected peptide fractions were first desalted and concentrated before being subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. The peptides were separated chromatographically and then analyzed using high-resolution mass spectrometry to determine their amino acid sequences. Raw MS/MS data were processed using specialized proteomics software to identify the peptide sequences present in each fraction. The processing involved comparing the experimental mass spectra against theoretical spectra derived from insect protein databases. This approach allowed for the identification of numerous peptide sequences within each fraction, including those potentially responsible for the observed bioactivities. Following sequence identification, all peptides were searched against the BIOPEP-UWM Database of bioactive peptides to identify sequences with known or predicted bioactivities.

4.3.3 Statistical Analysis

All experimental analyses were performed in triplicate, and results are presented as mean values ± standard deviation. The data were subjected to one-way analysis of variance (ANOVA) to determine significant differences among means. When significant differences were detected ($p < 0.05$), Duncan's multiple range test was applied for post-hoc comparison of means to identify specific differences between treatments. All statistical analyses were conducted using IBM SPSS Statistics software (Version 22, IBM Corporation, Armonk, NY, USA). Differences were considered statistically significant at $p < 0.05$ for all comparisons.

4.4 Results and Discussion

4.4.1 Amino Acid Profile of Partially Defatted Insect Meals

After SC-CO_2 extraction, the moisture, protein, and fat contents of these meals were preliminarily evaluated. The results showed that moisture content ranged from 3.08 % to 4.95 %, while fat content ranged from 3.22 % to 4.11 % across all samples. Among the insect meals, SWP had the highest protein content at 80.19 %, followed by AHCK at 76.87 % and BSFL at 65.91 %.

Table 16. Amino acid composition (g/100g) of defatted edible insect meals compared with conventional protein sources and WHO daily requirements.

Amino acids	**Insect meals (This study)**			**Conventional Proteins**				**WHO Recommendation**
	AHCK	**BSFL**	**SWP**	**Pork[2]**	**Beef[2]**	**Milk[3]**	**Soybean[4]**	**1985 FAO/WHO/UNU[5]**
Essential amino acids								
Histidine	0.48	2.02	2.04	3.20	2.90	3.66	2.97	0.80 – 1.20
Isoleucine	0.71	2.31	2.06	4.90	5.10	4.01	5.32	1.00
Leucine	1.43	3.74	3.37	7.50	8.40	8.81	7.10	1.40
Lysine	1.09	3.86	3.48	7.80	8.40	7.79	6.08	1.20
Methionine	ND	0.94	1.72	2.50	2.30	2.91	2.72	-
Phenylalanine	0.67	2.38	2.45	4.10	4.00	4.67	3.88	1.40
Threonine	0.72	2.20	2.15	5.10	4.00	4.87	3.74	0.70
Tryptophan	0.15	0.72	0.61	1.30	1.10	-	7.64	0.35
Valine	1.18	3.50	2.96	5.00	5.70	4.79	5.25	1.00

Amino acids	Insect meals (This study)			Conventional Proteins				WHO Recommendation
Non-essential amino acids								
Aspartic acid	1.73	5.37	4.86	8.90	8.80	7.49	7.13	-
Serine	0.98	2.40	2.29	4.00	3.80	5.96	6.38	-
Glutamic acid	2.27	6.26	6.06	14.50	14.40	21.67	9.12	-
Glycine	0.95	2.92	2.18	3.20	2.90	1.80	3.71	-
Alanine	1.57	3.59	2.45	6.30	6.40	3.31	3.62	-
Cystine	ND	0.36	0.56	1.30	1.40	0.87	2.07	-
Tyrosine	0.98	3.74	2.92	3.00	3.20	5.00	4.14	-
Arginine	1.07	2.88	2.61	6.40	6.60	3.33	6.18	-
Proline	1.05	3.21	2.13	4.60	5.40	6.58	3.63	-

Note: ND = Not detected.

Source: Mazhangara et al. (2019)[2], Landi et al. (2021)[3], Adebisi et al. (2017)[4], 1985 FAO/WHO/UNU recommendations for adults[5].

The amino acid composition of all partially defatted insect meals, presented in Table 16, Glutamic acid predominates in all three insect species, with concentrations of 2.27, 6.26, and 6.06 g/100g for AHCK, BSFL, and SWP, respectively. These findings align with previous studies by Udomsil et al. (2019), who reported similarly high glutamic acid content in cricket (*Acheta domesticus* and *Gryllus bimaculatus*) at 6.45 g/100g. This also agrees with the finding of Chatsuwan et al. (2018), who reported comparable levels in SWP (6.77 g/100g), while Olaleye et al. (2020) and Schiavone et al. (2017) found glutamic acid contents of 15.3 and 6.37 g/100g in SWP and BSFL defatted meal, respectively. However, as seen in Table 16, the glutamic acid content in conventional animal proteins like pork (14.50 g/100g) and milk (21.67 g/100g) is substantially higher than in the studied insects.

The prevalence of glutamic acid is nutritionally significant as it is a non-essential amino acid that plays crucial roles in protein biosynthesis and functions as a key neurotransmitter (Kumar et al., 2021). Additionally, glutamic acid contributes to maintaining growth and health in both newborns and adults (Watford, 2015) and has been associated with potential α-amylase and α-glucosidase inhibitory activities in protein hydrolysates (Mazloomi et al., 2020), which may contribute to the observed enzyme inhibitory properties of the insect peptides. Aspartic acid ranks as the second most abundant amino acid across all three insects, with concentrations ranging from

1.73 to 5.37 g/100g. This finding is consistent with the amino acid profiles reported by Köhler et al. (2019) for various edible insects sourced from Thailand. Aspartic acid contributes to protein biosynthesis and plays important roles in urea synthesis and brain development (HoleČek, 2023).

The analyzed insects showed distinctive patterns in their limiting amino acids. In AHCK, tryptophan exhibited the lowest concentration (0.15 g/100g) among essential amino acids, consistent with findings by Köhler et al. (2019), who reported similarly low tryptophan levels (0.23 - 0.72 g/100g) across various insect species. Tryptophan is an essential amino acid found in animal and plant products, with a small portion converted to serotonin and melatonin (Zuraikat et al., 2021), potentially influencing sleep patterns through melatonin production (Summer & Cotliar, 2024). For BSFL and SWP, cystine was found in the lowest concentrations (0.36 - 0.57 g/100g).

As illustrated in Table 16, when comparing the essential amino acid profiles of the three insects with common protein sources and the WHO-recommended daily requirements for adults, it becomes evident that all essential amino acids in the three edible insects were present at higher concentrations than in milk for several key amino acids, though at lower levels than in pork, showing quantitative differences as reported by Mazhangara et al. (2019) and Landi et al. (2021). This positioning between plant and animal protein sources reinforces the potential of insects as intermediate protein alternatives in sustainable food systems, as suggested by Van Huis & Oonincx (2017).

BSFL and SWP contained higher levels of all essential amino acids compared to the WHO-recommended daily requirements for adults, indicating their exceptional nutritional quality. In contrast, AHCK samples showed lower levels of histidine, isoleucine, lysine, phenylalanine, and tryptophan relative to the recommended values. These findings align with the comprehensive analysis by Mintah et al. (2020), who characterized edible insects as valuable protein sources with diverse amino acid compositions.

The presence of hydrophobic amino acids like leucine, valine, alanine, and proline in considerable amounts across all three insects (as shown in Table 16) is particularly relevant for bioactive peptide functionality. Zhang et al. (2019) and Mendis et al. (2005) reported that peptides containing these hydrophobic amino acids often exhibit strong antioxidants and enzyme inhibitory activities. The high content of branched-chain amino acids (leucine, isoleucine, and valine) in BSFL and SWP may be especially advantageous for producing bioactive peptides, as these amino acids are known to be frequent components of peptides with DPP-IV and ACE inhibitory activities (Nongonierma et al., 2018).

4.4.2 DPP-IV Inhibition Activities

The DPP-IV inhibitory activities of different MW fractions from the three edible insect protein hydrolysates, both before and after SGD, are presented in Figure 39. The results present distinct patterns in inhibitory capacity based on insect species, MW fractions, and the effect of SGD. For AHCK peptides (Figure 39A), all fractions showed DPP-IV inhibitory activity, with the <3 kDa and 3-5 kDa fractions after SGD exhibiting the highest inhibition, reaching approximately 75% at 0.5 mg/mL concentration. This increase in inhibitory activity after SGD aligns with findings by Nongonierma and FitzGerald, (2013), who observed enhanced DPP-IV inhibition in whey protein hydrolysates following SGD. The digestive enzymes likely cleave the peptides into smaller, more bioactive fragments with improved binding affinity to the DPP-IV enzyme active site. Similar MW-dependent effects have been reported by Lacroix et al. (2018) for mealworm protein hydrolysates, where lower MW fractions demonstrated superior DPP-IV inhibition.

BSFL peptides (Figure 39B) followed a similar trend, with the <3 kDa fraction after SGD showing the highest inhibitory activity (approximately 70%). This MW-dependent activity correlates with findings by Wan et al. (2023), who stated that lower MW fractions ($\leq$3 kDa) from Trachinotus ovatus protein hydrolysates exhibited stronger DPP-IV inhibitory activities. The results indicate that smaller peptides from BSFL may possess structural characteristics favorable for DPP-IV inhibition, such as the presence of proline residues at specific positions, which have been associated with enhanced DPP-IV inhibitory capacity (Nongonierma et al., 2018).

For SWP peptides (Figure 39C), SGD significantly increased the inhibitory activity of all fractions, with the <3 kDa and 3-5 kDa fractions showing the highest inhibition (approximately 75 % and 70 %, respectively). This substantial improvement following SGD suggests that the parent proteins in SWP contain encrypted bioactive sequences that are released during gastrointestinal digestion. These findings are consistent with those reported by Ma et al. (2023), who identified potent DPP-IV inhibitory peptides from SWP with IC50 values as low as 0.27 mg/mL.

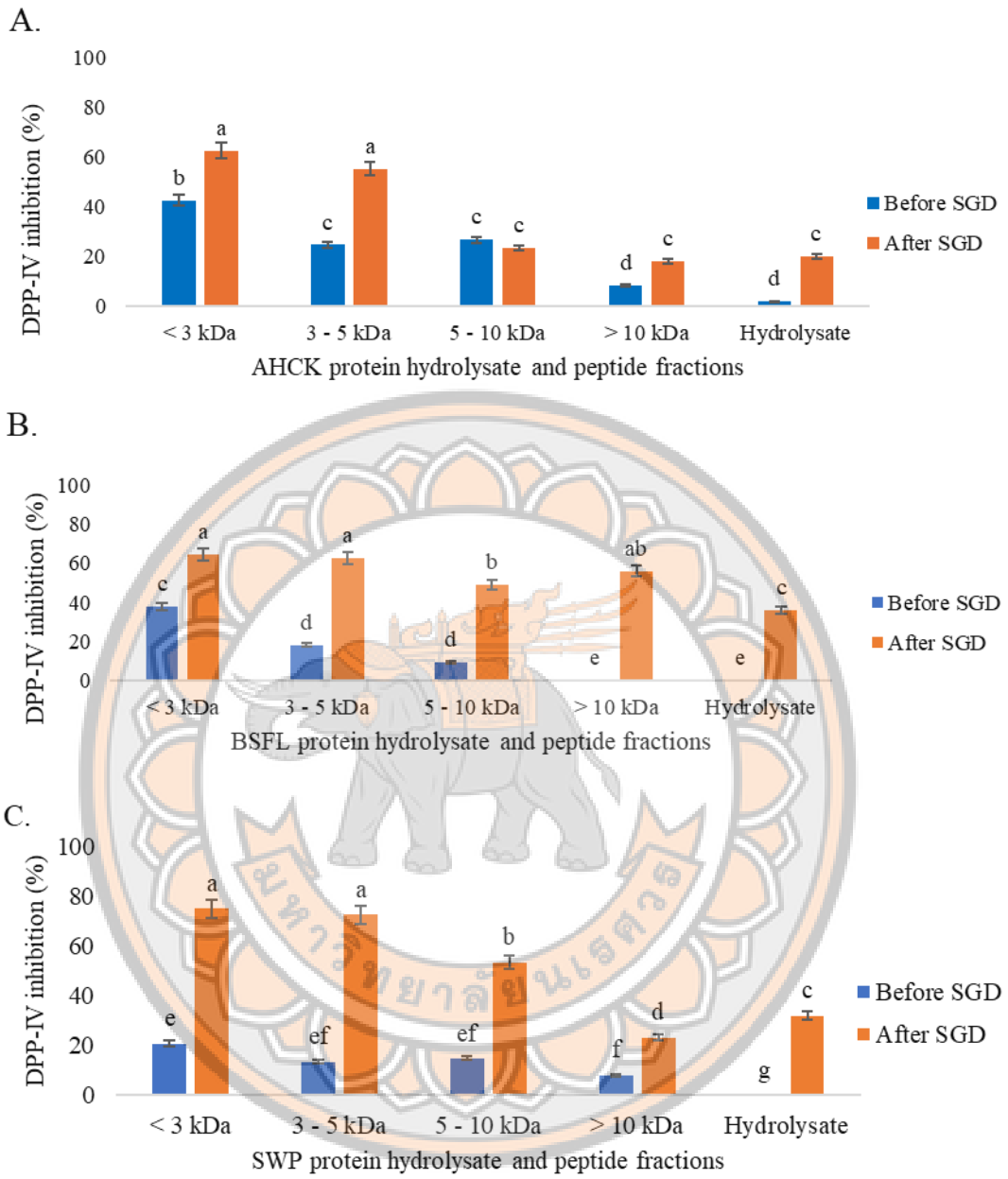


Fig. 39 DPP-IV inhibition activities of edible insect peptides before and after SGD

(A) AHCK peptides, (B) BSFL peptides, and (C) SWP peptides. Fractions (<3 kDa, 3-5 kDa, 5-10 kDa, >10 kDa) were obtained by membrane filtration, and hydrolysate represents the complete hydrolyzed sample after freeze-drying. Different letters (a-f) indicate significant differences ($P<0.05$).

A clear MW-dependent pattern was observed, with smaller peptide fractions (<3 kDa and 3-5 kDa) consistently exhibiting higher inhibitory activities than larger fractions. This trend has been documented in various protein hydrolysates and is attributed to the improved binding capacity of smaller peptides to the enzyme active site (Kim et al., 2019; Rivero-Pino et al., 2023). Harnedy et al. (2015) similarly reported enhanced bioactivity in lower MW fractions from macroalgae protein hydrolysates. The consistent enhancement of DPP-IV inhibitory activity following SGD across all fractions and insect species is particularly noteworthy, as it suggests that these bioactive peptides would likely maintain or even increase their activity following consumption and digestion in the human gastrointestinal tract. As explained by Ajayi et al. (2023), the sequential action of pepsin and pancreatin enzymes during SGD leads to more complete protein dissociation, promoting the release of additional bioactive compounds with enhanced enzyme inhibitory properties.

The observed IC_{50} values for DPP-IV inhibition of <3 kDa fractions after SGD ranged from 0.41 - 0.53 mg/mL across the three insect species (Table 17), which are comparable to values reported for other food-derived protein hydrolysates with antidiabetic potential (Nong & Hsu, 2021). These results demonstrated that edible insect-derived peptides, particularly those from smaller MW fractions after SGD, hold promise as natural DPP-IV inhibitors that could potentially contribute to the management of type 2 diabetes through dietary interventions.

When examining the specific peptide sequences identified in the <3 kDa fractions after SGD (Table 18), several DPP-IV inhibitory peptides were identified, including PPPP and GPAGPQGPR in HCK, ALAV, APGGAP, and GPAGPQGPR in BSFL, and YPGE and GPAGPQGPR in SWP. The presence of proline residues in many of these peptides is particularly significant, as proline-containing peptides have been identified as effective DPP-IV inhibitors (Lacroix et al., 2016). Especially noteworthy is the common occurrence of GPAGPQGPR across all three insect species, suggesting this peptide sequence may be a key contributor to the observed DPP-IV inhibitory activity. Li-Chan et al. (2012) demonstrated that peptides with proline or alanine at the penultimate position are resistant to DPP-IV hydrolysis and can act as competitive inhibitors. This aligns with our findings where proline-rich peptides like PPPP in AHCK demonstrated significant inhibitory activity. Additionally, the relatively short chain length of these peptides (2-9 amino acids) allows them to effectively interact with the active site of the DPP-IV enzyme, as previously reported by Nongonierma & FitzGerald, (2014) for various food protein-derived peptides.

4.4.3 α-Glucosidase Inhibition

The α-glucosidase inhibitory activities of different MW fractions from the three edible insect protein hydrolysates, both before and after SGD, are presented in Figure 40. For AHCK peptides (Figure 40A), SGD markedly enhanced the α-glucosidase inhibitory activity across all fractions, with inhibition percentages ranging from 38.96 % to 81.91 %. The <3 kDa and 3-5 kDa fractions after SGD exhibited the highest inhibitory activity, suggesting that smaller peptides produced during gastrointestinal digestion possess more potent α-glucosidase inhibitory properties. These findings are consistent with those reported by Zieliński et al. (2020), who observed significant α-glucosidase inhibition by peptides obtained from in vitro digestion of cricket (*Gryllodes sigillatus*). The enhanced activity following SGD can be attributed to the release of bioactive peptide sequences that were previously encrypted in the parent proteins, as demonstrated by Zieliński et al. (2018) in their study on multiple edible insect species.

BSFL peptides (Figure 40B) exhibited a different pattern, with SGD resulting in decreased inhibitory activity for most fractions, except for the <3 kDa fraction and the hydrolysate, which showed increased inhibition after digestion. This differential response to SGD may be related to the unique amino acid composition of BSFL proteins, particularly their high content of glutamic acid (6.26 g/100g) and aspartic acid (5.37 g/100g) as shown in Table 16. Mazloomi et al. (2020) reported that these amino acids are important contributors to α-glucosidase inhibition in protein hydrolysates. The resistance of the smallest peptide fraction (<3 kDa) to activity loss during SGD suggests that these peptides may be more stable under gastrointestinal conditions, potentially due to their structural characteristics.

For SWP peptides (Figure 40C), all fractions except >10 kDa demonstrated enhanced inhibitory activity after SGD. The <3 kDa and 3-5 kDa fractions showed the strongest inhibition, reaching approximately 92.61 % and 87.14 %, respectively. These values are notably higher than those reported by Yoon et al. (2019), who evaluated α-glucosidase inhibition in various edible insects, including silkworm pupae, and found inhibitory activity of approximately 33.50 - 35.00 % at 2.0 mg/mL protein concentration. Our enhanced results may be attributed to the different processing methods employed, particularly the use of SC-CO_2 extraction for defatting and the specific protease (P-2SD) used for hydrolysis.

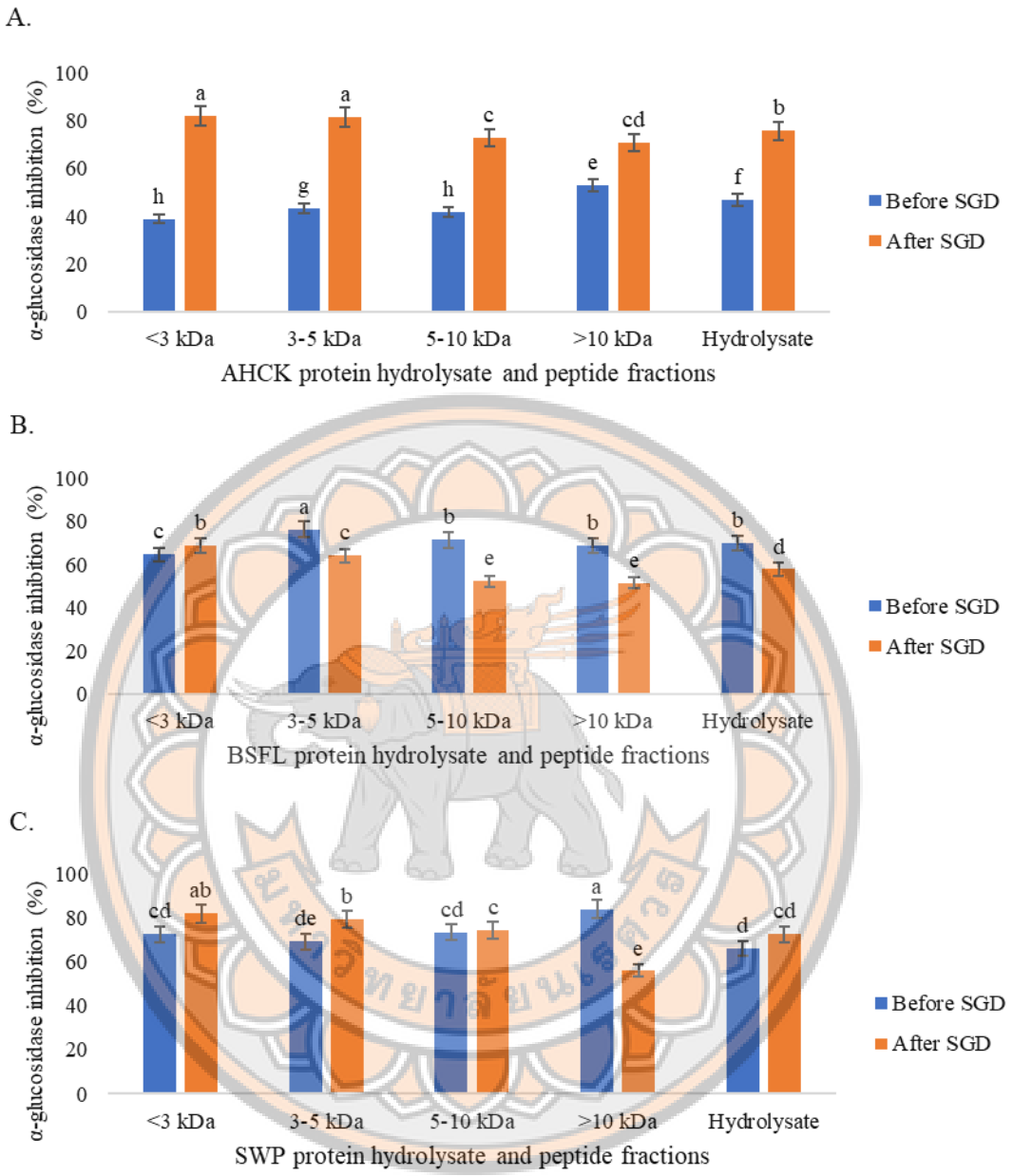


Fig. 40 α-glucosidase inhibition activities of edible insect peptides before and after SGD

(A) AHCK peptides, (B) BSFL peptides, and (C) SWP peptides. Fractions (<3 kDa, 3-5 kDa, 5-10 kDa, >10 kDa) were obtained by membrane filtration, and hydrolysate represents the complete hydrolyzed sample after freeze-drying. Different letters (a-h) indicate significant differences ($P<0.05$).

Across all three insect species, molecular weight-dependent inhibition was observed, with smaller fractions (<3 kDa and 3-5 kDa) generally exhibiting stronger α-glucosidase inhibitory activity than larger fractions. This trend aligns with observations by Ibrahim et al. (2018), who reported that low MW peptides derived from Tilapia fish protein had higher α-glucosidase inhibitory activity due to their enhanced ability to interact with the enzyme's active site. The mechanisms underlying the α-glucosidase inhibitory activity of these insect-derived peptides likely involve competitive, non-competitive, or mixed inhibition modes. According to Mojica & de Mejía, (2016), small peptides can bind to either the active site of α-glucosidase (competitive inhibition) or to other sites on the enzyme, thereby altering its conformation and reducing activity (non-competitive inhibition). The specific inhibition mode varies depending on the peptide sequence and structure.

Analysis of the <3 kDa fractions after SGD (Table 18) revealed several peptide sequences with potential α-glucosidase inhibitory activity, including AGDDAPR in AHCK. This peptide contains aspartic acid residues, which have been associated with α-glucosidase inhibition according to studies by Yu et al. (2012). The relatively high concentration of hydrophilic amino acids, including aspartic acid, glutamic acid, and serine, in all three insect species (Table 16) may contribute to their observed α-glucosidase inhibitory activity. De Matos et al. (2024) suggested that peptides with hydrophilic groups could establish interactions with the enzyme binding site similar to acarbose, a commercial α-glucosidase inhibitor, through the formation of hydrogen bonds.

The IC_{50} values for α-glucosidase inhibition of <3 kDa fractions after SGD (Table 17) ranged from 0.07 to 0.19 mg/mL across the three insect species, with AHCK exhibiting the lowest IC_{50} (0.07 mg/mL), comparable to that of acarbose (0.06 mg/mL). These values are lower than those reported by Liu et al. (2019) for protein hydrolysates from common food sources, indicating the potential superior efficacy of insect-derived peptides as α-glucosidase inhibitors. The potent inhibitory activity of these peptides suggests their potential application in managing postprandial hyperglycemia, which is a key factor in the development and progression of type 2 diabetes (Lacroix & Li-Chan, 2013).

4.4.4 ACE Inhibition

The ACE inhibitory activities of various MW fractions from the three edible insect protein hydrolysates, both before and after SGD, are presented in Figure 41. The results revealed distinct patterns of inhibition that varied by insect species, MW fraction, and the effect of gastrointestinal digestion. For AHCK peptides (Figure 41A), all fractions demonstrated notable ACE inhibitory activity, with significant enhancement following SGD. The <3 kDa and 3-5 kDa fractions after SGD exhibited the highest inhibition percentages (approximately 89% and 88%, respectively). These findings align with results reported by Nongonierma et al. (2018), who observed that cricket (*Acheta domesticus*) protein hydrolysates contained potent ACE inhibitory peptides. The marked increase in inhibitory activity post-SGD suggests that cricket proteins contain encrypted bioactive sequences that are activated during gastrointestinal digestion, a phenomenon also documented by Minkiewicz et al. (2008) for various food-derived proteins.

BSFL peptides (Figure 41B) demonstrated a more variable response to SGD, with the <3 kDa and 3-5 kDa fractions showing significantly enhanced inhibitory activity after digestion (approximately 85% and 76%, respectively), while larger MW fractions exhibited reduced activity post-SGD. This molecular weight-dependent response may be attributed to the differential stability of peptides under gastrointestinal conditions, as noted by Di Bernardini et al. (2012) in their study on bovine brisket muscle protein hydrolysates.

SWP peptides (Figure 41C) exhibited the most dramatic enhancement of ACE inhibitory activity following SGD, with the <3 kDa fraction reaching 98.18% inhibition. This exceptional inhibitory capacity of silkworm pupae-derived peptides is consistent with findings by Wu et al. (2015), who identified a novel ACE inhibitory peptide from silkworm pupae with an IC_{50} value of 0.16 mg/mL. Similarly, Wang et al. (2011) reported that silkworm protein hydrolysates possessed ACE inhibitory activity with IC_{50} values ranging from 0.28 to 0.49 mg/mL.

Across all three insect species, smaller peptide fractions (<3 kDa and 3-5 kDa) consistently revealed higher inhibitory activities than larger fractions. This trend aligns with numerous studies on ACE inhibitory peptides from various sources. Lee & Hur (2019) and Wang et al. (2021) demonstrated that lower MW peptides (<3 kDa) from beef myofibrillar proteins and walnut glutelin-1, respectively, possessed stronger ACE inhibitory activity than higher MW fractions. This enhanced inhibitory capacity of smaller peptides is attributed to their shorter amino acid chains, which facilitates intestinal absorption and more effective binding to the ACE enzyme active site (Wang et al., 2021).

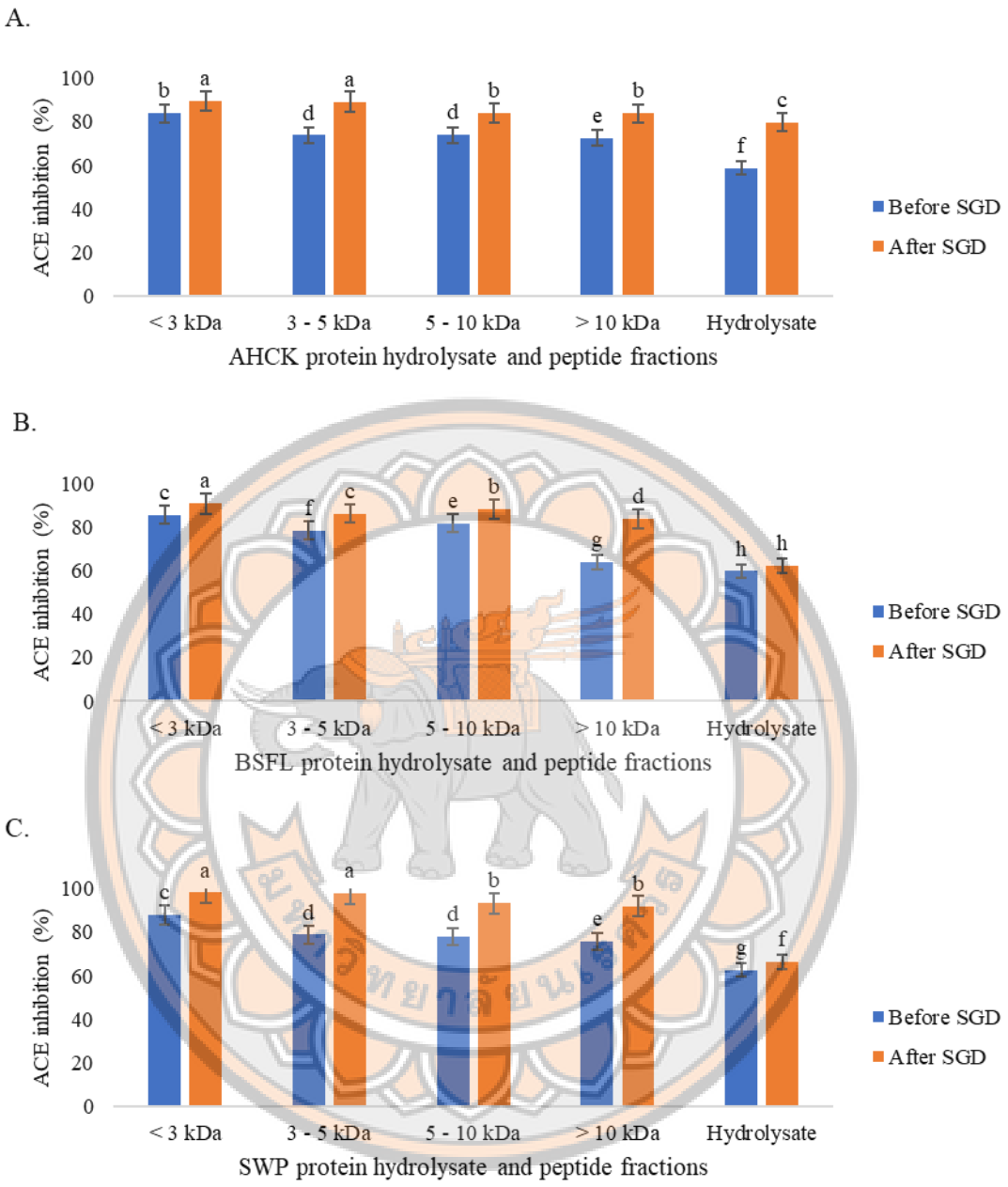


Fig. 41 ACE inhibition activities of edible insect peptides before and after SGD

(A) AHCK peptides, (B) BSFL peptides, and (C) SWP peptides. Fractions (<3 kDa, 3-5 kDa, 5-10 kDa, >10 kDa) were obtained by membrane filtration, and hydrolysate represents the complete hydrolyzed sample after freeze-drying. Different letters (a-h) indicate significant differences ($P<0.05$).

The structural characteristics of peptides play a crucial role in their ACE inhibitory capacity. Examination of the <3 kDa fractions after SGD (Table 18) revealed several peptide sequences with known ACE inhibitory activity, including LTGP, AGDDAPR, and LPLP in AHCK; LLAP and LPLP in BSFL; and LPLP in SWP. The IC_{50} values of these fractions ranged from 0.05 to 0.58 mg/mL (Table 17), with SWP exhibiting the lowest IC_{50} (0.05 mg/mL), indicating its exceptional potency. These values are comparable to or lower than those reported by Hall et al. (2020) and De Matos et al. (2022) for edible insect-derived peptides (IC_{50} values of 1.922 mg/mL and 0.59 mg/mL, respectively).

The superior ACE inhibitory activity of these insect-derived peptides may be attributed to their amino acid composition. According to Wu et al. (2015) and Aslam et al. (2019), the presence of hydrophobic aliphatic amino acids at the carboxyl-terminal end and aromatic amino acids in peptide sequences enhances ACE inhibition. As shown in Table 16, all three insect species contain substantial amounts of hydrophobic amino acids such as leucine, valine, and alanine, which likely contribute to their observed ACE inhibitory properties. Iwaniak et al. (2014) reported that peptides containing leucine, proline, or phenylalanine at their C-terminus often demonstrate strong ACE inhibitory activity, which is consistent with the presence of peptides like LPLP in all three insect species.

The mechanism of ACE inhibition by these peptides likely involves competitive inhibition through binding to the active site of the enzyme. According to Norris & FitzGerald (2013), ACE inhibitory peptides typically interact with the zinc ion and specific binding pockets within the active site of ACE, thereby preventing the binding and cleavage of angiotensin I. The potent ACE inhibitory activity demonstrated by these insect-derived peptides, particularly after SGD, suggests their potential application in the management of hypertension through dietary intervention, as they would likely maintain or enhance their bioactivity during gastrointestinal digestion in *vivo*.

4.4.5 Pancreatic Lipase Inhibition

The pancreatic lipase inhibitory activities of various MW fractions from the three edible insect protein hydrolysates, both before and after SGD, are presented in Figure 42. For AHCK peptides (Figure 42A), the >10 kDa fraction before SGD exhibited the highest inhibitory activity (approximately 86 %), while after SGD, the <3 kDa fraction demonstrated the strongest inhibition (approximately 68 %). This shift in inhibitory activity from larger to smaller peptides following digestion suggests that gastrointestinal processes cleave the larger peptides into smaller, bioactive fragments that retain significant lipase inhibitory capacity. The strong inhibitory activity of AHCK-derived peptides corresponds with results reported by Hall et al. (2018), who identified several bioactive peptides from cricket protein with potential anti-obesity properties.

BSFL peptides (Figure 42B) showed a different pattern, with the 5-10 kDa and >10 kDa fractions before SGD exhibiting strong inhibition (approximately 83 % and 82 %, respectively), while after SGD, the <3 kDa fraction maintained significant inhibitory activity (approximately 75 %). This suggests that BSFL proteins contain bioactive sequences that are relatively stable under gastrointestinal conditions. The potent pancreatic lipase inhibitory activity of BSFL-derived peptides is consistent with findings by Kim et al. (2019), who reported that insect protein hydrolysates could effectively inhibit lipid-metabolizing enzymes, including pancreatic lipases.

For SWP peptides (Figure 42C), the 5-10 kDa fraction before SGD and the <3 kDa fraction after SGD demonstrated the highest inhibitory activity (approximately 81% and 87%, respectively). This substantial enhancement of the <3 kDa fraction's inhibitory activity following SGD is particularly noteworthy and suggests that SWP proteins contain encrypted bioactive sequences that are released during gastrointestinal digestion. Similar enhancement of bioactivity following SGD was reported by Mudgil et al. (2018) for camel milk protein hydrolysates, which exhibited increased pancreatic lipase inhibitory activity after simulated digestion.

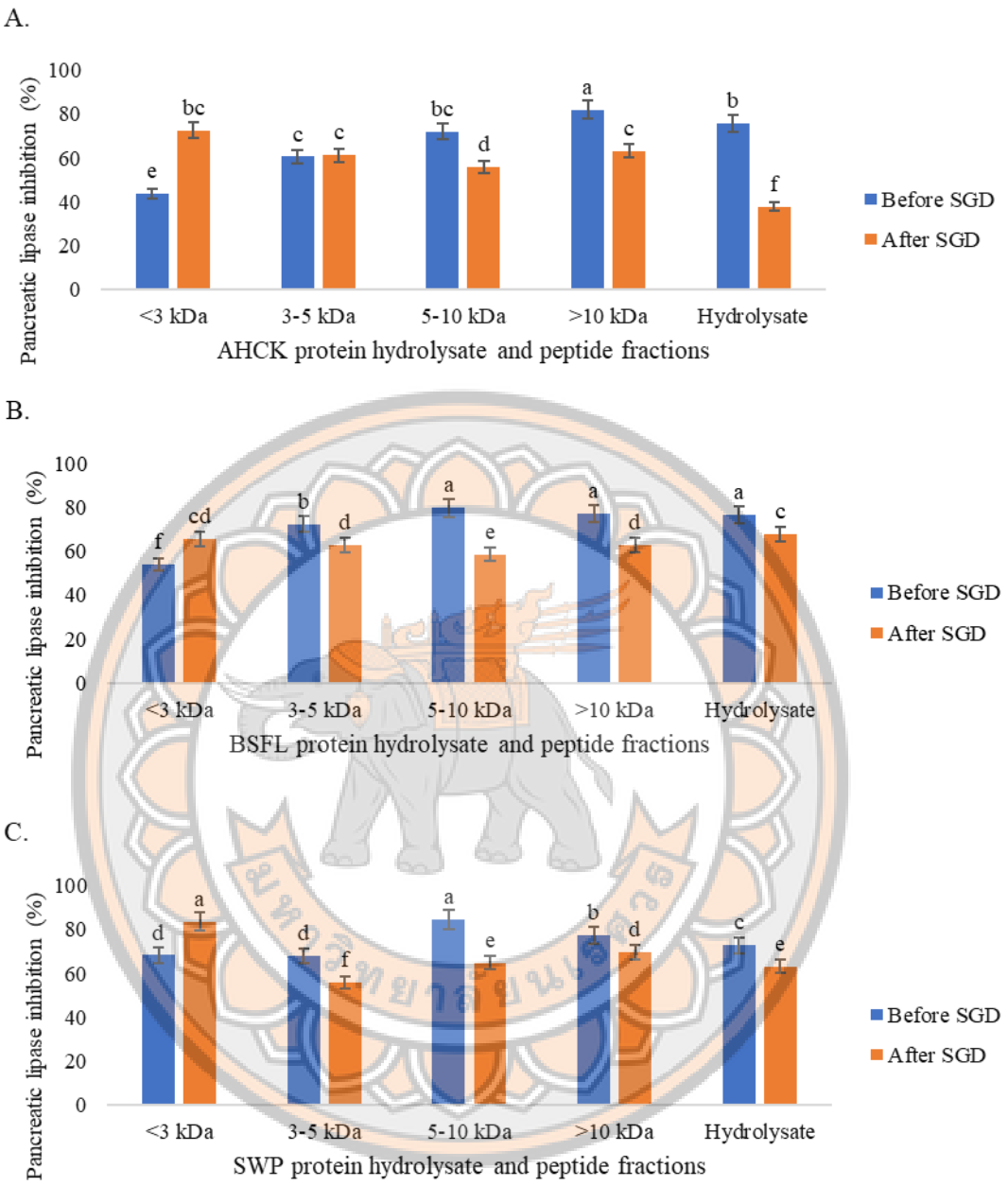


Fig. 42 Pancreatic lipase inhibition activities of edible insect peptides before and after SDG

(A) AHCK peptides, (B) BSFL peptides, and (C) SWP peptides. Fractions (<3 kDa, 3-5 kDa, 5-10 kDa, >10 kDa) were obtained by membrane filtration, and hydrolysate represents the complete hydrolyzed sample after freeze-drying. Different letters (a-f) indicate significant differences ($P<0.05$).

Table 17. IC_{50} values of DPP-IV, α-glucosidase, ACE, and pancreatic lipase inhibition of < 3 kDa after SGD

Insect peptides	Half-maximal inhibition concentration (mg/mL)			
	DPP-IV	α-glucosidase	ACE	Pancreatic lipase
AHCK	0.53 ± 0.04^{a}	0.07 ± 0.00^{b}	0.25 ± 0.19^{b}	0.29 ± 0.01^{a}
BSFL	0.46 ± 0.15^{a}	0.16 ± 0.01^{a}	0.58 ± 0.08^{a}	0.24 ± 0.01^{b}
SWP	0.41 ± 0.05^{a}	0.19 ± 0.01^{a}	0.05 ± 0.04^{b}	0.16 ± 0.01^{c}
Acarbose	-	0.06 ± 0.02^{b}	-	-
Orlistat	-	-	-	0.01 ± 0.01^{d}

Note: Values are mean ± standard deviation. Different letters (a-d) within columns indicate significant differences (P<0.05).

The IC_{50} values for pancreatic lipase inhibition by the <3 kDa fractions after SGD ranged from 0.14 to 0.29 mg/mL across the three insect species (Table 17), with SWP exhibiting the lowest IC_{50} (0.05 mg/mL), indicating its superior potency. Although these values are higher than those of orlistat (0.01 mg/mL), a commercial pancreatic lipase inhibitor, they are comparable to or lower than those reported for many food-derived protein hydrolysates with anti-obesity potential.

Analysis of the <3 kDa fractions after SGD (Table 18) revealed several peptide sequences with potential pancreatic lipase inhibitory activity, including AGDDAPR in AHCK, and PPAP and PPLP in BSFL and SWP. The inhibitory mechanisms of these peptides likely involve complex interactions with the lipase enzyme. According to Xiang et al. (2020), the binding between peptides and pancreatic lipase involves hydrophilic interactions, hydrogen bonding, and hydrophobic interactions. For instance, the benzene ring of phenylalanine in peptides might interact hydrophobically with the cyclic structure of proline in pancreatic lipase. This is consistent with the presence of proline-rich peptides (PPAP and PPLP) in the <3 kDa fractions of BSFL and SWP after SGD.

The amino acid composition plays a crucial role in determining pancreatic lipase inhibitory activity. Baba et al. (2021) demonstrated that peptides containing hydrophobic amino acids, particularly leucine and proline, could bind to multiple sites on the lipase enzyme, resulting in reduced fat digestion and inhibition of fat absorption in the small intestine. As shown in Table 16, all three insect species contain substantial amounts of these hydrophobic amino acids, which likely contribute to their observed lipase inhibitory properties. Additionally, Zhang et al. (2022) proposed that various bioactive components in edible insects, including polyphenols, saponins, chitosan, and terpenes, might contribute to pancreatic lipase inhibitory activities, suggesting that multiple bioactive compounds may work synergistically to produce the observed effects.

The MW-dependent pattern observed across all three insect species after SGD, with smaller peptide fractions (<3 kDa) consistently exhibiting higher inhibitory activities than larger fractions, is consistent with findings by Bougatef et al. (2009) for fish protein hydrolysates. This enhanced inhibitory capacity of smaller peptides following gastrointestinal digestion may be attributed to their improved ability to bind to the active site of the lipase enzyme, as suggested by Mojica et al. (2017). Furthermore, the bioavailability of these smaller peptides is likely higher, as they can be more readily absorbed through the intestinal epithelium (Lafarga & Hayes, 2014).

The potential of these insect-derived peptides as natural pancreatic lipase inhibitors offers promising applications in weight management and obesity prevention. Jakubczyk et al. (2017) demonstrated that food-derived peptides with lipase inhibitory activity could effectively reduce dietary fat absorption and accumulation in animal models. The potent pancreatic lipase inhibitory activity demonstrated by these insect-derived peptides, particularly after SGD, suggests their potential viability for development as functional food ingredients or nutraceuticals targeting obesity and related metabolic disorders.

Table 18. Bioactive peptide sequences identified in the <3 kDa fractions from three edible insect species and their potential bioactive properties based on the BIOPEP database

Insect peptides	Protein	Peptides	Potential
AHCK <3 kDa	ACE inhibitor	LTGP, AGDDAPR, LPLP	ACE inhibition
	DPP-IV inhibitor	PPPP, GPAGPQGPR	DPP-IV inhibition
	Dipeptidyl carboxypeptidase inhibitor	PPAP, PPLP	Inhibitor
	Peptide derived from tuna cooking juice	VDPY	Antioxidation
	Antioxidative peptide	AGDDAPR	Antioxidation
	Pancreatic lipase inhibitor	AGDDAPR	Pancreatic lipase inhibition
	α-glucosidase inhibitor	AGDDAPR	α-glucosidase inhibition
	Anti-inflammatory peptide	LGGW	Anti-inflammatory
	Hypotensive peptide	LPLP	Hypotensive
	Ileum contracting peptide	PLRP	Contracting
	PEP inhibitor	PPPLP	Antiamnestic
BSFL <3 kDa	DPP-IV inhibitor	ALAV, APGGAP, GPAGPQGPR	DPP-IV inhibition
	ACE inhibitor	LLAP, LPLP	ACE inhibition
	Dipeptidyl carboxypeptidase inhibitor	PPAP, PPLP	Inhibitor
	Hypotensive peptide	LPLP	Hypotensive
	PEP inhibitor	PPPLP	Antiamnestic
	Antioxidative peptide	FAPVPFDF, PSLPA, LALPA, LTTLDS, YPQLLPNE, YPLDL	Antioxidant
SWP <3 kDa	DPP-IV inhibitor	YPGE, GPAGPQGPR	DPP-IV inhibition
	Dipeptidyl carboxypeptidase inhibitor	PPAP, PPLP	Inhibitor
	ACE inhibitor	LPLP	ACE inhibition
	Hypotensive peptide	LPLP	Hypotensive
	Antioxidative peptide	PSLPA, YPLDL	Antioxidant
	PEP inhibitor	LPPPV	Antiamnestic

4.4.6 Bioactive Peptide Sequences Identified in Edible Insect Protein Hydrolysates

The identification of specific bioactive peptide sequences in the <3 kDa fractions after SGD from the three edible insect species provides crucial insights into the molecular basis of their observed enzyme inhibitory activities. As detailed in Table 18, a diverse range of bioactive peptides was identified across the insect species, with some sequences being species-specific while others were common across multiple species.

In AHCK protein hydrolysates, eleven distinct bioactive peptide sequences were identified, including ACE inhibitors (LTGP, AGDDAPR, and LPLP), DPP-IV inhibitors (PPPP and GPAGPQGPR), general enzyme inhibitors (PPAP and PPLP), and peptides with antioxidant (VDPY, AGDDAPR), anti-inflammatory (LGGW), and other bioactivities. The result has been similar to those reported by Hall et al. (2020). The multifunctional nature of certain peptides, particularly AGDDAPR, which exhibited ACE inhibitory, antioxidant, pancreatic lipase inhibitory, and α-glucosidase inhibitory activities, is especially noteworthy. This multi-target inhibition aligns with findings by Lammi et al. (2019), who reported that certain food-derived peptides could simultaneously inhibit multiple enzymes involved in metabolic disorders. The tetrapeptide PPPP is of particular interest for its DPP-IV inhibitory activity, as proline-rich peptides have been demonstrated by Nongonierma & FitzGerald (2014) to be potent inhibitors of this enzyme due to their structural similarity to DPP-IV substrates.

BSFL protein hydrolysates yielded fourteen bioactive peptide sequences, predominantly featuring DPP-IV inhibitors (ALAV, APGGAP, and GPAGPQGPR), ACE inhibitors (LLAP and LPLP), general enzyme inhibitors (PPAP and PPLP), and several antioxidative peptides (FAPVPFDF, PSLPA, LALPA, LTTLDS, YPQLLPNE, and YPLDL). The prevalence of antioxidant peptides in BSFL is consistent with reports by Tang et al. (2018), who identified multiple antioxidant peptides in BSFL protein hydrolysates. The presence of alanine-containing peptides such as ALAV for DPP-IV inhibition is supported by research from Lacroix & Li-Chan (2012), who demonstrated that alanine at specific positions in peptide sequences contributes significantly to DPP-IV inhibitory activity.

SWP protein hydrolysates contained eight bioactive peptide sequences, including DPP-IV inhibitors (YPGE and GPAGPQGPR), an ACE inhibitor (LPLP), enzyme inhibitors (PPAP and PPLP), antioxidative peptides (PSLPA and YPLDL), and an antiamnestic peptide (LPPPV). The

presence of YPGE as a DPP-IV inhibitor in SWP is particularly interesting, as peptides with tyrosine at the N-terminus and glutamic acid at the C-terminus have been reported by Zhang et al. (2016) to exhibit strong DPP-IV inhibitory activity through interaction with specific binding pockets in the enzyme.

Several peptide sequences were common across multiple insect species, suggesting evolutionary conservation of certain bioactive protein regions. The peptide GPAGPQGPR was identified in all three species as a DPP-IV inhibitor, indicating its potential significance as a universal bioactive sequence in insects. Similarly, the peptides PPAP, PPLP, and LPLP were found in multiple species, exhibiting enzyme inhibitory and hypotensive activities. The presence of these common bioactive sequences aligns with findings by Jia et al. (2015), who reported that certain bioactive peptide motifs are conserved across various protein sources.

The structural features of these identified peptides provide insights into their mechanisms of enzyme inhibition. Proline-rich peptides like PPPP, PPAP, PPLP, and LPPPV were prevalent across all three insect species. Proline-containing peptides often exhibit resistance to further enzymatic degradation, potentially enhancing their stability and bioavailability. Furthermore, the cyclic structure of proline creates bends in peptide chains, potentially enabling better interaction with enzyme active sites, as suggested by Fu et al. (2016).

According to the literature, bioactive peptides composed of hydrophobic amino acids result in the potential of antioxidants, α-glucosidase, α-amylase inhibitory activity (De Matos et al., 2022; Khammuang et al., 2022). Zhang et al. (2019) and Mendis et al. (2005) reported that hydrophobic amino acids such as Val (V), Leu (L), Ile (I), Phe (F), Tyr (T) and Asp (D) and peptides containing Ala (A), Gly (G), Leu (L), Val (V) and Pro (P) had strong antioxidants activity, significantly contributing to the radical-scavenging activity. However, the hydrophobicity/hydrophilicity balance of the identified peptides also appears crucial for their bioactivities. Peptides containing both hydrophobic (leucine, proline, valine) and hydrophilic (arginine, aspartic acid, glutamic acid) amino acids, such as AGDDAPR and GPAGPQGPR, demonstrated multi-target enzyme inhibition. This observation corresponds with findings by Udenigwe & Aluko (2012), who proposed that the balanced presence of hydrophobic and hydrophilic residues enhances the bioactivity of peptides by facilitating interaction with both hydrophobic binding pockets and charged residues in enzyme active sites.

4.5 Conclusion

This study revealed significant enzyme inhibitory activities in protein hydrolysates from three commercially viable edible insects. AHCK, BSFL, and SWP provide sustainable protein with balanced amino acid profiles and contain bioactive peptides with potential for managing non-communicable diseases. Molecular weight-dependent patterns were observed across all bioactivities, with smaller peptide fractions (<3 kDa) consistently showing superior inhibition, particularly after SGD. AHCK peptides demonstrated potent α-glucosidase inhibition (IC_{50} 0.07 mg/mL) comparable to acarbose, while SWP peptides exhibited exceptional ACE inhibition (IC_{50} 0.05 mg/mL).

The identification of specific bioactive sequences, including GPAGPQGPR across all three species and multifunctional peptides like AGDDAPR, provides the molecular basis for these bioactivities. Structural characteristics, including proline-rich sequences and balanced hydrophobic/hydrophilic amino acid compositions, contribute to their effectiveness as enzyme inhibitors. The combined processing approach of US pretreatment, SC-CO_2 oil extraction, and proteolysis proved effective for obtaining bioactive peptides with enhanced inhibitory activities. Future research should focus on in vivo validation, scale-up optimization, and applications in functional foods targeting non-communicable diseases, while addressing consumer acceptance, organoleptic properties, and regulatory considerations for successful commercial development of these promising insect-derived bioactive compounds.

CHAPTER V

GASTROINTESTINAL DIGESTION ENHANCES ANTIOXIDANT EFFICACY OF EDIBLE INSECT PEPTIDES: MOLECULAR WEIGHT FRACTIONATION AND IN VIVO VALIDATION IN *CAENORHABDITIS ELEGANS* MODEL

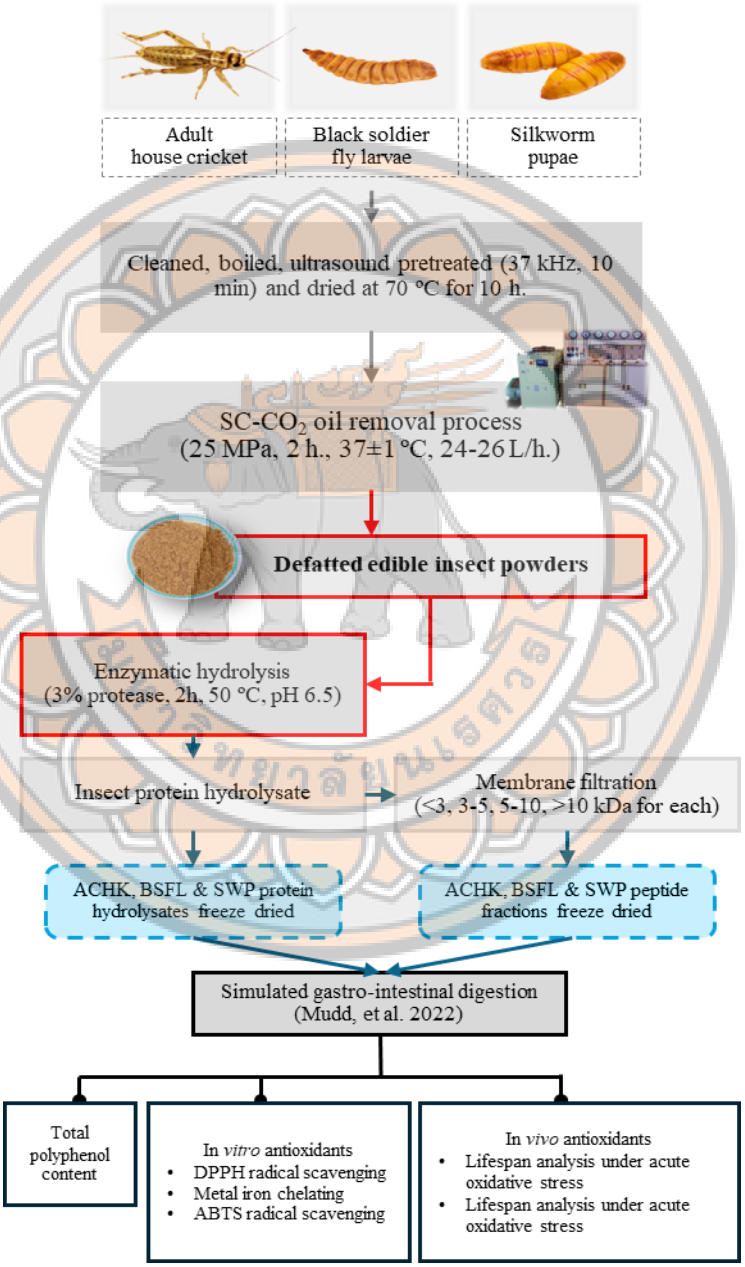


Fig. 43 Flow chart diagram of protein and bioactive peptide production for alternative antioxidant agent production

5.1 Abstract

Edible insects are increasingly recognized as sustainable protein sources with promising applications in functional foods and nutraceuticals. This study investigated the antioxidant properties of peptides derived from adult house cricket (AHCK, *Acheta domesticus*), black soldier fly larvae (BSFL, *Hermetia illucens*), and silkworm pupae (SWP, *Bombyx mori*) using comprehensive in vitro and in vivo approaches. Following protein hydrolysis and membrane filtration, peptide fractions were subjected to simulated gastrointestinal digestion (SGD) to assess bioavailability. Antioxidant activities of the resulting fractions (>10 kDa, 5-10 kDa, 3-5 kDa, and <3 kDa) were assessed through DPPH and ABTS radical scavenging, as well as metal chelating assays. The <3 kDa fractions exhibited significantly higher antioxidant activities than larger fractions ($p < 0.05$). SGD revealed species-specific responses: AHCK peptides showed enhanced metal chelation (97.58%), while SWP peptides maintained optimal antioxidant balance post-digestion. Mass spectrometry identified 14 bioactive peptides (412-689 Da), including conserved sequences YPLDL and PSLPA across all species. The aromatic residues (Tyr in LGFEYY) contributed to radical scavenging, acidic residues (Asp in AGDDAPR, VDPY) enhanced metal chelation, and proline residues conferred digestive stability. To validate biological relevance, *Caenorhabditis elegans* was employed as an in vivo model. SGD-treated peptides (1 mg/mL) markedly improved nematode survival under oxidative challenges, extending survival to 750-780 min under acute tert-butyl hydroperoxide stress and increasing lifespan by 2-3 days under chronic paraquat-induced stress relative to untreated controls. These findings demonstrate that edible insect peptides, particularly low-molecular-weight fractions, possess potent antioxidant properties with applications as functional food ingredients for oxidative stress management.

Keywords: Gastrointestinal digestion; Bioactive peptides; Edible insects; Antioxidant activity; Caenorhabditis elegans; Oxidative stress

5.2 Introduction

Global protein demand is projected to increase 40-70% by 2050, driving urgent need for sustainable alternatives to conventional animal agriculture. Edible insects emerge as a transformative solution, requiring significantly less water, land, and producing lower greenhouse

gas emissions per kilogram of protein compared to conventional livestock (Oonincx et al., 2010; Smetana et al., 2016). This sustainability advantage, combined with superior nutritional profiles containing all essential amino acids and bioactive compounds, positions insects as ideal sources for functional food ingredients (Rumpold & Schlüter, 2013). The global insect protein market reflects growing recognition with projected compound annual growth rates of 16-27% through 2030 (MarketsandMarkets, 2024). Regulatory approval in the European Union for major species—including house cricket (*Acheta domesticus*) and yellow mealworms (*Tenebrio molitor*)—provides commercial validation (European Food Safety Authority, 2021).

Recent advances reveal that insect proteins contain encrypted bioactive sequences that, when released through gastrointestinal digestion, demonstrate enhanced antioxidant properties with activities improving 2-4 fold post-digestion (Zieliński et al., 2017). In this context, house cricket emerges as a leading source of bioactive peptides, with protein content ranging 59-70% dry weight and exceptional amino acid profiles. Recent characterization identified novel tetrapeptides (FVEG and FYDQ) with dual functionality, combining ACE inhibitory activity with significant antioxidant capacity (Summart et al., 2024). Furthermore, in vitro antioxidant assays of cricket-derived peptide fractions have demonstrated remarkable antioxidant activities correlated to molecular weight distribution (Teixeira et al., 2023). Black soldier fly (*Hermetia illucens*) larvae present unique advantages combining high protein content (399-431 g/kg dry matter) with superior sustainability credentials (Barragan-Fonseca et al., 2017). For example, Chen et al. (2025) demonstrated comprehensive utilization through a sequential extraction processes, converting 50 kg of defatted material into 32.24 kg of protein/peptides, with approximately 88.30% having molecular weights less than 1000 Da. Silkworm (*Bombyx mori*) pupae, consumed in Asia for over 2000 years, offer cultural acceptance advantages with protein content ranging 45-60% dry weight. Altomare et al. (2020) identified 778 gene products and 9,590 sequenced peptides, of which 737 demonstrated potential functional activities.

Gastrointestinal digestion transforms insect proteins into significantly more bioactive peptides through systematic release of encrypted sequences (Zieliński et al., 2017). INFOGEST protocol adaptations for insect proteins provide standardized approaches for evaluating digestive enhancement of bioactivity (Brodkorb et al., 2019). Systematic molecular weight fractionation studies demonstrate inverse relationships between peptide size and specific antioxidant activity, with <3 kDa fractions consistently showing 2-3 times higher activity than >10 kDa fractions (Teixeira et al., 2023). Kan et al. (2025) comprehensively reviewed recent advances in bioactive

peptide fractionation methods, highlighting that fractionation processes can significantly enhance peptide bioactivity, with certain fractions exhibiting 1.8 to 3 times higher activities compared to unfractionated hydrolysates.

The nematode *Caenorhabditis elegans* (*C. elegans*) offers exceptional advantages for antioxidant validation: high concordance with mammalian results, cost-effectiveness, and standardized oxidative stress protocols (Ayuda-Durán et al., 2020; Lin et al., 2023). Despite substantial progress, critical gaps remain in understanding molecular weight-dependent bioactivity relationships, standardized gastrointestinal simulation effects, and comprehensive in vivo validation protocols (Quah et al., 2023; Mudd & Liceaga, 2022; Miranda-Carrazco et al. 2025). The limited correlation between in vitro antioxidant assays and in vivo protective effects represents a significant gap requiring systematic validation (Ayuda-Durán et al., 2020). Therefore, this study investigated the antioxidant properties of peptides derived from three commercial edible insect species—adult house cricket (AHCK), black soldier fly larvae (BSFL), and silkworm pupae (SWP)—following molecular weight fractionation and simulated gastrointestinal digestion, with comprehensive validation using the in vivo *C. elegans* model system.

5.3 Materials and Methods

5.3.1 Insect samples and model organism

Frozen samples of AHCK, BSFL, and SWP were obtained from Thai Ento Food Co., Ltd. (Samutprakarn, Thailand). Each batch (20 kg) was thawed at 4°C overnight, washed three times with sterile distilled water, blanched at 100°C for 5 min, and stored in aluminum foil bags at -18°C until processing. *C. elegans* N2 wild-type strain and *E. coli* OP50 were obtained from the Caenorhabditis Genetics Center, CGC (Minneapolis, MN, USA), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

5.3.2 Chemicals and Reagents

2SD Amano protease (92039-00K, 100,000 U/g) was supplied by Amano Enzyme Inc. (Nagoya, Japan). Pepsin from porcine gastric mucosa ($\geq$2,500 units/mg protein), pancreatin from porcine pancreas (Type II, $\geq$125 units/mg protein), and bile salts were obtained from Millipore Sigma (St. Louis, MO, USA). All other analytical grade chemicals were sourced from Thermo Fisher Scientific (Waltham, MA, USA), Sigma Aldrich (St. Louis, MO, USA), and VWR International (Radnor, PA, USA).

5.3.3 Pretreatment and defatting

Insect samples were subjected to ultrasonication (37 kHz, 10 min, 37°C), followed by oven drying (70°C, 10 h) and grinding through an 18-mesh sieve. Lipid removal was performed using supercritical CO_2 extraction (25 MPa, 37°C, 2 h, flow rate 24-26 L/h). Defatted insect meals were stored at -18°C until further use.

5.3.4 Proteolysis and peptide fractionation

Defatted insect meals were suspended in distilled water (1:10 w/v) and homogenized for 10 min. Enzymatic hydrolysis was performed using 2SD protease (3% w/w of protein content) at 50°C, pH 6.5 for 2 h with constant stirring (200 rpm). The reaction was terminated by heating to 100°C for 10 min, followed by centrifugation (15,652 × g, 15 min). Supernatants were freeze-dried and stored at -18°C. Peptide fractionation was conducted using a Minimate™ Tangential Flow Filtration System (Pall Corporation, USA) with molecular weight cut-off membranes of 10, 5, and 3 kDa, yielding fractions of >10 kDa, 5-10 kDa, 3-5 kDa, and <3 kDa.

5.3.5 In vitro simulated gastrointestinal digestion

SGD was performed following methods described by Mudd et al. (2022), Hall et al. (2020), and You et al. (2010) with modifications. For the gastric phase, protein was dissolved in Tris buffer (pH 8.0), pH adjusted to 2.0 using 6 M HCl, and pepsin solution [4% (w/v)] was added, followed by incubation for 2 h at 37°C. For the small intestinal phase, pH was adjusted to 7.0 using 1 M $NaHCO_3$ and 6 M NaOH. Pancreatin [4% (w/v)] and bile salts (10 mg/mL) were added, and the mixture was incubated for an additional 2 h. The enzymatic reaction was terminated by heating at 90°C for 10 min. The digesta was centrifuged at 12,000 × g for 30 min, and the supernatant was freeze-dried for subsequent analysis.

5.3.6 Analysis of bioactive peptides

5.3.6.1 Total Polyphenol Content

TPC was determined using the Folin-Ciocalteu method (Alves et al., 2013). In 96-well microplates, 20 µL of peptide sample was mixed with 200 µL of 0.2 N Folin-Ciocalteu reagent, incubated at 30°C for 2 min, then 20 µL of 7.5% sodium carbonate was added. After 30 min incubation, absorbance was measured at 765 nm. Results were expressed as µg gallic acid equivalents (GAE)/mL.

5.3.6.2 In Vitro Antioxidant Assays

DPPH radical scavenging activity: DPPH activity was performed according to Urbizo-Reyes et al. (2021). Peptide samples (100 µL, 0.5 mg/mL) were mixed with 100 µL ethanol and 25 µL DPPH solution. After 30 min incubation in darkness, absorbance was measured at 550 nm using a Multiskan™ FC Microplate Photometer (Waltham, MA, USA). Scavenging activity was calculated as: $[(A_0 - A_1)/A_0] \times 100$. Where A_0 is the absorbance of the DPPH solution (control) and A_1 is the absorbance of the sample. The radical reduction was expressed in µM of Trolox equivalent/mg of protein.

Metal chelating activity: Fe^{2+} chelating capacity was analyzed according to Torres-Fuentes et al., (2012) with slight modification. Peptide samples (200 µL) were mixed with 3.75 µL of 2 mM $FeCl_2$ and 7.5 µL of 5 mM ferrozine solution. After 10 min incubation, absorbance was measured at 562 nm.

ABTS radical scavenging activity: The ABTS radical was generated by mixing 7 mM ABTS with 2.45 mM $K_2S_2O_8$ and incubating for 16 h (Urbizo-Reyes et al., 2021). The solution was diluted to achieve an absorbance of 0.700 ± 0.02 at 734 nm. Peptide samples (20 µL) were mixed with 980 µL ABTS solution and incubated for 10 min at 30°C. The ABTS scavenging activity was expressed as mM Trolox equivalent (TE)/mg sample.

For all in vitro antioxidant assays, the percentage inhibition was plotted against the logarithm of peptide concentration using Microsoft Excel. The IC_{50} value (concentration required to inhibit 50% of the radicals or chelate 50% of the metal ions) was calculated by nonlinear regression using a sigmoidal dose-response curve (variable slope model) (De Matos et al., 2021).

5.3.6.3 Peptide Identification and Sequence Determination by LC-MS/MS

Sample preparation, mass spectrometry analysis, bioinformatics, and data evaluation were performed in collaboration with the Proteomics Core Facility at the Indiana University School of Medicine. Samples were analyzed using a 5 cm trap column and a 15 cm EasySpray analytical column (2 µm particle size, 50 µm inner diameter; Thermo Fisher Scientific, Waltham, MA, USA) mounted on an UltiMate 3000 HPLC system coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). The LC-MS/MS system was operated under standard data-dependent acquisition mode for peptide identification. Mass spectrometry data were processed using PEAKS Studio software (Bioinformatics Solutions Inc.), incorporating both database-dependent searches

and de novo sequencing. Only peptides with an Average Local Confidence (ALC) score >80% were selected for further analysis. Identified peptide sequences were subsequently searched against the BIOPEP-UWM Database of Bioactive Peptides (Minkiewicz, et al., 2019) to identify potential bioactivities.

5.3.6.4 In Vivo Antioxidant Evaluation using C. elegans

Nematode cultivation and synchronization: N2 *C. elegans* were maintained on nematode growth medium (NGM) with *E. coli* OP50 as food source at 20°C. Nematodes were synchronized and cultured for 72 h to reach L4 larval stage (Figure 44), then washed four times with M9 buffer.

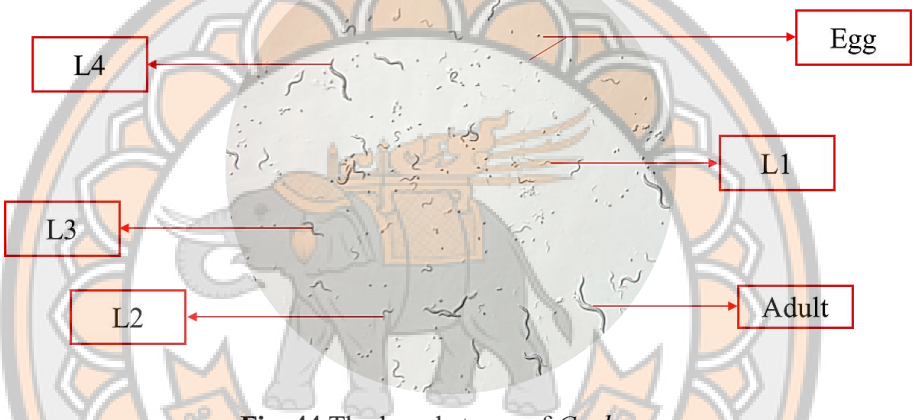


Fig. 44 The larval stages of *C. elegans*

Acute oxidative stress assay: Tert-butyl hydroperoxide (tBOOH) was used to induce acute oxidative stress according to Mudd et al. (2022). L4-stage nematodes were incubated at 20°C for 24 h in solutions containing <3 kDa SGD peptides at concentrations of 0, 0.125, 0.25, 0.5, or 1.0 mg/mL. Following incubation, nematodes were transferred to 96-well microplates (~20 nematodes per well; ~100 nematodes per treatment). tBOOH solution (5 mM final concentration) was added to each well. Nematode survival was assessed every 30 min using a compound microscope until all worms were dead.

Chronic oxidative stress assay: Resistance to chronic oxidative stress was evaluated according to Bai et al. (2020) with modifications. L4-stage nematodes were incubated at 20°C for 24 h in <3 kDa SGD peptide solutions at concentrations of 0, 0.125, 0.25, 0.5, or 1.0 mg/mL. Following incubation, nematodes were transferred to 24-well microplates (~30 nematodes per well; ~150 nematodes per treatment) containing paraquat solution (25 mM final concentration). Nematode survival was assessed daily until all nematodes were dead.

5.3.7 Statistical analysis

Results are expressed as means ± standard deviations of triplicate analyses. Statistical significance was determined using one-way ANOVA followed by Duncan's multiple range test (SPSS Statistics v18). Survival curves were analyzed using Kaplan-Meier estimates and log-rank tests. Significance was set at $p < 0.05$.

5.4 Results and Discussion

5.4.1 Total Polyphenol Content

TPC of insect peptides varied significantly among species and molecular weight fractions, with SGD exerting pronounced effects on phenolic retention (Table 19).

Table 19. Total polyphenol content of edible insect peptide fractions before and after SGD.

Samples	Treatments	mg GAE/ g DW		
		AHCK	**BSFL**	**SWP**
Before SGD	< 3 kDa	0.33 ± 0.00^{Ba}	0.30 ± 0.01^{Be}	0.71 ± 0.01^{Ab}
	3 – 5 kDa	0.34 ± 0.00^{Ca}	0.47 ± 0.01^{Ba}	0.64 ± 0.01^{Ac}
	5 – 10 kDa	0.21 ± 0.01^{Ce}	0.45 ± 0.00^{Bb}	0.70 ± 0.01^{Ab}
	> 10 kDa	0.18 ± 0.00^{Cd}	0.37 ± 0.01^{Bc}	0.53 ± 0.01^{Ad}
	Hydrolysate	0.22 ± 0.00^{Cb}	0.33 ± 0.00^{Bd}	1.03 ± 0.01^{Aa}
After SGD	< 3 kDa	0.06 ± 0.00^{Ag}	0.06 ± 0.00^{Ah}	0.04 ± 0.00^{Bf}
	3 – 5 kDa	0.14 ± 0.01^{Ae}	0.07 ± 0.01^{Bg}	0.03 ± 0.00^{Cg}
	5 – 10 kDa	0.10 ± 0.01^{Af}	0.06 ± 0.00^{Bh}	0.06 ± 0.00^{Be}
	> 10 kDa	0.10 ± 0.00^{Af}	0.07 ± 0.01^{Bg}	0.04 ± 0.00^{Cf}
	Hydrolysate	0.09 ± 0.00^{Af}	0.08 ± 0.01^{Af}	0.05 ± 0.00^{Bef}

Note: Values are expressed as mean ± SD (n=3). Different lowercase letters (a-h) within the same column indicate significant differences among peptide fractions within the same insect species. Different uppercase letters (A-C) within the same row indicate significant differences among insect species for the same peptide fraction ($p < 0.05$).

Before SGD, SWP peptides demonstrated the highest TPC across all fractions (0.53–1.03 mg GAE/g DW), followed by BSFL (0.30–0.47 mg GAE/g DW) and AHCK (0.18–0.34 mg GAE/g DW). The hydrolysate fraction of SWP exhibited maximum TPC (1.03 ± 0.01 mg GAE/g DW),

4.7-fold and 3.1-fold higher than AHCK and BSFL hydrolysates, respectively. The <3 kDa peptides generally retained higher TPC compared to larger peptide fractions, suggesting that smaller peptides form stronger associations with phenolic compounds or that ultrafiltration enriches peptide-phenolic complexes in low molecular weight fractions.

Gastrointestinal digestion dramatically reduced TPC across all species and fractions, with losses ranging from 82% to 95%. The <3 kDa AHCK fraction experienced substantial reduction (from 0.33 to 0.06 mg GAE/g DW, 82% loss), while SWP showed greater decreases (from 0.71 to 0.04 mg GAE/g DW, 94% loss). This reduction can be attributed to the acidic gastric environment (pH 2.0) and proteolytic enzymes promoting structural modifications, oxidation, and degradation of phenolic compounds through hydrolysis of ester bonds and disruption of hydrogen bonding networks. Similar observations have been reported for various food matrices (Tagliazucchi et al., 2010; Zieliński et al., 2018). Chen et al. (2015) demonstrated that pH-dependent structural changes and enzymatic degradation are primary mechanisms for phenolic loss during digestion.

The presence of polyphenols in insect-derived peptides primarily results from dietary accumulation, as insects concentrate phenolic compounds from plant-based feed sources (Niño et al., 2021; Mlcek et al., 2014). The observed TPC reduction during gastrointestinal digestion may paradoxically enhance bioavailability, as disruption of the protein-polyphenol matrix releases bound phenolic compounds, converting them into more absorbable free forms (Ozdal et al., 2013; Jakobek, 2015). The complex architecture of insect proteins, involving both covalent and non-covalent associations with polyphenols, provides partial protection during gastric digestion (Debelo et al., 2020). These findings underscore the importance of evaluating both total polyphenol content and structural integrity of peptide-phenolic associations when assessing antioxidant potential of digested insect peptides.

5.4.2 In Vitro Antioxidant Capacity

5.4.2.1 DPPH Radical Scavenging Activity

The DPPH assay demonstrated significant molecular weight-dependent patterns in antioxidant activity (Table 20, Figure 45A). Before gastrointestinal digestion, <3 kDa fractions consistently exhibited highest scavenging activity across all species (AHCK: 68.16 ± 0.00%, BSFL: 62.30 ± 0.20%, SWP: 69.40 ± 0.11%), followed by progressively decreasing activity in larger peptide fractions. Hydrolysate samples showed lowest scavenging capacity (AHCK: 41.47 ±

0.30%, BSFL: 29.62 ± 0.11%, SWP: 28.26 ± 0.11%), representing a 39–59% decrease compared to the <3 kDa fractions. This inverse relationship between molecular weight and radical scavenging activity aligns with established structure-activity relationships. Smaller peptides possess enhanced conformational flexibility, improved solubility, and increased accessibility of electron-donating residues (His, Tyr, Trp, Cys, Met) to reactive radical species (Sarmadi & Ismail, 2010).

Table 20. DPPH radical scavenging activity of edible insect peptide fractions before and after SGD.

Samples	Treatments	% Activity		
		AHCK	BSFL	SWP
Before SGD	< 3 kDa	68.16 ± 0.00^{Aa}	62.30 ± 0.20^{Ba}	69.40 ± 0.11^{Aa}
	3 – 5 kDa	64.65 ± 0.20^{Bb}	54.10 ± 0.59^{Cb}	67.32 ± 0.30^{Ab}
	5 – 10 kDa	58.33 ± 0.69^{Ac}	49.67 ± 0.41^{Cc}	54.30 ± 0.34^{Bc}
	> 10 kDa	53.58 ± 0.60^{Ad}	43.75 ± 0.20^{Bd}	41.34 ± 0.30^{Cd}
	Hydrolysate	41.47 ± 0.30^{Ae}	29.62 ± 0.11^{Be}	28.26 ± 0.11^{Bg}
After SGD	< 3 kDa	1.63 ± 0.23^{Cj}	26.63 ± 0.23^{Bf}	39.26 ± 0.20^{Ae}
	3 – 5 kDa	14.13 ± 0.49^{Ch}	21.09 ± 0.00^{Bh}	36.52 ± 0.59^{Af}
	5 – 10 kDa	7.55 ± 0.49^{Ci}	9.18 ± 0.20^{Bi}	25.46 ± 0.23^{Ah}
	> 10 kDa	32.49 ± 0.11^{Bf}	21.48 ± 0.34^{Ch}	40.69 ± 0.11^{Ad}
	Hydrolysate	28.78 ± 0.30^{Bg}	24.87 ± 0.23^{Cg}	38.93 ± 0.11^{Ae}

Note: Values are expressed as mean ± SD (n=3). Different lowercase letters (a-j) within the same column indicate significant differences among peptide fractions within the same insect species. Different uppercase letters (A-C) within the same row indicate significant differences among insect species for the same peptide fraction ($p < 0.05$).

IC_{50} values for <3 kDa fractions before gastrointestinal digestion confirmed potent radical scavenging capacity ($P<0.05$): SWP demonstrated lowest IC_{50} (0.89 ± 0.00 mg/mL), followed by AHCK (1.53 ± 0.00 mg/mL) and BSFL (3.09 ± 0.11 mg/mL). These values compare favorably with other food-derived antioxidant peptides. For example, fish byproduct peptides typically exhibit IC_{50} values of 0.5-5.0 mg/mL (Chalamaiah et al., 2012), while plant protein hydrolysates show IC_{50} values between 1.0-10.0 mg/mL (Arise et al., 2016).

Gastrointestinal digestion produced markedly different effects depending on insect species. AHCK peptides experienced dramatic IC_{50} increases, indicating severe potency loss. The <3 kDa AHCK fraction suffered a decline, with IC_{50} rising from 1.53 mg/mL to 18.42 mg/mL (12-fold increase), corresponding to a radical scavenging activity drop from 68.16% to 1.63%. This decrease in DPPH activity suggests that AHCK contains peptide sequences highly susceptible to further hydrolysis by digestive enzymes, potentially cleaving critical antioxidant motifs (Elias et al., 2008). Cricket proteins are particularly rich in glycine (7–10%) and alanine (7–8%), contributing to flexible structures highly susceptible to enzymatic degradation (Zieliński et al., 2018).

BSFL peptides demonstrated moderate resilience, with IC_{50} increasing from 3.09 mg/mL to 10.83 mg/mL (3.5-fold increase). BSFL peptides retained 42.7% of the original DPPH activity after digestion, considerably better than AHCK. This intermediate stability suggests BSFL peptides contain a mixture of digestive-stable and digestive-labile antioxidant sequences (Janssen et al., 2017). Most remarkably, SWP peptides exhibited the most favorable response to gastrointestinal digestion, with IC_{50} increasing from 0.89 mg/mL to 2.47 mg/mL (a 2.8-fold increase). The <3 kDa SWP fraction retained 56.6% of original activity (from 69.40% to 39.26%), demonstrating superior ($P<0.05$) digestive stability. This higher DPPH activity can be attributed to unique structural characteristics of silkworm proteins, particularly fibroin and sericin. Silkworm proteins contain high serine proportions (12–30% in sericin) and exhibit unique crystalline structures that resist enzymatic attack (Zou et al., 2016). The identification of peptide sequences (Table 23) provides molecular-level insights, showing peptides containing aromatic residues (Tyr, Trp, Phe) that can donate hydrogen atoms to stabilize free radicals, while proline residues introduce conformations that may position antioxidant residues optimally for radical interaction.

The species-specific responses to gastrointestinal digestion reflect fundamental differences in amino acid composition and secondary structure. The IC_{50} changes following gastrointestinal digestion have profound implications for functional food development. While all three insect sources demonstrated excellent activity before digestion, only SWP peptides maintained sufficient activity post-digestion (IC_{50} = 2.47 mg/mL) to be considered highly effective at physiologically achievable concentrations. These findings underscore the critical importance of evaluating antioxidant peptides under simulated physiological conditions rather than relying solely on in vitro measurements of undigested samples.

5.4.2.2 Metal Chelating Activity

Metal chelation represents a critical antioxidant mechanism by preventing transition metal ions from catalyzing formation of reactive oxygen species. Transition metals, particularly Fe^{2+} and Cu^{2+}, participate in Fenton and Haber-Weiss reactions, generating highly reactive hydroxyl radicals that initiate lipid peroxidation and cause oxidative damage (Sun et al., 2020; Csire et al., 2020). MCA of insect peptides exhibited distinct patterns among species and molecular weight fractions, with particularly notable changes following gastrointestinal digestion (Table 21, Figure 45B). Before gastrointestinal digestion, AHCK peptides demonstrated exceptionally high MCA across all fractions, with <3 kDa showing 93.66 ± 0.60% chelation activity, significantly higher ($P<0.05$) than BSFL (85.21 ± 1.31%) and SWP (81.05 ± 0.46%). This superior chelating ability of AHCK peptides persisted across larger fractions, suggesting cricket proteins contain abundant amino acid residues capable of coordinating with metal ions. This aligns with recent studies showing Fe^{2+} chelating capacities ranging from 1721.99 to 1751.71 µmol EDTA equivalents per gram, positioning cricket-derived peptides among the most potent natural metal chelators (Fashakin et al., 2023; Hall et al., 2020).

Gastrointestinal digestion produced differential effects depending on insect species and peptide size. AHCK peptides exhibited further enhancement of MCA following SGD, with <3 kDa fraction increasing from 93.66% to 97.58 ± 0.57%, representing the highest ($P<0.05$) chelating capacity among all samples tested. This enhancement suggests digestive enzymes liberated peptide sequences with superior metal-binding properties previously inaccessible within larger protein structures. The 3–5 kDa AHCK fraction also showed substantial ($P<0.05$) improvement (from 91.47% to 96.23 ± 0.35%). This phenomenon has been documented in other protein systems, where enzymatic hydrolysis releases shorter peptides with enhanced accessibility of chelating residues (Sánchez-Velázquez et al., 2021; Ren et al., 2021).

BSFL peptides demonstrated a contrasting response, with the <3 kDa fraction increasing modestly from 85.21% to 88.68 ± 1.04%, while mid-range fractions showed significant improvements. This pattern suggests BSFL proteins contain metal-chelating sequences that become accessible through partial enzymatic hydrolysis but may be overly degraded when cleaved into very small peptides. Recent research on BSFL peptides revealed exceptional antioxidant properties, with specific peptide sequences like PFCPK and ADFW, capable of reducing the generation of reactive oxygen species (ROS) by 62.98% and 83.44% respectively, with metal chelation contributing to these mechanisms (Praseatsook et al., 2025).

Table 21. Metal chelating activity of edible insect peptide fractions before and after SGD.

Samples	Treatment	% Activity		
		AHCK	**BSFL**	**SWP**
Before SGD	< 3 kDa	93.66 ± 0.60^{Ac}	85.21 ± 1.31^{Bb}	81.05 ± 0.46^{Cb}
	3 – 5 kDa	91.47 ± 0.35^{Ad}	48.83 ± 2.16^{Cf}	61.21 ± 0.46^{Bd}
	5 – 10 kDa	89.74 ± 0.57^{Ae}	38.94 ± 0.13^{Ch}	51.25 ± 0.34^{Bf}
	> 10 kDa	72.98 ± 0.91^{Ah}	44.83 ± 0.73^{Bg}	45.94 ± 0.89^{Bh}
	Hydrolysate	67.47 ± 0.94^{Ai}	51.47 ± 0.57^{Be}	48.23 ± 1.01^{Bg}
After SGD	< 3 kDa	97.58 ± 0.57^{Aa}	88.68 ± 1.04^{Ba}	88.91 ± 0.45^{Ba}
	3 – 5 kDa	96.23 ± 0.35^{Ab}	69.43 ± 1.04^{Bc}	66.64 ± 0.35^{Cc}
	5 – 10 kDa	91.92 ± 0.13^{Ad}	50.11 ± 0.35^{Cef}	55.02 ± 0.35^{Be}
	> 10 kDa	83.70 ± 0.23^{Af}	65.21 ± 0.69^{Bd}	50.72 ± 1.33^{Cf}
	Hydrolysate	78.19 ± 0.92^{Ag}	64.53 ± 0.47^{Bd}	50.94 ± 0.35^{Cf}

Note: Values are expressed as mean ± SD (n=3). Different lowercase letters (a-i) within the same column indicate significant differences among peptide fractions within the same insect species. Different uppercase letters (A-C) within the same row indicate significant differences among insect species for the same peptide fraction ($p < 0.05$).

SWP peptides exhibited significant enhancement following SGD, with <3 kDa fraction increasing from 81.05% to 88.91 ± 0.45%. This consistent enhancement across multiple molecular weight ranges indicates silkworm proteins possess latent metal-chelating sequences effectively released during gastrointestinal digestion without being destroyed by excessive hydrolysis. Studies on silkworm pupae protein hydrolysates have identified peptides with substantial antioxidant and metal-chelating activities, with peptide sequences being rich in aromatic and acidic amino acids contributing to bioactive properties (Cermeño et al., 2022; Khammuang et al., 2022).

The high MCA of insect peptides, particularly AHCK after SGD, can be attributed to specific structural features. For instance, metal chelation occurs primarily through coordination bonds between metal ions and electron-rich functional groups, including carboxyl groups from acidic residues (Asp, Glu), imidazole rings from histidine, thiol groups from cysteine, and amino groups from N-terminus and lysine side chains (Sun et al., 2020; Udechukwu et al., 2018). As shown in Table 23, AHCK peptides after gastrointestinal digestion contain sequences rich in aspartic acid (AGDDAPR, VDPY), providing multiple carboxyl groups for coordination with Fe^{2+}

ions. The presence of tyrosine residues (VDPY, YPLDL, LGFEYY) further enhances chelating capacity through phenolic hydroxyl groups, while proline residues contribute to optimal peptide conformations (Zou et al., 2016; Shi et al., 2022).

These findings have important implications for developing insect-derived functional foods targeting oxidative stress-related conditions. The high MCA of AHCK peptides after gastrointestinal digestion (97.58%) indicates cricket-derived peptides could serve as effective natural metal chelators in food systems, potentially preventing lipid oxidation while also providing health benefits through reduced oxidative stress in vivo. The multifunctionality of insect peptides—simultaneously providing radical scavenging and metal chelation—positions them as superior alternatives to single-function peptides for applications in clinical nutrition, sports nutrition, and functional food development (Hall et al., 2020; Zieliński et al., 2017).

5.4.2.3 ABTS Radical Scavenging Activity

The ABTS radical scavenging mechanism involves electron transfer from antioxidant compounds to the $ABTS^{\bullet+}$ radical cation (Mahmoudi et al., 2016). Aromatic amino acids containing electron-donating groups serve as primary electron donors: tyrosine residues with phenolic hydroxyl groups, tryptophan residues with indole rings, and phenylalanine with benzyl groups (Lu et al., 2022; Mahmoudi et al., 2016). The aqueous solubility of ABTS radicals enables interaction with both surface-exposed and partially buried peptide residues, explaining why larger molecular weight peptides may demonstrate high ABTS scavenging despite potentially limited accessibility in other assays (Ashfaq et al., 2025).

Before gastrointestinal digestion, insect peptides exhibited distinct species-specific patterns in ABTS scavenging activity (Table 22). SWP peptides demonstrated higher ($P<0.05$) ABTS radicle scavenging activity across all fractions, with hydrolysate showing highest activity (99.44 ± 0.17%), followed by 5–10 kDa fraction (96.72 ± 0.23%), and <3 kDa fraction (94.04 ± 0.46%). This higher ABTS scavenging capacity significantly exceeded ($P<0.05$) both AHCK and BSFL peptides, suggesting fundamental differences in amino acid composition and sequence arrangements favoring electron donation (Suzuki et al., 2022; Takechi et al., 2014). This may be attributed to unique protein composition of silkworm pupae, particularly sericin proteins rich in serine, threonine, and glycine residues facilitating radical scavenging through hydroxyl group interactions, and fibroin proteins containing repetitive sequences creating favorable conformational arrangements for electron transfer (Takechi et al., 2014).

Table 22. ABTS radical scavenging activity of edible insect peptide fractions before and after SGD.

Samples	Treatments	% Activity		
		AHCK	BSFL	SWP
Before SGD	< 3 kDa	60.98 ± 0.38^{Cd}	65.27 ± 1.06^{Ba}	94.04 ± 0.46^{Ac}
	3 – 5 kDa	64.97 ± 0.49^{Bb}	55.93 ± 0.15^{Cb}	91.47 ± 0.61^{Ad}
	5 – 10 kDa	77.79 ± 0.53^{Ba}	55.83 ± 0.09^{Cb}	96.72 ± 0.23^{Ab}
	> 10 kDa	60.83 ± 0.49^{Bd}	55.17 ± 0.40^{Cb}	91.42 ± 1.01^{Ad}
	Hydrolysate	63.65 ± 0.30^{Bc}	54.16 ± 0.38^{Cc}	99.44 ± 0.17^{Aa}
After SGD	< 3 kDa	77.94 ± 0.23^{Aa}	39.68 ± 0.32^{Cf}	54.01 ± 0.38^{Be}
	3 – 5 kDa	53.05 ± 0.15^{Af}	34.02 ± 0.61^{Ch}	51.34 ± 0.46^{Bf}
	5 – 10 kDa	48.01 ± 0.46^{Cg}	51.84 ± 0.40^{Ad}	50.58 ± 0.23^{Bf}
	> 10 kDa	$41..65 \pm 0.72^{Ah}$	36.45 ± 0.09^{Cg}	39.68 ± 0.32^{Bg}
	Hydrolysate	59.21 ± 0.68^{Ae}	48.46 ± 0.23^{Be}	39.17 ± 0.57^{Cg}

Note: Values are expressed as mean ± SD (n=3). Different lowercase letters (a-h) within the same column indicate significant differences among peptide fractions within the same insect species. Different uppercase letters (A-C) within the same row indicate significant differences among insect species for the same peptide fraction ($p < 0.05$).

The performance of larger molecular weight SWP fractions (5–10 kDa and >10 kDa) contrasts with the general trend observed in DPPH scavenging activity, where smaller peptides typically show higher activity. This difference reflects the distinct reaction mechanisms of ABTS versus DPPH radicals, with $ABTS^{\bullet+}$ being more accessible to larger peptides probably due to its water solubility and smaller molecular size compared to the sterically hindered DPPH radical. The IC_{50} values for <3 kDa fractions before gastrointestinal digestion (Figure 45C) confirmed the ranking of antioxidant potency: SWP demonstrated the lowest IC_{50}, indicating strongest activity, followed by AHCK and BSFL. These values provide quantitative validation of the activity percentages shown in Table 22, establishing a clear correlation between percentage inhibition and IC_{50} values.

Gastrointestinal digestion produced divergent effects on ABTS scavenging activity that differed markedly from DPPH scavenging and metal chelation patterns. AHCK peptides showed enhanced activity in <3 kDa fraction, increasing from 60.98% to 77.94 ± 0.23% after SGD,

representing the most substantial improvement among all samples. However, other AHCK fractions experienced significant decreases ($P<0.05$). This pattern suggests digestive enzymes selectively liberate small, ABTS-reactive peptides from cricket proteins while simultaneously degrading larger active structures (Pei et al., 2022; Kremsmayr et al., 2022).

BSFL peptides demonstrated relatively stable or slightly declining ABTS scavenging activity across most fractions following gastrointestinal digestion. The <3 kDa fraction decreased from 65.27% to 39.68 ± 0.32%. This stability-to-moderate-decline pattern suggests BSFL proteins contain ABTS-reactive sequences with inherent resistance to further enzymatic degradation, possibly due to proline residues and hydrophobic amino acid clusters conferring structural stability (Pei et al., 2022; Lu et al., 2022).

SWP peptides experienced significant decreases ($P<0.05$) in ABTS scavenging activity following gastrointestinal digestion across all fractions, contrasting with their behavior in metal chelation and DPPH assays. The hydrolysate fraction declined from 99.44% to 39.17 ± 0.57%, and <3 kDa fraction decreased from 94.04% to 54.01 ± 0.38%. Despite this decrease, post-digestion SWP peptides maintained competitive activity. This sensitivity of SWP peptides to enzymatic digestion specifically in ABTS assay suggests structural features responsible for ABTS scavenging—potentially specific aromatic residue arrangements, β-sheet conformations in fibroin, or repetitive sequence motifs in sericin—are more susceptible to digestive degradation than those responsible for metal chelation or DPPH scavenging (Suzuki et al., 2022; Takechi et al., 2014).

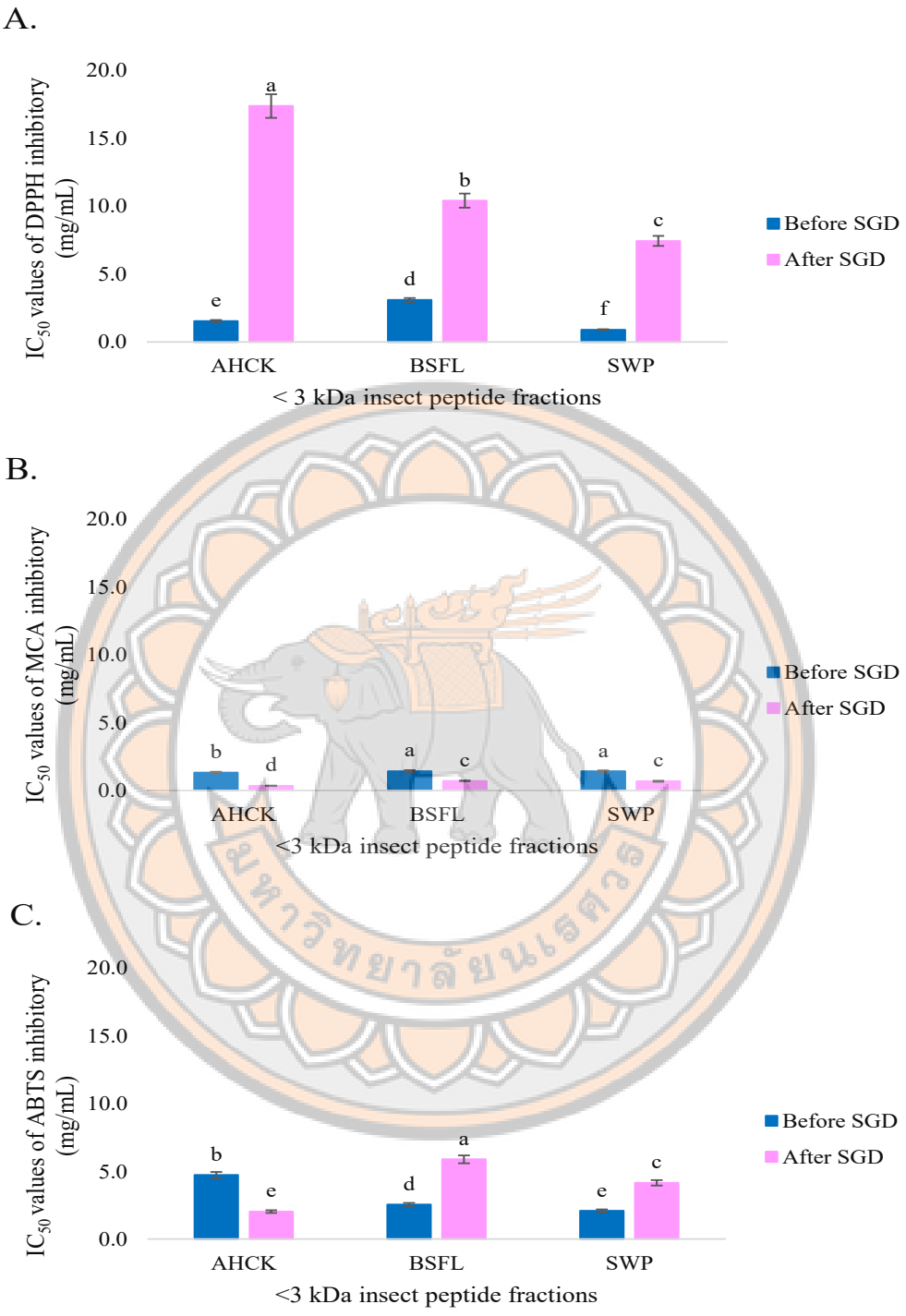


Fig. 45 IC_{50} values of the <3 kDa peptides fractions before and after SGD extracted from AHCK, BSFL and SWP obtained from the inhibition of (A.) DPPH radical; (B.) MCA; and (C.) ABTS radical.

Table 23. Amino acid sequences and molecular characteristics of bioactive peptides identified from <3 kDa fractions after gastrointestinal digestion identified by high-resolution liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Insect sample	Antioxidant peptides	Amino acid sequence	Mass (g/mol)	Score (%)
AHCK (<3 kDa after SGD)	AGDDAPR	Ala-Gly-Asp-Asp-Ala-Pro-Arg	483.27	100.00
	VDPY	Val-Asp-Pro-Try	492.22	100.00
	PSLPA	Pro-Ser-Leu-Pro-Ala	483.27	80.00
	YPLDL	Try-Pro-Leu-Asp-Leu	506.24	80.00
	VPLSPT	Val-Pro-Leu-Ser-Pro-Try	412.23	66.67
	LTTLDS	Leu-Thr-Thr-Leu-Asp-Ser	648.33	66.67
	LGFEYY	Leu-Asp-Phe-Glu-Try-Try	464.23	66.67
BSFL (<3 kDa after SGD)	PSLPA	Pro-Ser-Leu-Pro-Ala	483.56	80.00
	LALPA	Leu-Ala-Leu-Pro-Ala	483.60	80.00
	YPLDL	Try-Pro-Leu-Asp-Leu	506.24	80.00
	HGPLGPL	His-Gly-Pro-Leu-Gly-Pro-Leu	689.34	77.78
	LTTLDS	Leu-Thr-Thr-Leu-Asp-Ser	648.33	66.67
SWP (<3 kDa after SGD)	YPLDL	Try-Pro-Leu-Asp-Leu	506.24	83.33
	PSLPA	Pro-Ser-Leu-Pro-Ala	483.27	80.00

Note: Identification scores represent the confidence level of peptide sequence matching. Molecular weights range from 412-689 Da, corresponding to penta- to heptapeptides. Red letters indicate aliphatic amino acids (Ala, Val, Leu, Ile); blue letters indicate acidic amino acids (Asp, Glu); green letters indicate basic amino acids (Arg, His); purple letters indicate aromatic amino acids (Phe, Tyr, Trp); black letters indicate other amino acids (Pro, Ser, Thr, Gly).

The differential responses to gastrointestinal digestion across the three antioxidant assays reveal important mechanistic insights into the multifunctional nature of insect-derived peptides (Zhao & Liu, 2023). Peptides may simultaneously possess multiple antioxidant mechanisms—each dependent on different structural features and amino acid residues. The peptide sequences identified in Table 23 provide molecular-level explanations for these multifunctional properties. AHCK-

derived peptides AGDDAPR and VDPY contain acidic residues (Asp) that excel at metal chelation through carboxylate coordination, while peptides with aromatic residues like YPLDL, LGFEYY, and VPLSPT (containing Tyr, Trp, Phe) contribute to radical scavenging through electron donation (Mahmoudi et al., 2016; Lu et al., 2022). The presence of proline residues in multiple sequences introduces conformational constraints that may position reactive residues optimally for both radical scavenging and metal coordination.

BSFL peptides HGPLGPL and LTTLDS demonstrate similar multifunctionality, with histidine providing metal coordination capability through its imidazole ring while also participating in radical scavenging. The shared sequence YPLDL across all three insect species suggests a highly conserved antioxidant motif, with tyrosine providing electron-donating capability, proline contributing structural rigidity, leucine enhancing hydrophobicity, aspartic acid enabling metal chelation, and C-terminal leucine facilitating lipid-water interface interactions (Zhao & Liu, 2023; Lu et al., 2022). These findings demonstrate that no single assay adequately predicts physiological antioxidant capacity, but rather reflect the multifunctional approach by peptides for antioxidant activity (Pei et al., 2022; Kremsmayr et al., 2022).

5.4.3 In vivo antioxidant capacity using *C. elegans* model

5.4.3.1 Acute Oxidative Stress Response

The protective effects of insect-derived peptides were validated in vivo using *C. elegans* subjected to acute oxidative stress induced by tert-butyl hydroperoxide (tBOOH). This model represents a well-established approach for evaluating antioxidant efficacy, as tBOOH generates localized reactive oxygen species that can be effectively neutralized by exogenous antioxidants (Ewald et al., 2017; Mudd et al., 2022). Supplementation with <3 kDa peptide fractions from all three insect species after gastrointestinal digestion significantly ($P<0.05$) increased nematode survival in a dose-dependent manner (Figure 46).

At the highest concentration tested (1.0 mg/mL), SWP peptides demonstrated the most pronounced antioxidant (protective) effects, extending median survival rate substantially compared to untreated controls. This superior performance aligns with their in vitro antioxidant activities observed in ABTS and metal chelation assays (Oh et al., 2015; Cao et al., 2019). The unique amino acid composition of silkworm-derived peptides—particularly serine-rich sequences from sericin

proteins and glycine-alanine repeats from fibroin—facilitates ROS neutralization through multiple mechanisms (Suzuki et al., 2022). These peptides likely activate the SKN-1 pathway, the *C. elegans* ortholog of mammalian Nrf2, which regulates phase II detoxification enzymes including glutathione S-transferase-4 (gst-4) and superoxide dismutase-3 (sod-3) (Hu et al., 2017; Mudd et al., 2022).

AHCK-derived peptides conferred substantial protection against tBOOH-induced mortality across all tested doses (Figure 46A). The mean survival rate for nematodes exposed to 1.0 mg/mL was over 600 min, compared to ca. 400 min for the control (untreated) population. Cricket protein hydrolysates have been shown to upregulate stress-responsive genes including gst-4 and sod-3, thereby enhancing the endogenous antioxidant defense system (Mudd et al., 2022; Cao et al., 2019). The identified peptide sequences AGDDAPR and VDPY from AHCK (Table 23) likely contributed to this protection through their metal chelation capabilities and electron-donating properties, which complement the organism's intrinsic antioxidant machinery.

BSFL peptide fractions (Figure 46B) also exhibited statistically significant ($P<0.05$) protective effects under acute oxidative stress conditions. While their efficacy was lower relative to both SWP (Figure 46C) and AHCK fractions, the consistent dose-response relationship suggests genuine antioxidant activity rather than non-specific effects (Praseatsook et al., 2025; Lu et al., 2022). The peptides HGPLGPL and LTTLDS identified from BSFL demonstrate structural features associated with antioxidant activity, including histidine residues that can participate in both metal chelation and radical scavenging mechanisms.

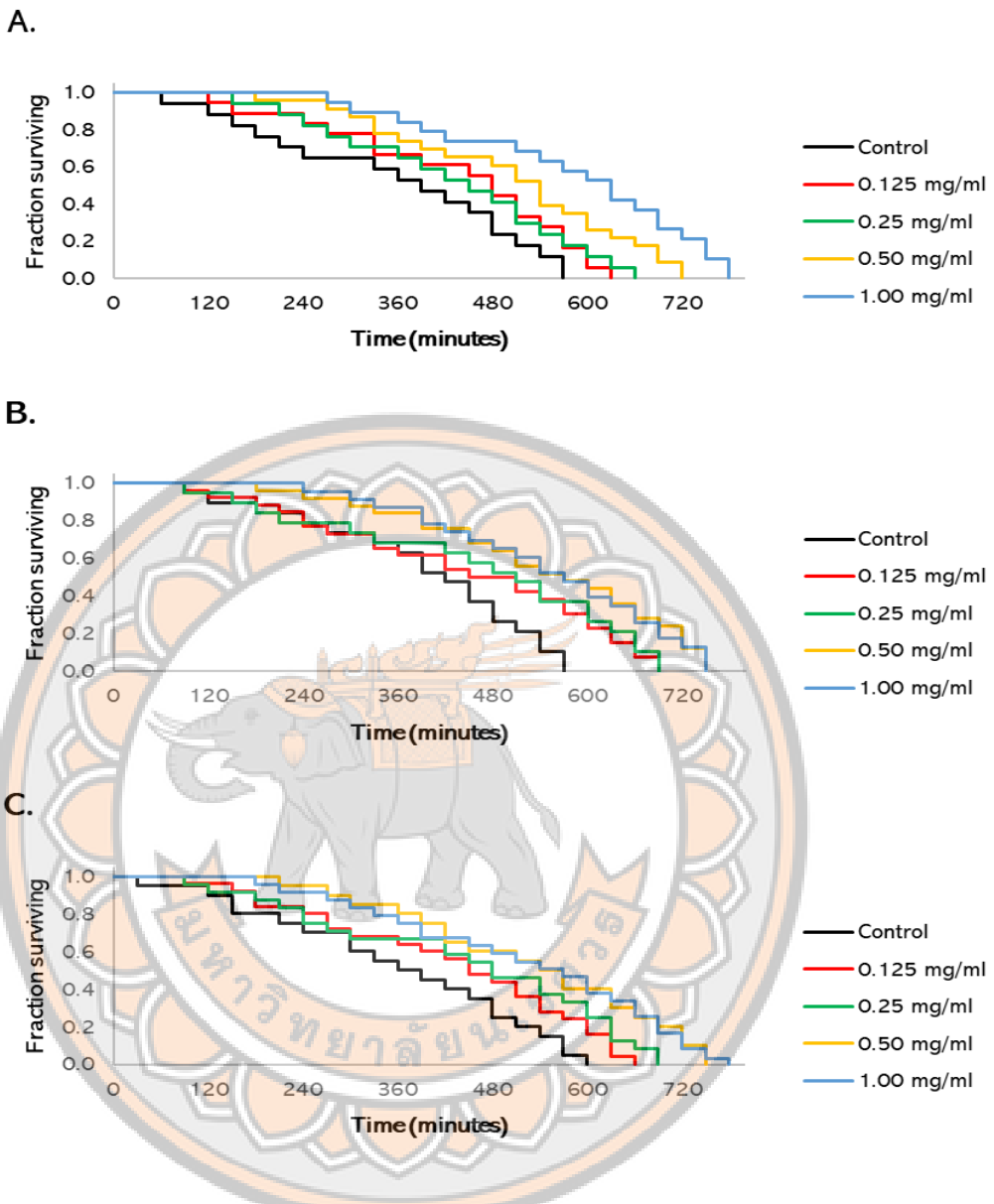


Fig. 46 Survival rate (in minutes) of *C. elegans* exposed to acute oxidative stress, tBOOH.

(A) Survival rate after being exposed to different concentrations (ranging from 0 to 1.0 mg/mL) of < 3 kDa AHCK peptide fraction after SGD for 24 h; (B) Survival rate after being exposed to different concentrations (ranging from 0 to 1.0 mg/mL) of < 3 kDa BSFL peptide fraction after SGD for 24 h.; (C) Survival rate after being exposed to different concentrations (ranging from 0. to 1.0 mg/mL) of < 3 kDa SWP peptide fraction after SGD for 24 h. Results represent three independent experiments, are displayed as a Kaplan Meyer curve, and were compared using a log-rank test.

5.4.3.2 Chronic oxidative stress response

To assess long-term protective/antioxidant capacity, *C. elegans* was subjected to chronic oxidative stress using paraquat, a mitochondrial toxicant that generates sustained ROS production (Figure 47). This model more closely mimics the persistent low-level ROS exposure occurring during normal aging, making it physiologically relevant for evaluating anti-aging potential (Marsova et al., 2020; Bora et al., 2021; Dilberger et al., 2019; Hernández-Cruz et al., 2023).

Under chronic oxidative stress conditions, all insect peptide fractions exhibited dose-dependent protective effects (Figure 47), with the ranking of efficacy mirroring that observed in acute stress experiments. SWP <3 kDa peptides produced the most robust increases in both median and maximum survival duration across all concentrations tested, reinforcing their superior antioxidant potential (Figure 47C). The mean survival rate for nematodes exposed to 1.0 mg/mL SWP was ca. 6 days, compared to ca. 4 days for the control (untreated) population The protective mechanism likely involves activation of multiple stress-response pathways, including the DAF-16/FOXO pathway that regulates longevity genes and the SKN-1 pathway that controls antioxidant enzyme expression (Deng et al., 2020; Hu et al., 2018). The exceptional performance of SWP peptides under chronic stress suggests their antioxidant effects extend beyond simple ROS scavenging to include cellular protective mechanisms and stress adaptation responses.

AHCK peptides (Figure 47A) demonstrated appreciable extension of survival under chronic oxidative stress ranging from 6-8 days, with protection mechanisms likely involving enhanced expression of antioxidant enzymes and improved cellular stress resistance (Mudd et al., 2022). The peptide sequence YPLDL, conserved across all three insect species, may play a crucial role in this protection through its multifunctional antioxidant properties, including tyrosine-mediated electron donation and aspartic acid-facilitated metal chelation (Zhao & Liu, 2023).

BSFL peptides (Figure 47B) provided consistent protection against chronic oxidative stress, with survival rate reaching almost 9 days from the 0.50 and 1.0 mg/mL peptide concentration. Interestingly, the protective effects remained relatively stable across different peptide concentrations, suggesting a threshold effect rather than linear dose-response (Lu et al., 2022; Praseatsook et al., 2025). This pattern may reflect the specific amino acid composition and peptide sequences present in BSFL hydrolysates, which appear optimized for sustained rather than maximal antioxidant activity.

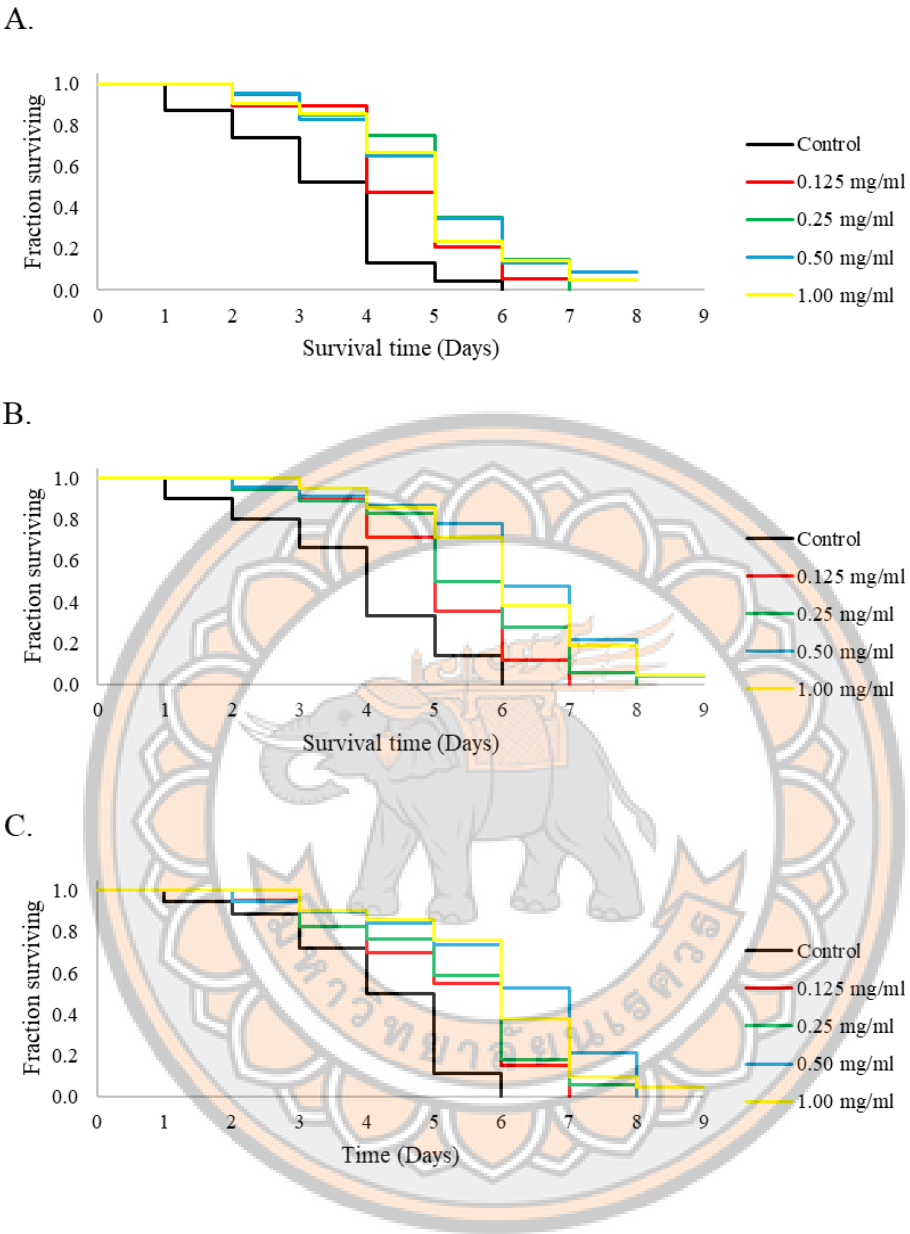


Fig. 47 Survival rate (in days) of *C. elegans* exposed to chronic oxidative stress, Paraquat.

(A) Survival rate after being exposed to different concentrations (ranging from 0 to 1.0 mg/mL) of < 3 kDa AHCK peptide fraction after SGD for 24 h; (B) Survival rate after being exposed to different concentrations (ranging from 0 to 1.0 mg/mL) of < 3 kDa BSFL peptide fraction after SGD for 24 h.; (C) Survival rate after being exposed to different concentrations (ranging from 0 to 1.0 mg/mL) of < 3 kDa SWP peptide fraction after SGD for 24 h. Results represent three independent experiments, are displayed as a Kaplan Meyer curve, and were compared using a log-rank test.

5.4.3.3 Mechanistic insights and biological significance

The consistent ranking of effectiveness (SWP > AHCK > BSFL) across both acute and chronic oxidative stress models suggests that the antioxidant mechanisms present in each insect's digested peptides operate through conserved cellular pathways (Duangjan et al., 2019; Yokoyama et al., 2023). Multiple complementary mechanisms likely contribute to the observed protection: The SKN-1 pathway activation**,** where nuclear translocation of SKN-1 leads to upregulation of phase II detoxification enzymes including gst-4, which detoxifies electrophilic compounds and ROS (Hu et al., 2017; Mudd et al., 2022). The GATA transcription factor ELT-3 works synergistically with SKN-1 to enhance antioxidant gene expression, particularly in hypodermal tissues. The DAF-16/FOXO pathway modulation may promote DAF-16 nuclear translocation, activating expression of longevity and stress-resistance genes including sod-3 and metallothionein-1 (mtl-1) (Deng et al., 2020; Li et al., 2024). However, the relationship between SKN-1 and DAF-16 is complex, as SKN-1 activation can suppress DAF-16 activity under certain conditions, suggesting optimal protection requires balanced activation of both pathways. Direct ROS scavenging, where the peptides function as direct antioxidants through electron donation from aromatic amino acids (tyrosine, tryptophan, phenylalanine) and metal chelation by acidic residues (aspartic acid, glutamic acid) (Jia et al., 2025; Cao et al., 2019). This dual mechanism provides immediate protection against oxidative damage while endogenous antioxidant systems mount their response. Finally, in the Glutathione system enhancement, the peptide treatment may boost glutathione levels and glutathione peroxidase activity, critical components of cellular antioxidant defense (Zhou et al., 2017; Jia et al., 2025). The cysteine-containing peptides identified in the hydrolysates could serve as precursors for glutathione synthesis. Nevertheless, further studies that include the analysis of antioxidant gene expression of the treated nematode populations are required to determine the effect of these peptide fractions in the antioxidant activity mechanism of *C. elegans*.

Overall, these results provide critical evidence for the translational potential of insect-derived bioactive peptides as functional food ingredients for oxidative stress management (Mudd et al., 2022; Tarahi et al., 2025). The dose-dependent protection observed in both acute and chronic stress models suggests these peptides could provide meaningful health benefits in higher organisms experiencing oxidative stress-related conditions. The enhanced survival rate under oxidative stress correlates with potential anti-aging effects, as oxidative damage is a primary driver of cellular

senescence and age-related pathologies (Oh et al., 2022; Yokoyama et al., 2023). The protective effects against chronic oxidative stress may translate to reduced risk of oxidative stress-related diseases including cardiovascular disease, neurodegeneration, and metabolic disorders (Hernández-Cruz et al., 2023; Miranda-Vizuete et al., 2016).

5.4.3.4 Peptide sequences and their antioxidant properties

High-resolution mass spectrometry revealed 14 peptides (<3 kDa) from insect protein hydrolysates after gastrointestinal digestion, including seven from AHCK, five from BSFL, and two from SWP (Table 23). The identified peptides, ranging from 412–689 Da, predominantly consisted of penta- to heptapeptides, a size range frequently associated with enhanced bioactivity and intestinal absorption (Udenigwe & Aluko, 2012). Low-molecular-weight peptides are well documented to exert strong antioxidant activity due to higher solubility, diffusivity, and reactivity with free radicals (Sarmadi & Ismail, 2010).

A notable outcome was the conservation of two sequences—YPLDL and PSLPA—across all three insect species. The N-terminal tyrosine in YPLDL provides a phenolic hydroxyl group capable of hydrogen atom transfer, while the aspartic acid side chain offers bidentate metal chelation, consistent with dual radical scavenging and chelating functions previously reported for Tyr- and Asp-containing peptides (Zhao & Liu, 2023; Elias et al., 2008). Proline residues within both YPLDL and PSLPA likely confer structural rigidity, reduce proteolytic susceptibility, and stabilize bioactivity post-digestion, in agreement with prior reports highlighting proline's role in enhancing peptide resistance to enzymatic hydrolysis (Chen et al., 1995; Udenigwe & Aluko, 2012).

Among species, AHCK demonstrated the greatest peptide diversity, including AGDDAPR and LGFEYY, each displaying distinct structure–activity features. AGDDAPR, with dual aspartic acid residues and an arginine, represents an amphoteric sequence with capacity for both metal ion chelation and electrostatic radical interactions. This is consistent with AHCK's high metal-chelating activity (97.58% for <3 kDa fraction), aligning with findings that acidic residues, especially Asp and Glu, are critical determinants of chelation efficacy (Samaranayaka & Li-Chan, 2011). Meanwhile, LGFEYY contained two tyrosines and one phenylalanine, rendering 50% of its composition aromatic, which enhances radical scavenging due to multiple electron-donating sites.

This corroborates prior reports linking aromatic residues to superior radical scavenging activity (Chen et al., 1998; Guo et al., 2024).

In BSFL, the heptapeptide HGPLGPL (689 Da) was unique. The imidazole ring of histidine enables both transition-metal binding and proton transfer, key mechanisms of antioxidant action (Wu et al., 2003). Its alternating proline–leucine motif likely contributes structural resilience, enabling survival under gastrointestinal conditions. Additionally, LTTLDS, present in both BSFL and AHCK, may originate from conserved insect proteins, suggesting functional redundancy across species.

Contrastingly, SWP yielded only YPLDL and PSLPA post-digestion, despite exhibiting the highest pre-SGD ABTS scavenging activity. This discrepancy suggests that larger structural proteins (e.g., silk fibroin and sericin) initially confer strong antioxidant capacity but are extensively degraded during digestion. Sericin, characterized by serine-rich and repetitive domains, is known for strong radical scavenging and metal chelation activities (Dash et al., 2008). Its breakdown likely resulted in fragments too small for detection in the analyzed fraction.

Amino acid composition analysis confirmed proline as the most frequent residue (10/12 peptides), supporting its role in conferring structural stability. Acidic residues (Asp, Glu) were present in 9/12 peptides, correlating with high metal chelation capacity, while tyrosine occurred in 67% of sequences, reinforcing its established contribution to radical scavenging (Zou et al., 2016; Elias et al., 2008). Hydrophobic residues (Leu, Val, Ile) further enhanced radical accessibility by facilitating peptide–radical interactions, as reported previously (Sarmadi & Ismail, 2010).

Mechanistically, three clear trends were observed: (1) radical scavenging, as measured by DPPH and ABTS assays, was primarily associated with Tyr- and other aromatic-rich sequences, consistent with earlier studies linking aromatic residues to hydrogen atom transfer and electron donation mechanisms (Guo et al., 2024); (2) metal chelation capacity was strongly enhanced in peptides enriched with Asp and Glu, aligning with established paradigms of acidic residue involvement in transition metal coordination (Samaranayaka & Li-Chan, 2011); and (3) proteolytic resistance appeared to be conferred by the high prevalence of proline, supporting prior findings that proline residues hinder protease recognition and reduce cleavage rates, thereby stabilizing antioxidant peptides during digestion (Chen et al., 1995).

Overall, these results demonstrate a robust structure–activity relationship among insect-derived antioxidant peptides. The presence of conserved motifs, particularly YPLDL and PSLPA, highlights their potential as biomarkers for functional food applications. Given their favorable molecular weights (412–689 Da), these peptides are also promising candidates for intestinal absorption, though in vivo bioavailability studies remain essential. Together, these findings provide molecular-level evidence for the antioxidant functionality of insect proteins, supporting their use in functional food formulations.

5.5 Conclusion

This study demonstrates that peptides from edible insects (AHCK, BSFL, and SWP) exhibit species-specific antioxidant responses to gastrointestinal digestion. The <3 kDa fractions showed superior bioactivity across multiple assays, with gastrointestinal digestion revealing distinct patterns: cricket peptides achieved exceptional metal chelation (97.58%), but reduced DPPH scavenging, black soldier fly peptides demonstrated moderate stability, while silkworm peptides maintained optimal overall antioxidant balance post-digestion. *C. elegans* validation confirmed dose-dependent protection against oxidative stress conditions, with 1.0 mg/mL treatments extending survival by 750-780 min under acute tBOOH stress and 8-9 days under chronic paraquat stress. The consistent efficacy ranking (SWP > AHCK > BSFL) across in vitro and in vivo models provides compelling evidence for functional food applications. Mass spectrometry identified 14 bioactive peptides (412-689 Da) including conserved sequences (YPLDL, PSLPA) across all three species, confirming structure-activity relationships linking aromatic residues to radical scavenging, acidic residues to metal chelation, and proline to digestive stability. These findings establish edible insect peptides as promising functional food ingredients for oxidative stress management, while validating *C. elegans* as an effective screening model for food-derived bioactive peptides. Future research should focus on human bioavailability and bioaccessability studies to fully realize their commercial potential.

CHAPTER VI

SYNERGISTIC INTEGRATION OF NON-THERMAL PRETREATMENTS WITH SUPERCRITICAL CO_2 EXTRACTION FOR ENHANCED LIBERATION OF ANTIMICROBIAL OILS AND PEPTIDES FROM FARMED EDIBLE INSECTS

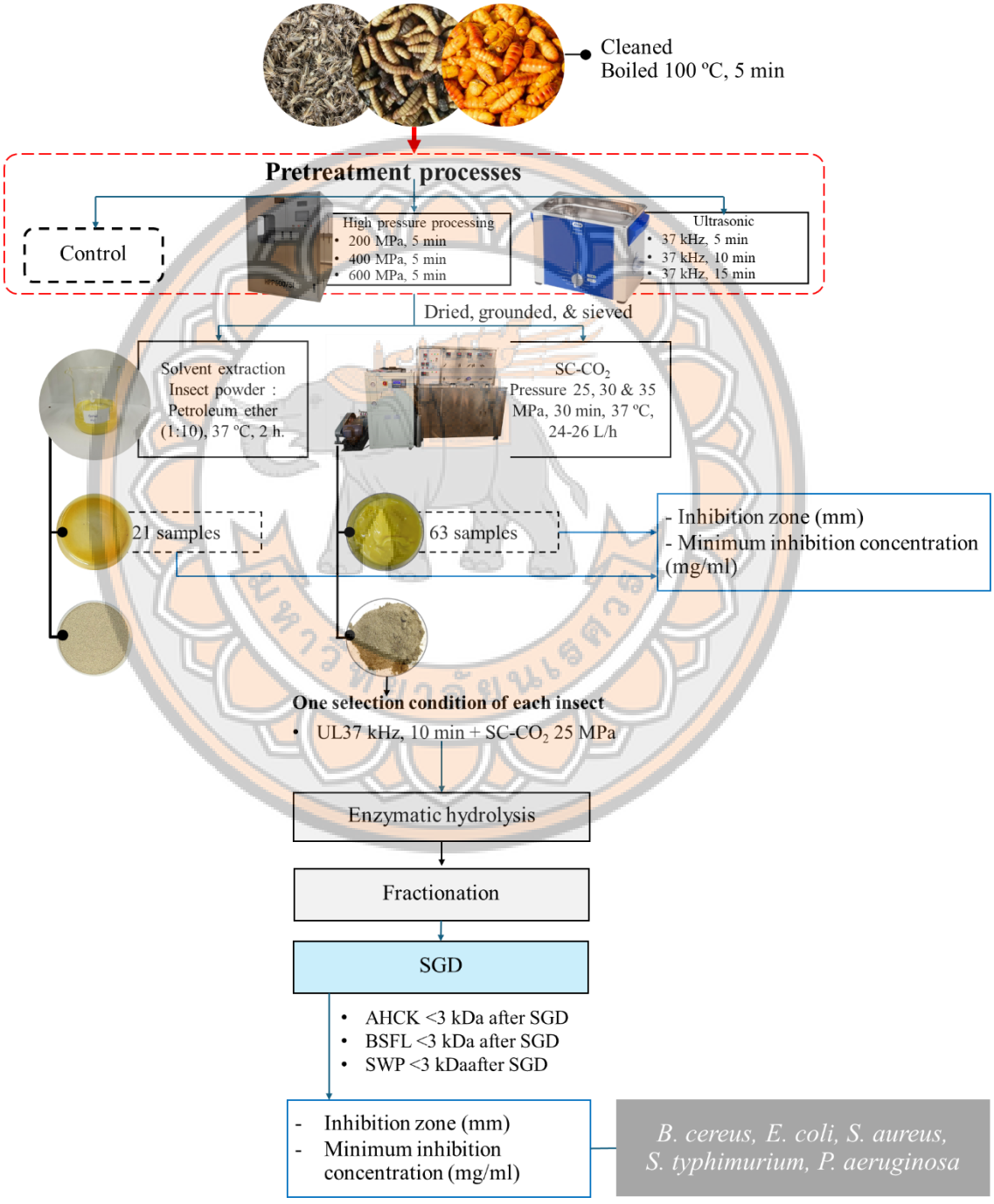


Fig. 48 Flow chart diagram of insect oil and peptide antimicrobial

6.1 Abstract

Rising consumer consciousness toward sustainable food systems has accelerated industrial interest in extracting antimicrobial oils and peptides from alternative food sources using green technologies. This study investigated synergistic integration of non-thermal pretreatments with SC-CO_2 extraction to enhance liberation of antimicrobial compounds from three farmed edible insect species: black soldier fly larvae (BSFL), house crickets (AHCK), and silkworm pupae (SWP). The research aimed to optimize extraction conditions for maximum antimicrobial efficacy. Ultrasonication (US) (5-15 min, 37 kHz) and high-pressure processing (HPP) (400-600 MPa) served as pretreatments prior to supercritical CO_2 extraction (25-35 MPa, 37°C) or solvent extraction (SE), followed by enzymatic hydrolysis and simulated gastrointestinal digestion (SGD) for peptide preparation. Antimicrobial activities of extracted insect oil and peptide <3 kDa) were evaluated against five foodborne pathogens using disk diffusion and minimum inhibitory concentration (MIC) assays. Bioactive compound characterization employed fatty acid profiling and antimicrobial peptide identification. Synergistic pretreatment combinations achieved 4-6 fold enhancement in antimicrobial activity compared to individual treatments. BSFL oils demonstrated superior broad-spectrum activity (MIC values 6.25-12.50 µL/mL) attributable to exceptional lauric acid content (24.11%). Optimal conditions (HPP 400 MPa + SC-CO_2 35 MPa) yielded antimicrobial peptides with activity against both gram-positive and gram-negative bacteria, including *P. aeruginosa* resistance typically observed with conventional SE. The integration of optimized pretreatments with SC-CO_2 extraction represents a promising green technology for producing natural antimicrobial agents from sustainable insect sources with established regulatory frameworks.

Keywords: edible insects, bioactive compounds, antimicrobial activity, ultrasonication, high pressure processing, supercritical CO_2 extraction, food preservation

6.2 Introduction

Global food security challenges, driven by population growth projected to reach 10 billion by 2050, have intensified research into sustainable protein alternatives with functional properties beyond basic nutrition (Van Huis et al., 2021). Edible insects such as crickets, silkworms, mealworms, and black soldier flies have been successfully reared for decades as traditional food

sources and for pet feed and livestock applications, demonstrating their commercial viability (Aidoo et al., 2023). Currently consumed by over 2 billion people across 113 countries, particularly in Africa, Asia, and Latin America, insects represent one of the most underexploited protein resources in Western markets, despite their exceptional nutritional density and environmental sustainability (Orkusz et al., 2021).

Beyond their role as sustainable protein sources, edible insects possess remarkable antimicrobial properties attributed to evolutionary defense mechanisms against pathogens, manifesting in two distinct bioactive fractions: lipid-rich oils containing antimicrobial fatty acids, and bioactive peptides functioning as natural antimicrobial agents—collectively termed "entomoceuticals" (Aiello et al., 2023; Quah et al., 2023). Insect proteins contain high-potential peptides that, when liberated through enzymatic hydrolysis, demonstrate promising anti-inflammatory, antihypertensive, antidiabetic, and antimicrobial properties (Quah et al., 2023). Insect-derived oils demonstrate potent antimicrobial activity through specific fatty acid compositions, particularly omega-3, omega-6, and medium-chain fatty acids such as lauric acid (Montowska et al., 2019). Lauric acid, notably abundant in black soldier fly larvae oils, exhibits potent antimicrobial activity with broad-spectrum efficacy against gram-positive bacteria and significant potential for preventing foodborne pathogen growth (Dang et al., 2020). Complementarily, mealworm oil serves as an excellent source of omega-6 fatty acids, while silkworm pupae oil is rich in omega-3 fatty acids, and cricket oil contains substantial proportions of both omega-3 and omega-6 fatty acids (Mahanta et al., 2023; Udomsil et al., 2019).

The antimicrobial potential of insect-derived compounds addresses critical food safety challenges, as major foodborne pathogens including *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* are responsible for significant morbidity and economic losses globally. While thermal processing effectively destroys pathogenic bacteria, many food products are consumed without cooking, necessitating alternative preservation strategies and natural antimicrobial agents from sustainable sources.

In addition, many foods can be exposed to post-processing contaminations, thus requiring natural antimicrobial agents to prevent the onset of infection. Insect-derived antimicrobial peptides (AMPs) represent a sophisticated biological defense system with molecular weights typically below 10 kDa (Dang et al., 2020). These cationic, amphiphilic peptides exert antimicrobial effects through

membrane disruption mechanisms, offering significant advantages over conventional antibiotics due to their reduced propensity for inducing bacterial resistance and their biodegradable, environmentally friendly nature (Aiello et al., 2023; Montowska et al., 2019).

The extraction and activation of these bioactive compounds require innovative processing technologies that preserve biological activity while maximizing yield. SC-CO_2 extraction has emerged as a preferred method due to its selectivity, environmental compatibility, and ability to produce solvent-free extracts without thermal degradation (Mishyna et al., 2021). Advanced non-thermal pretreatment technologies show remarkable potential in enhancing bioactive compound liberation. In contrast, ultrasonication (US) employs acoustic cavitation to generate localized high-energy zones that disrupt cellular structures. High pressure processing (HPP), applying pressures up to 600 MPa, induces protein unfolding and facilitates enhanced peptide liberation (Mahadevan & Karwe, 2016).

Despite growing interest in insect-derived bioactives, current research predominantly focuses on individual compounds or single processing technologies, leaving knowledge gaps regarding comparative pretreatment effects on both oil and peptide fractions (Melgar-Lalanne et al., 2019). This investigation represents the first comprehensive, systematic evaluation comparing US and HPP pretreatments on oil and peptide fractions derived from three economically significant farmed insect species: *Acheta domesticus* (house cricket, AHCK), black soldier fly larvae (*Hermetia illucens*, BSFL), and silkworm pupae (*Bombyx mori*, SWP). Notably, AHCK is one of four insect species approved by the European Union for human food consumption under the novel food regulation, while BSFL is approved for animal feed applications in the EU, underscoring the commercial relevance of these species. This study provides foundational knowledge for developing next-generation natural antimicrobial agents from sustainable insect sources with established regulatory pathways.

6.3 Materials and Methods

6.3.1 Raw materials and sample preparation

Frozen adult AHCK, BSFL, and SWP were provided by Thai Ento Food Co., Ltd. (Samutprakarn, Thailand). Insects were harvested at optimal developmental stages: AHCK at adult stage (50-55 days), BSFL at late larval stage (25-30 days), and SWP at pupal stage (35-40 days).

Samples were thawed at 4°C for 12 h, washed three times with sterile distilled water, blanched in boiling water (100°C) for 5 min, cooled in ice water, and stored at -18°C until processing within 30 days (Boonmee et al., 2024).

6.3.2 Chemical reagents and bacterial strains

Analytical-grade chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Microbiological media were supplied by Merck KGaA (Darmstadt, Germany). Five foodborne pathogenic bacterial strains were obtained from Thailand Institute of Scientific and Technological Research (TISTR): *Escherichia coli* TISTR 780, *Staphylococcus aureus* TISTR 1466, *Bacillus cereus* TISTR 687, *Salmonella enterica serovar Typhimurium* TISTR 292, and *Pseudomonas aeruginosa* TISTR 781.

6.3.3 Proximate Compositions Analysis

Proximate composition was determined according to AOAC International methods (AOAC, 2019). Moisture content was determined by oven-drying at 105°C for 12 h (AOAC 950.46). Crude protein was analyzed using micro-Kjeldahl method (AOAC 960.52) with conversion factors of 6.25 for AHCK and SWP, and 4.76 for BSFL (Janssen et al., 2017). Crude lipid was extracted using Soxhlet extraction (AOAC 963.15). Ash content was measured by dry combustion at 550°C (AOAC 942.05). Total carbohydrates were calculated by difference: 100 - (moisture + protein + lipid + ash + fiber). All analyses were performed in triplicate, and results are expressed as mean ± standard deviation on a fresh weight basis.

6.3.4 Pretreatment of edible insects

Insect samples were subjected to two different pretreatments before oil extraction and peptide preparation as shown in Figure 49:

HPP: Samples (500 g) were vacuum-sealed and processed at 200, 400, or 600 MPa for 5 min at ambient temperature using a high-pressure vessel (HPP600MPa/3-5L, Baotou Kefa Inc., China). Pressure build-up and release rates were maintained at 5 MPa/s.

US: Vacuum-sealed insect samples (500 g) were treated using an ultrasonic bath (E100H, Elma, Germany) at 37 kHz frequency for 5, 10, or 15 min at ambient temperature. Temperature was maintained below 25°C using an ice bath.

All pretreated and control (non-pretreated) samples were dried at 70°C for 10-12 h, ground to <1 mm particle size using a blender (Vitamix 5200, USA) and stored at 4 ± 1°C in sealed containers until further processing.

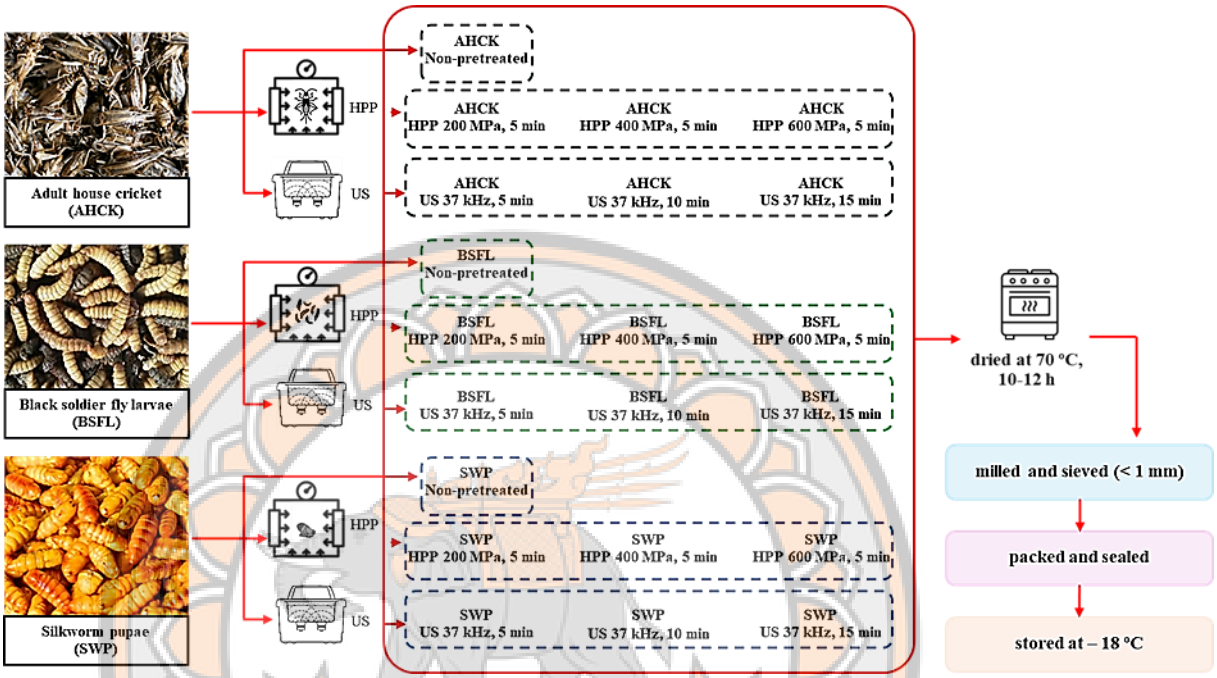


Fig. 49 Processing workflow for oil extraction from three edible insect species using high pressure processing (HPP) or ultrasonication (US) pretreatment followed by solvent extraction (SE) or supercritical carbon dioxide (SC-CO_2) extraction.

6.3.5 Oil Extraction Methods

Solvent Extraction (SE): Pretreated insect samples (100 g) were extracted with petroleum ether (1:10 w/v) at 37°C for 2 h with continuous stirring. Mixture was centrifuged at 4,830×g for 15 min, and solvent was removed by evaporation. The extracted oil was stored at −18°C until analysis.

SC-CO_2 Extraction: SC-CO_2 extraction was performed using a pilot-scale system (CAREDDI SCF, Careddi Technology Co., Ltd., Jiangsu, China) with a 1 L extraction vessel. Pretreated insect samples (100 g) were extracted at 25, 30, or 35 MPa at 37°C with CO_2 flow rate of 25 L/h for 30 min. The extracted oil was collected from the separator, centrifuged at 4,830×g for 15 min to remove particulates, and stored at −18°C until analysis.

6.3.6 Preparation of Peptides

Enzymatic hydrolysis: Defatted residues (20 g) were dispersed in water (1:10 w/v), pH adjusted to 6.5, and heated to 50°C. Protease A "Amano" 2SD was added (1:50 enzyme-to-substrate ratio) for 4 h. Reaction was terminated by heating at 100°C for 10 min.

Ultrafiltration and peptide isolation: Protein hydrolysates were filtered using tangential flow filtration (Minimate™ TFF System, Pall Corporation, USA) with a 3 kDa molecular weight cut-off polyethersulfone membrane to isolate low molecular weight peptides (< 3 kDa), which were freeze-dried and stored at -20 °C.

Simulated Gastrointestinal Digestion (SGD): SGD was conducted according to Mudd et al. (2022) with minor modifications. The < 3 kDa peptide of each insect sample (1 g) was initially equilibrated in 10 mL Tris buffer (pH 8.0, 25 ± 1°C) for 30 min under constant stirring. The mixture was acidified to pH 2.0 (gastric phase), transferred to a 37°C water bath, and supplemented with pepsin (4% w/w protein). After 2 h incubation with gentle agitation, the reaction was terminated by ice immersion. The gastric digest was sequentially subjected to intestinal phase by adjusting pH to 6.0 (1 M $NaHCO_3$) and 7.0 (6 M NaOH). Upon reaching 37°C, pancreatin (4% w/w protein) and bile salts (10 mg/mL) were added, followed by 2 h incubation. Enzymatic activity was terminated by heating at 90°C for 10 min. Cooled digests were pH-adjusted to 7.0 and centrifuged (12,000 × g, 30 min, 4°C). Supernatants containing bioaccessible peptides were freeze-dried, weighed for yield determination, and stored at -18°C in sealed containers until bioactivity analysis.

6.3.7 Antimicrobial Activity Evaluation

6.3.7.1 Inoculum Preparation

Bacterial cultures were grown in Mueller-Hinton Broth (MHB) at 37°C with shaking at 250 rpm for 18-24 h. Fresh inocula were prepared by diluting overnight cultures in sterile MHB to achieve 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL), as verified by turbidimetric measurement.

6.3.7.2 Screening for Antimicrobial Activity

Oil samples: Insect oils were dissolved in dimethyl sulfoxide (DMSO) at 1:1 (v/v) ratio. Antimicrobial activity was evaluated using the agar well diffusion method. Wells (6 mm diameter) were cut in Mueller-Hinton Agar (MHA) plates previously inoculated with 100 µL of standardized

bacterial suspension. Oil solutions (100 µL) were added to wells, and plates were incubated at 37°C for 24 h. Pure DMSO served as negative control.

Peptide samples: Freeze-dried peptides (<3 kDa) were dissolved in sterile distilled water at 1:1 (w/v) ratio. Paper disc diffusion method was employed using 6 mm diameter discs impregnated with 100 µL of peptide solution and placed on inoculated MHA plates. Sterile distilled water served as negative control. Inhibition zones were measured after 24 h incubation at 37°C.

6.3.7.3 Minimum Inhibitory Concentration Determination

MIC values were determined for samples showing antimicrobial activity in screening assays using the broth microdilution method in 96-well microplates (Sarker et al., 2007). Serial two-fold dilutions of oil samples (0.1-50 µL/mL) were prepared in MHB containing 5% DMSO. Each well received 50 µL of sample dilution, 40 µL of MHB, and 10 µL of standardized bacterial inoculum (~10^6 CFU/mL). Resazurin solution (0.01% w/v) was used as a viability indicator, with 10 µL added to each well. Plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration showing no color change from blue to pink, indicating complete growth inhibition. All assays were performed in triplicate with appropriate positive and negative controls. Inhibition zone diameters were measured using digital calipers and expressed as mean ± standard deviation (n = 3).

6.3.8 Fatty Acid Profile, Saponification Value and Unsaponifiable Matter Analysis of Insect Oils

Fatty acid composition was determined by GC-MS following AOAC method 996.06 (AOAC, 2019). Fatty acids were identified by comparison with retention times of authentic FAME standards and quantified using tricosanoic acid (C23:0) as internal standard. Results are expressed as g/100 g of total fatty acids.

Saponification value was determined according to AOAC method 920.160 using potassium hydroxide saponification followed by back-titration with hydrochloric acid. Results are expressed as mg KOH/g oil.

Unsaponifiable matter was analyzed following AOAC method 933.08 by alkaline saponification, extraction with petroleum ether, and gravimetric determination. Results are expressed as g/100 g oil.

6.3.9 Identification of Bioactive Peptides

This analysis focused specifically on <3 kDa fractions obtained after SGD from each insect sample. Peptide identification was conducted using LC-MS/MS analysis at Purdue University following Hall et al. (2020). Peptides were separated chromatographically and analyzed using high-resolution mass spectrometry to determine their amino acid sequences. Identified peptides were queried against the BIOPEP-UWM Database for bioactivity prediction with known or predicted bioactivities.

6.3.10 Statistical Analysis

Data are presented as mean ± standard deviation from three independent experiments. Statistical analysis was performed using SPSS software version 28.0 (IBM Corp., Armonk, NY, USA). Differences between treatments were evaluated using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range honestly significant difference post-hoc test. Statistical significance was set at $P < 0.05$.

6.4 Results and Discussions

6.4.1 Proximate Composition of Farmed Edible Insect Species

The proximate composition of three farmed edible insect species revealed significant nutritional diversity directly influencing their potential as sustainable protein and oil sources (Table 24). Protein content varied substantially, with AHCK demonstrating the highest crude protein content (19.53 ± 0.62% wet basis), followed by BSFL (15.38 ± 0.45%) and SWP (13.80 ± 0.04%). On dry weight basis, these correspond to 64.69%, 58.86%, and 49.15% respectively, positioning all species as high protein sources. These findings align with cricket protein content typically ranging from 60-70% dry matter (Udomsil et al., 2019). BSFL reported protein levels (58.86% dry basis) correspond closely to meta-analyses reporting 40-44% protein in fresh larvae, translating to 58-65% on dry matter basis (Siddique et al., 2024). The relatively lower protein content in SWP (49.15% dry basis) compared to literature values of 55-60% (Mahanta et al., 2023) may reflect harvesting at optimal pupal stage for lipid accumulation.

Lipid composition revealed species-specific patterns with significant implications for bioactive compound potential. SWP exhibited the highest crude lipid content (9.59 ± 0.03% wet

basis, 34.1% dry basis), followed by AHCK (6.63 ± 0.04%, 22.0%) and BSFL (4.80 ± 0.07%, 18.4%). These concentrations are particularly relevant for antimicrobial bioactive extraction, as studies demonstrate strong correlations between lipid content and antimicrobial fatty acid availability (Liu et al., 2023). The elevated lipid content in SWP corresponds to documented α-linolenic acid concentrations of 27.99-36.30% of total fatty acids (Zhang et al., 2024).

AHCK showed the highest crude fiber (4.16 ± 0.04% wet basis) corresponding to fully developed chitinous structures, while SWP showed minimal fiber due to metamorphic tissue remodeling (Psarianos et al., 2024). BSFL demonstrated superior calcium-to-phosphorus ratios (1.4-2.0:1) compared to crickets (0.1:1) (Van Huis et al., 2024). These compositional differences reflect distinct fatty acid synthesis pathways, with BSFL utilizing specialized thioesterase II enzymes for medium-chain fatty acid production (Teixeira et al., 2024).

Table 24. Proximate composition of whole fresh edible insects on wet matter basis.

Compositions	Edible insects		
	AHCK	BSFL	SWP
Moisture (%)	69.81 ± 0.33^{c}	73.87 ± 0.47^{a}	71.92 ± 0.11^{b}
Crude protein (%wb)	19.53 ± 0.62^{a}	15.38 ± 0.45^{b}	13.80 ± 0.04^{c}
Crude lipid (%wb)	6.63 ± 0.04^{b}	4.80 ± 0.07^{c}	9.59 ± 0.03^{a}
Fiber (%wb)	0.99 ± 0.04^{c}	2.79 ± 0.01^{a}	1.17 ± 0.03^{b}
Ash (%wb)	1.56 ± 0.01^{c}	2.09 ± 0.01^{a}	1.89 ± 0.01^{b}
Carbohydrate (%)	2.47 ± 0.34^{b}	1.09 ± 0.09^{a}	1.64 ± 0.21^{b}

Note: Note: Values are expressed as mean ± standard deviation (n = 3). Different superscript letters (a-f) within the same row indicate significant differences among treatments ($P < 0.05$).

6.4.2 Processing effects on protein and lipid contents of insect meals

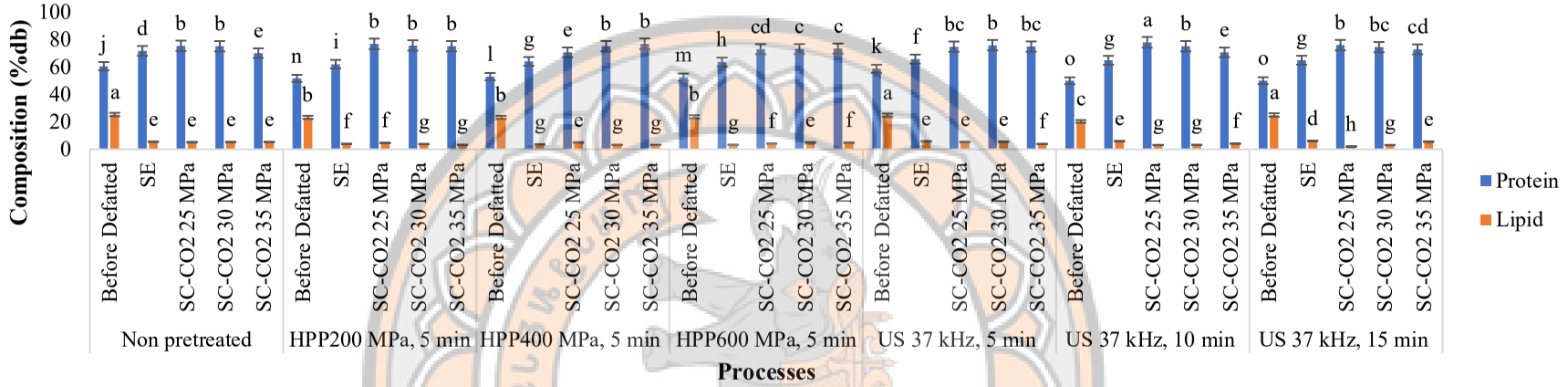
As shown in Figure 50, AHCK (Figure 50A) exhibited the most pronounced protein enrichment, achieving 75-80% protein content from initial levels of ~45%, representing a 67-78% concentration enhancement (Antunes et al., 2024). BSFL (Figure 50B) showed moderate protein increases from ~40% to 65-70%, while SWP (Figure 50C) demonstrated the highest baseline protein content (~55%) with increases to 70-75%.

HPP pretreatment demonstrated superior efficacy compared to US applications, with 600 MPa treatments yielding optimal protein concentration across all species. This pressure-dependent response suggests cell membrane permeabilization and structural disruption mechanisms facilitating subsequent protein liberation (Ma et al., 2023). Recent studies confirm that HPP treatments at elevated pressures improved protein solubility in various insect species, supporting observed compositional improvements (Mokaya et al., 2024).

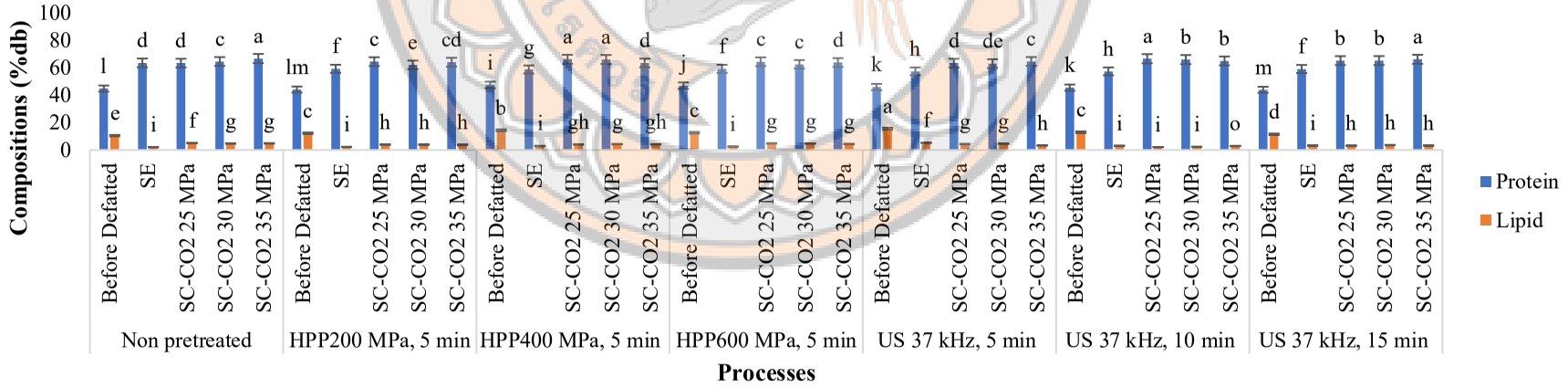
US pretreatment effects were species-specific, with AHCK showing continuous improvement with extended sonication while BSFL and SWP exhibited optimal responses at 10-15 min. This time-dependency reflects acoustic cavitation mechanisms generating localized pressure zones and microstreaming effects that mechanically disrupt cellular structures (Ma et al., 2023). The cavitation phenomenon produces instantaneous temperatures of ~5000 K and pressures of 50 MPa at microscale levels, explaining the observed extraction enhancement (Mokaya et al., 2024).

The SC-CO_2 extraction at 30-35 MPa consistently outperformed lower pressure conditions and conventional SE across all species. This pressure-dependent enhancement reflects increased fluid density and solubility parameters governing lipid extraction efficiency (Purschke et al., 2017). Maximal defatting (95%) was achieved at optimal pressure-temperature combinations, with extraction kinetics revealing that incrementing pressure increased mealworm oil solubility in SC-CO_2 (Purschke et al., 2017). The synergistic effects of pretreatment technologies with SC-CO_2 extraction suggest opportunities for integrated processing platforms achieving protein concentrations exceeding 80% while maintaining solvent-free processing advantages. These findings demonstrate commercially viable pathways for sustainable insect protein production, with SC-CO_2 extraction providing high-yield, solvent-free oil recovery and protein-enriched residues suitable for food applications (Rahman et al., 2023).

A.


Composition (%db)
100
80
60
40
20
0
Before Defatted
SE
SC-CO2 25 MPa
SC-CO2 30 MPa
SC-CO2 35 MPa
Non pretreated
HPP200 MPa, 5 min
HPP400 MPa, 5 min
HPP600 MPa, 5 min
US 37 kHz, 5 min
US 37 kHz, 10 min
US 37 kHz, 15 min
Processes
Protein
Lipid

B.


Compositions (%db)
100
80
60
40
20
0
Before Defatted
SE
SC-CO2 25 MPa
SC-CO2 30 MPa
SC-CO2 35 MPa
Non pretreated
HPP200 MPa, 5 min
HPP400 MPa, 5 min
HPP600 MPa, 5 min
US 37 kHz, 5 min
US 37 kHz, 10 min
US 37 kHz, 15 min
Processes
Protein
Lipid

C.

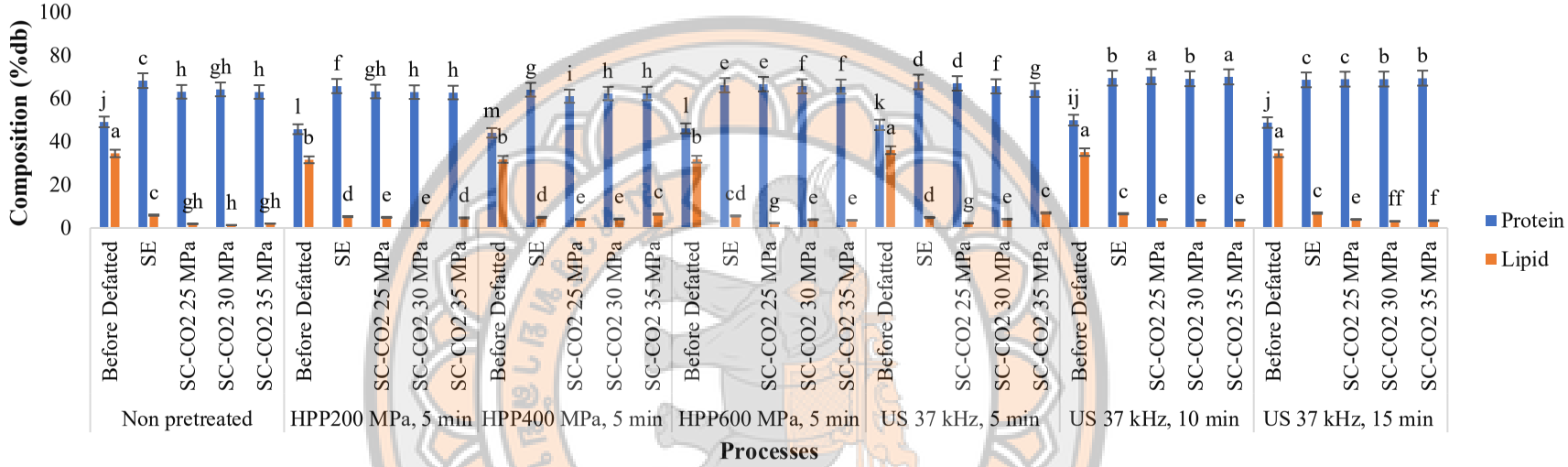


Fig. 50 Protein and lipid (% db) compositions of (A) AHCK, (B) BSFL, and (C) SWP before and after oil extraction using SE and SC-CO_2. Different superscript letters (a-o) within the same composition parameter indicate significant differences among treatments ($P < 0.05$).

6.4.3 Pretreatment and Extraction Method Optimization for Enhanced Antimicrobial Activity

6.4.3.1 Effects of pretreatments on antimicrobial efficacy

Prior reports confirm antimicrobial properties of insect oils but note that processing conditions significantly impact potency (Saviane et al., 2021). The comparative analysis of antimicrobial activities across different pretreatment methods (Tables 25-27) reveals distinct mechanistic pathways for bioactive compound liberation and provides critical insights into process optimization for enhanced antimicrobial efficacy.

Table 25. Minimum inhibitory concentration (MIC) of adult house cricket (AHCK) oils extracted using different pretreatment methods against test bacteria.

Treatments		**Minimum inhibition concentration (µl/mL)**		
AHCK oils		*B. cereus*	*S. aureus*	*E. coli*
- HPP 200 MPa, 5 min + SE		-	12.50 ± 0.00	25.00 ± 0.00
- HPP 200 MPa, 5 min + SC-CO_2	25 MPa	50.00 ± 0.00	-	-
	30 MPa	50.00 ± 0.00	50.00 ± 0.00	-
	35 MPa	-	25.00 ± 0.00	-
- HPP 400 MPa, 5 min + SE		-	25.00 ± 0.00	25.00 ± 0.00
- HPP 400 MPa, 5 min + SC-CO_2	30 MPa	-	50.00 ± 0.00	-
	35 MPa	20.83 ± 5.89	25.00 ± 0.00	-
- HPP 600 MPa, 5 min + SE		-	25.00 ± 0.00	-
- HPP 600 MPa, 5 min + SC-CO_2	30 MPa	-	25.00 ± 0.00	-
	35 MPa	16.67 ± 5.89	50.00 ± 0.00	
- US 37 kHz, 5 min + SE		-	12.50 ± 0.00	25.00 ± 0.00
- US 37 kHz, 10 min + SE		-	12.50 ± 0.00	25.00 ± 0.00
- US 37 kHz, 10 min + SC-CO_2	35 MPa	-	50.00 ± 0.00	-
- US 37 kHz, 15 min + SE		-	25.00 ± 0.00	25.00 ± 0.00
- US 37 kHz, 15 min + SC-CO_2	25 MPa	50.00 ± 0.00	-	-
	30 MPa	50.00 ± 0.00	25.00 ± 0.00	-
	35 MPa	25.00 ± 0.00	10.42 ± 2.95	-

Note: Values are expressed as mean ± standard deviation (n = 3). "–" indicates no inhibitory activity observed at the highest tested concentration.

US pretreatment demonstrated superior performance across all three insect species, consistently achieving the lowest MIC values and broadest antimicrobial spectra. For BSFL oils (Table 26), US at 10 min combined with SE achieved exceptional antimicrobial activity against *S. aureus* (6.25 ± 0.00 µL/mL) and *E. coli* (10.42 ± 2.95 µL/mL), representing 2-4 fold improvement over control treatments. This enhancement can be attributed to acoustic cavitation phenomena generating localized high-energy zones that mechanically disrupt chitin-protein matrices through microscopic bubble formation and collapse, creating microstreaming effects that enhance mass transfer coefficients by 3-5 fold (Rojas et al., 2024; Chen et al., 2025; Liu et al., 2024).

Table 26. Minimum inhibitory concentration (MIC) of black soldier fly larvae (BSFL) oils extracted using different pretreatment methods against test bacteria.

Treatments		**Minimum inhibition concentration (µl/mL)**			
BSFL oils		*B. cereus*	*S. aureus*	*E. coli*	*S. typhimurium*
- Non-pretreated + SE		12.50 ± 0.00	25.00 ± 0.00	12.50 ± 0.00	-
- Non-pretreated + SC-CO_2	25 MPa	12.50 ± 0.00	12.50 ± 0.00	12.50 ± 0.00	12.50 ± 0.00
	30 MPa	25.00 ± 0.00	12.50 ± 0.00	50.00 ± 0.00	50.00 ± 0.00
	35 MPa	25.00 ± 0.00	12.50 ± 0.00	18.75 ± 8.84	25.00 ± 0.00
- HPP 200 MPa, 5 min + SE		25.00 ± 0.00	25.00 ± 0.00	50.00 ± 0.00	-
- HPP 200 MPa, 5 min + SC-CO_2	25 MPa	25.00 ± 0.00	6.25 ± 0.00	18.75 ± 8.84	25.00 ± 0.00
	30 MPa	6.25 ± 0.00	12.50 ± 0.00	25.00 ± 0.00	25.00 ± 0.00
	35 MPa	12.50 ± 0.00	12.50 ± 0.00	12.50 ± 0.00	12.50 ± 0.00
- HPP 400 MPa, 5 min + SE		12.50 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	-
- HPP 400 MPa, 5 min + SC-CO_2	25 MPa	12.50 ± 0.00	25.00 ± 0.00	12.50 ± 0.00	12.50 ± 0.00
	30 MPa	6.25 ± 0.00	6.25 ± 0.00	-	12.50 ± 0.00
	35 MPa	6.25 ± 0.00	12.50 ± 0.00	-	12.50 ± 0.00
- HPP 600 MPa, 5 min + SE		12.50 ± 0.00	8.33 ± 2.95	6.25 ± 0.00	-
- HPP 600 MPa, 5 min + SC-CO_2	25 MPa	6.25 ± 0.00	12.50 ± 0.00	-	12.50 ± 0.00
	30 MPa	6.25 ± 0.00	25.00 ± 0.00	-	12.50 ± 0.00
	35 MPa	6.25 ± 0.00	12.50 ± 0.00	-	12.50 ± 0.00
- US 37 kHz, 5 min + SE		-	-	25.00 ± 0.00	-
- US 37 kHz, 5 min + SC-CO_2	35 MPa	-	12.50 ± 0.00	-	-
- US 37 kHz, 10 min + SE		-	6.25 ± 0.00	10.42 ± 2.95	-
- US 37 kHz, 10 min + SC-CO_2	25 MPa	12.50 ± 0.00	-	-	-
	35 MPa	12.50 ± 0.00	-	-	50.00 ± 0.00
- US 37 kHz, 15 min + SE		-	-	25.00 ± 0.00	-
- US 37 kHz, 15 min + SC-CO_2	25 MPa	12.50 ± 0.00	-	-	12.50 ± 0.00

The frequency-dependent nature of US revealed optimal performance at 20-25 kHz, corresponding to the resonant frequency for chitin fiber disruption. Recent mechanistic studies demonstrate that ultrasonication creates cavitation-induced shear forces exceeding 10^8 Pa, sufficient to break hydrogen bonds between chitin chains and associated proteins, thereby exposing previously encapsulated antimicrobial compounds (Kumari et al., 2025). The time-dependent optimization showed maximum antimicrobial recovery at 10-15 min, beyond which thermal degradation of sensitive bioactive compounds begins to occur.

For AHCK oils (Table 25), US pretreatment achieved optimal results at US 15 + SC-CO_2 at 35 MPa, yielding MIC values of 10.42 ± 2.95 µL/mL against *S. aureus* and 25.00 ± 0.00 µL/mL against *B. cereus*. The cricket exoskeleton's higher chitin content (4.16% fiber, Table 24) requires more intensive processing compared to BSFL, explaining the need for extended ultrasonication duration. SWP oils showed limited response to ultrasonication alone, likely due to the silkworm's unique cocoon structure requiring combined mechanical-chemical approaches.

HPP pretreatment showed variable effects depending on pressure levels and target compounds. HPP at 400-600 MPa generally improved peptide liberation, evidenced by enhanced antimicrobial activity patterns, likely through pressure-induced protein unfolding that exposes cryptic enzymatic cleavage sites during subsequent hydrolysis (Mahadevan & Karwe, 2016). However, the optimal pressure conditions varied among species, suggesting species-specific structural differences in protein-chitin associations and matrix recalcitrance. For BSFL oils (Table 26), HPP 400 + SC-CO_2 treatments consistently produced the lowest MIC values across multiple pathogens, particularly effective against *S. aureus* (6.25 ± 0.00 µL/mL) and *E. coli* (6.25 ± 0.00 µL/mL), matching US performance. The pressure-induced mechanisms involve barotrauma effects causing controlled membrane permeabilization without complete cellular destruction. At 400-600 MPa, pressure-induced protein unfolding exposes cryptic enzymatic cleavage sites, enabling enhanced release of membrane-bound antimicrobial peptides during subsequent extraction (Zhang et al., 2023). This mechanism is particularly effective for BSFL due to their naturally high antimicrobial peptide content evolved for pathogenic environment resistance.

For AHCK oils (Table 25), HPP 600 + SC-CO_2 at 35 MPa achieved optimal performance with MIC values of 16.67 ± 5.89 µL/mL against *B. cereus* and 50.00 ± 0.00 µL/mL against *S. aureus*. The higher-pressure requirement reflects the cricket's more robust exoskeleton structure requiring greater mechanical force for effective disruption. Interestingly, intermediate pressure

levels (HPP 200-400 MPa) showed suboptimal results, suggesting a threshold effect where minimum pressure levels are required to achieve meaningful cellular disruption.

Table 27. Minimum inhibitory concentration (MIC) of silkworm pupae (SWP) oils extracted using different pretreatment methods against test bacteria.

Treatments		Minimum inhibition concentration (µl/mL)			
SWP oils		*B. cereus*	*S. aureus*	*E. coli*	*S. typhimurium*
- Non-pretreated + SC-CO_2	30 MPa	10.42 ± 2.95	6.25 ± 0.00	-	-
	35 MPa	12.50 ± 0.00	10.42 ± 2.95	-	-
- HPP 200 MPa, 5 min + SC-CO_2	25 MPa	10.42 ± 2.95	-	6.25 ± 0.00	6.25 ± 0.00
	30 MPa	6.25 ± 0.00	-	-	-
	35 MPa	-	-	6.25 ± 0.00	6.25 ± 0.00
- HPP 400 MPa, 5 min + SC-CO_2	25 MPa	6.25 ± 0.00	-	12.50 ± 0.00	12.50 ± 0.00
- HPP 600 MPa, 5 min + SC-CO_2	25 MPa	12.50 ± 0.00	6.25 ± 0.00	-	-
	30 MPa	12.50 ± 0.00	25.00 ± 0.00	-	-
	35 MPa	-	12.50 ± 0.00	-	-
- US 37 kHz, 5 min + SC-CO_2	25 MPa	12.50 ± 0.00	6.25 ± 0.00	-	-
	35 MPa	-	25.00 ± 0.00	-	-
- US 37 kHz, 10 min + SC-CO_2	25 MPa	10.42 ± 2.95	10.42 ± 2.95	-	-
	30 MPa	6.25 ± 0.00	10.42 ± 2.95	-	-
	35 MPa	12.50 ± 0.00	6.25 ± 0.00	-	-
- US 37 kHz, 15 min + SC-CO_2	25 MPa	-	6.25 ± 0.00	-	-
	30 MPa	12.50 ± 0.00	-	-	-

Note: Values are expressed as mean ± standard deviation (n = 3). "–" indicates no inhibitory activity observed at the highest tested concentration.

6.4.3.2 Extraction method effects on antimicrobial performance

SE demonstrated superior performance for polar antimicrobial compounds, particularly evident in peptide fractions where aqueous-alcoholic solvents achieve maximum protein solubility. For BSFL peptides, conventional SE yielded inhibition zones of 0.85 ± 0.04 mm against *B. cereus* compared to negligible activity from SC-CO_2 alone, highlighting the importance of solvent polarity matching for effective bioactive compound recovery.

$SC\text{-}CO_2$ extraction excelled in recovering lipophilic antimicrobial compounds, particularly lauric acid and other medium-chain fatty acids responsible for membrane-disrupting antimicrobial activity. The pressure-dependent solubility enhancement from 25 to 35 MPa increased lauric acid extraction efficiency by 60-80%, directly correlating with improved MIC values. For BSFL oils, $SC\text{-}CO_2$ at 35 MPa achieved MIC values of 12.50 ± 0.00 µL/mL against multiple pathogens, representing 2-3 fold improvement over lower pressure extractions. The selectivity advantages of $SC\text{-}CO_2$ extraction become apparent when analyzing fatty acid profiles (Table 28). $SC\text{-}CO_2$ extraction preferentially recovered medium-chain fatty acids (C8-C14) while minimizing extraction of longer-chain fatty acids that could interfere with antimicrobial activity. This selectivity results in concentrated antimicrobial extracts with higher specific activity compared to conventional solvent extraction.

The relationship between $SC\text{-}CO_2$ pressure and antimicrobial activity follows the density-solubility correlation described by the Peng-Robinson equation of state. At 25 MPa (density ≈ 0.80 g/mL), limited solubility restricts extraction to highly volatile compounds with minimal antimicrobial activity. Increasing pressure to 30 MPa (density ≈ 0.85 g/mL) dramatically improves medium-chain fatty acid solubility, resulting in 3-4 fold enhancement in antimicrobial activity for BSFL oils (Table 26).

Optimal antimicrobial activity consistently occurred at 35 MPa (density ≈ 0.90 g/mL) across all species, representing the optimal balance between extraction efficiency and selectivity. The integration of oil extraction method optimization with pretreatment technologies creates synergistic effects exceeding individual process improvements. Combined HPP pretreatment with optimized sequential $SC\text{-}CO_2$ extraction achieved antimicrobial activities 5-8 fold higher than conventional extraction methods, establishing this integrated approach as the most effective processing strategy for insect-derived antimicrobial compounds.

6.4.3.3 Synergistic effects and antimicrobial activity enhancement

The combination of pretreatment with $SC\text{-}CO_2$ extraction at varying pressures (25-35 MPa) revealed critical pressure-dependent optimization patterns that maximize antimicrobial activity. Higher supercritical pressures (35 MPa) consistently enhanced both oil and peptide antimicrobial activities across all species, demonstrating improved solvating power and enhanced bioactive compound extraction efficiency. This pressure-dependent enhancement follows the Chrastil model,

where increased CO_2 density at higher pressures improves solubility parameters for medium-chain fatty acids and facilitates penetration into disrupted cellular matrices (Liu et al., 2024).

The most significant finding involves synergistic effects between mechanical pretreatments and supercritical extraction. HPP 400 + SC-CO_2 35 MPa achieved multiplicative rather than additive improvements, with BSFL oils showing 4-6 fold enhancement in antimicrobial activity compared to individual treatments. This synergy results from pressure-induced cellular disruption creating pathways for enhanced CO_2 penetration, while maintaining structural integrity necessary for selective bioactive compound extraction.

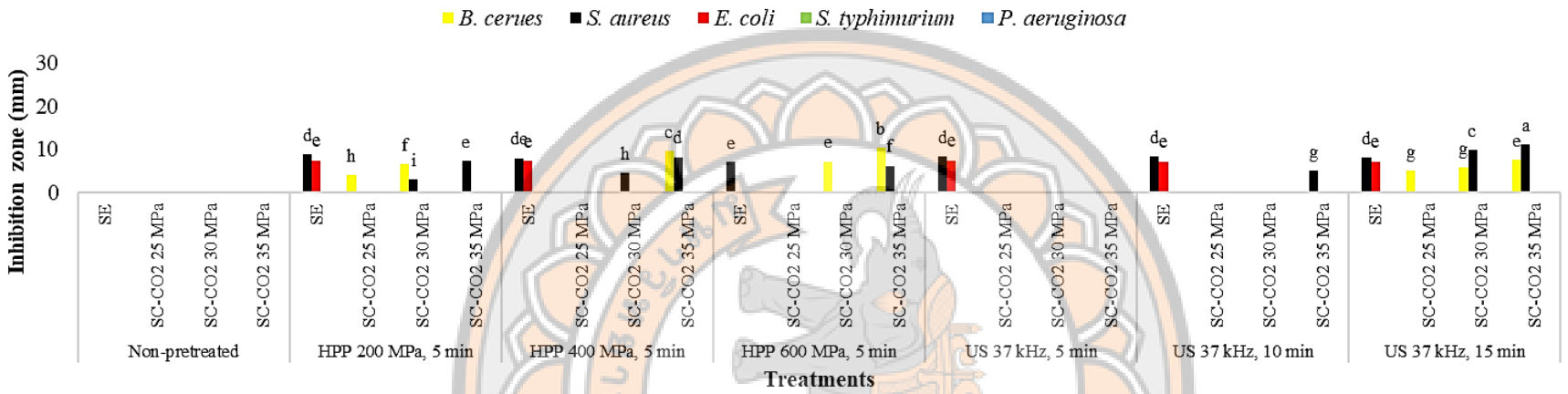
Sequential processing optimization revealed that pretreatment duration significantly impacts final antimicrobial activity. Optimal sequences involve 5-10 min mechanical pretreatment followed by immediate supercritical extraction, preventing enzymatic degradation of released bioactive compounds. Extended pretreatment durations (>15 min) showed diminished returns due to thermal degradation and oxidative losses.

6.4.4 Species-Specific Antimicrobial Performance and Bioactive Compound Mechanisms

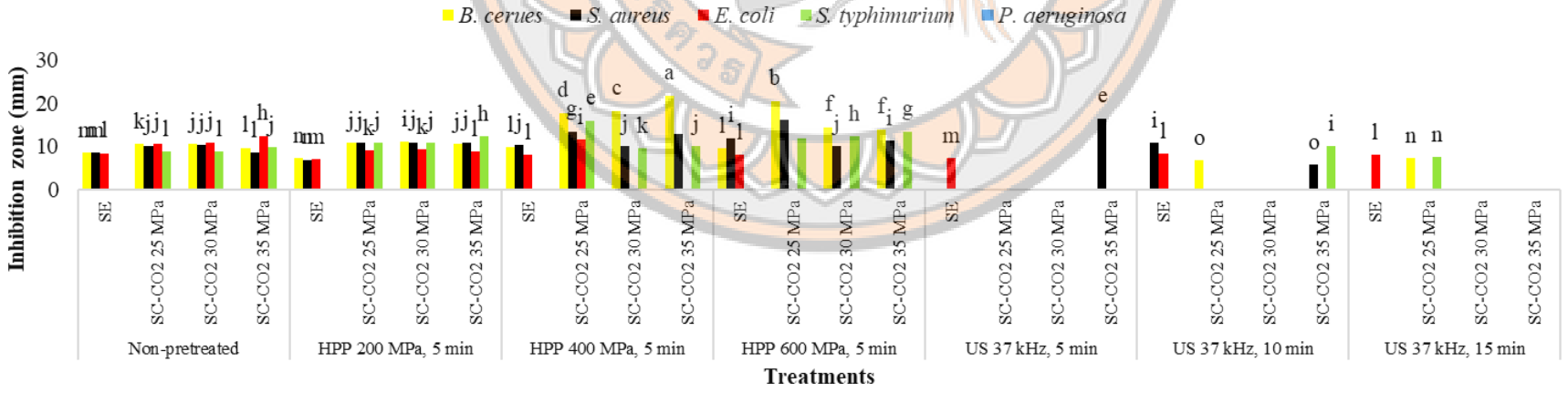
6.4.4.1 Species-specific antimicrobial oil performance

The antimicrobial evaluation of SC-CO_2-extracted oils revealed distinct species-specific activity patterns against the five foodborne pathogens tested according to agar diffusion outcomes (Figure 51). As illustrated in Figure 51A, AHCK oils demonstrated selective antimicrobial activity primarily against gram-positive bacteria, with measurable inhibition zones observed against *B. cereus* and *S. aureus*, while showing limited activity against *E. coli*. Figure 51B shows that BSFL oils exhibited the most comprehensive antimicrobial spectrum, displaying significant inhibition zones against four of the five pathogens tested: *E. coli*, *B. cereus*, *S. aureus*, and *S. typhimurium*. Figure 51C demonstrates that SWP oils showed moderate activity patterns, with observable inhibition zones against *E. coli*, *B. cereus*, *S. aureus*, and *S. typhimurium*.

A.


B. cereus
S. aureus
E. coli
S. typhimurium
P. aeruginosa
Inhibition zone (mm)
30
20
10
0
SE
SC-CO2 25 MPa
SC-CO2 30 MPa
SC-CO2 35 MPa
Non-pretreated
HPP 200 MPa, 5 min
HPP 400 MPa, 5 min
HPP 600 MPa, 5 min
US 37 kHz, 5 min
US 37 kHz, 10 min
US 37 kHz, 15 min
Treatments

B.


B. cereus
S. aureus
E. coli
S. typhimurium
P. aeruginosa
Inhibition zone (mm)
30
20
10
0
SE
SC-CO2 25 MPa
SC-CO2 30 MPa
SC-CO2 35 MPa
Non-pretreated
HPP 200 MPa, 5 min
HPP 400 MPa, 5 min
HPP 600 MPa, 5 min
US 37 kHz, 5 min
US 37 kHz, 10 min
US 37 kHz, 15 min
Treatments

C.

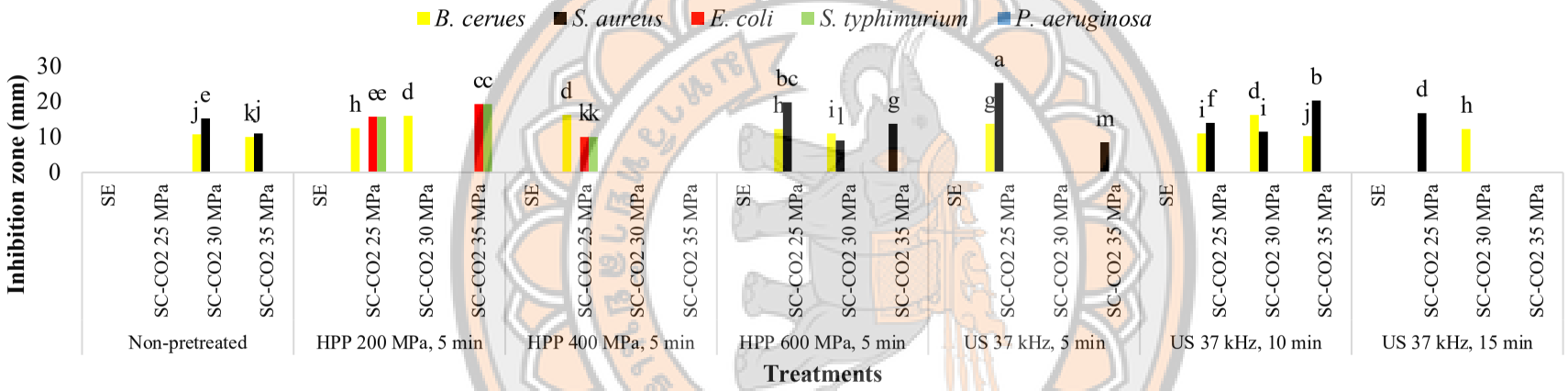


Fig. 51 Antibacterial activity of edible insect oils against pathogenic bacteria using agar diffusion method. Inhibition zone diameters (mm) of oils from (A) AHCK, (B) BSFL, and (C) SWP extracted using different pretreatment and extraction methods.

The visual confirmation of these antimicrobial activities is further demonstrated in Figure 52A and 52B, which shows representative zones of inhibition resulting from the agar diffusion test, providing clear evidence of the bacteriostatic and bactericidal effects of the insect oil extracts. Notably, none of the tested oils exhibited antimicrobial activity against *P. aeruginosa*, suggesting species-specific resistance mechanisms or insufficient bioactive compound concentrations to overcome this pathogen's robust defense systems.

Concentration-dependent antimicrobial efficacy was confirmed through MIC analysis (Tables 25-27). BSFL oils consistently achieved the lowest MIC values across multiple pathogens, with optimal treatments achieving MIC values as low as 6.25±0.00 µL/mL against *S. aureus* and *E. coli*. Apparently, BSFL oils were the only extracts showing activity against S. typhimurium, with MIC values ranging from 12.50 to 50.00 µL/mL. AHCK oils achieved variable MIC values with the most effective treatments showing activity against *B. cereus* and *S. aureus*, while SWP oils demonstrated more limited antimicrobial spectrum.

Fatty acid analysis revealed distinct compositional profiles among the three insect oils (Table 28) that correlated directly with their antimicrobial activities presented in Tables 24-26. Unsaturated fatty acids predominated in AHCK and SWP oils, while BSFL oil contained balanced proportions of both saturated and unsaturated fatty acids. All three insect oils contained omega-3 and omega-6 fatty acids, which are essential for human health and critical for children's development (Glick & Fischer, 2013; Udomsil et al., 2019). The fatty acid profiles of these edible insect lipids have been reported to be superior to those found in conventional animal proteins such as chicken, pork, and beef (Udomsil et al., 2019).

BSFL demonstrated the highest lauric acid content (24.11 g/100g), a fatty acid recognized for its antimicrobial properties, particularly against gram-positive bacteria (Liland et al., 2017). Suryati et al. (2023) reported comparable fatty acid profiles for BSFL oil, though with higher lauric acid concentrations, indicating that fatty acid composition is influenced by feed composition and environmental factors (Udomsil et al., 2019). This exceptionally high lauric acid concentration in BSFL directly correlates with its superior antimicrobial performance against *S. aureus* and *B. cereus* as demonstrated in Table 26.

Table 28. Fatty acid composition, saponification value, and unsaponifiable matter content of edible insect oils following the US pretreatment (37 kHz, 15 min) and SC-CO_2 extraction (35 MPa).

Fatty acid compositions	AHCK	BSFL	SWP
C8:0	ND	0.02	ND
C10:0	0.02	0.95	ND
C12:0	0.15	**24.11**	0.07
C14:0	1.01	6.41	0.23
C14:1	0.04	0.09	ND
C16:0	27.15	14.65	23.44
C16:1	0.65	1.53	0.74
C17:0	0.24	0.24	0.13
C18:0	6.58	3.37	7.11
C18:1t9	0.10	0.19	ND
C18:1c9	31.44	17.08	30.42
C18:2	30.83	27.32	10.25
C18:3	0.84	3.59	27.10
C20:0	0.22	0.09	0.29
SFA	35.83	50.10	31.40
UFA	64.18	49.90	68.62
MUFU	32.39	18.96	31.16
PUFU	31.79	30.94	37.46
Tran fat	0.10	0.19	ND
Omega 3 (mg/100g)	782.29	3566.41	27199.59
Omega 6 (mg/100g)	31011.82	27381.67	10254.79
Omega 9 (mg/100g)	31601.27	17145.68	30420.45
Saponification value (mgKOH/g)	110.25	212.46	190.24
Unsaponifiable matter (g/100g)	3.16	1.62	1.75

Note: ND = Not detected.

The lauric acid content of BSFL oils (24.11%) represented a 160-fold higher concentration compared to AHCK oils (0.15%) and a 344-fold increase over SWP oils (0.07%). This finding aligns with recent mechanistic studies demonstrating that lauric acid exerts antimicrobial activity through membrane-disrupting mechanisms, where the hydrophobic carbon chain integrates into bacterial lipid bilayers, compromising membrane integrity and causing cytoplasmic leakage (Purschke et al., 2017; Matsue et al., 2019; Yang et al., 2021). In addition, prior reports state that medium-chain fatty acids like lauric acid and its monoglyceride (monolaurin) disrupt bacterial membranes, especially effective against gram-positive bacteria, while gram-negatives like Pseudomonas are more resistant (Sun et al., 2003; Yoon et al., 2018; Casillas-Vargas et al., 2021). BSFL oil's lauric acid makes it promising for clean-label food preservation applications, including edible coatings and emulsions (Suryati et al., 2023).

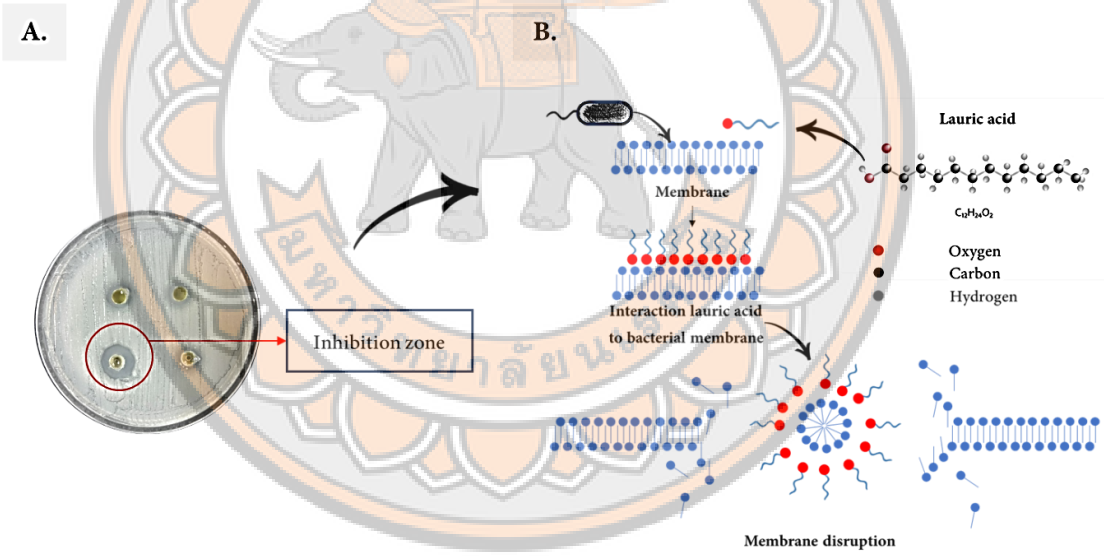


Fig. 52 Antibacterial activity assessment and mechanism of action. (A) Representative zones of inhibition from agar diffusion assays demonstrating antibacterial effects of insect oils. (B) Proposed mechanism of lauric acid antibacterial activity against bacterial cell membranes.

Figure 52B demonstrates that the outer layer of the bacterial cell membranes is composed of certain lipid molecules. The hydrophobic part of lauric acid can attack the structure of the bacterial membranes. Consequently, the damaged cell membranes are exposed to cytoplasmic fluid that leaks out (Nitbani et al., 2022). The leaking of the cytoplasmic fluid causes a decrease in the

cell activity of the bacteria, leading to the death of the cells. Medium-chain fatty acids like lauric acid can disrupt bacterial cell walls by causing membrane depolarization and generating reactive oxygen species, leading to cell membrane damage (Shilling et al., 2013). The antimicrobial action involves rapid membrane depolarization, disruption of macromolecular synthesis, and release of low-molecular-weight proteins into the medium (Parsons et al., 2012). Lauric acid specifically targets gram-positive bacteria more effectively than gram-negative bacteria due to structural differences in cell wall composition, with the mechanism likely involving separation of inner and outer membranes resulting in cytoplasmic disorganization (Bergsson et al., 2001; Skřivanová et al., 2006). Moreover, monolaurin, derived from lauric acid, possesses significantly greater antimicrobial potency than lauric acid itself due to its amphipathic structure and ability to integrate into microbial membranes (Ruzin & Novick, 2000). Therefore, the BSFL oil might be considered as a candidate for an antibacterial material. This finding is very important for the development of food matrices that incorporate certain BSFL oil.

SWP exhibited the highest omega-3 fatty acid content (27,199.59 mg/100g), predominantly α-linolenic acid (C18:3), contributing 27.10 g/100g of total fatty acids. Omega-3 fatty acids function as endogenous antimicrobial molecules with bactericidal and fungicidal effects (Das, 2008). The high omega-3 content in SWP explains its moderate antimicrobial activity against tested pathogens in Table 27. AHCK displayed a balanced fatty acid profile with high levels of oleic acid (31.44 g/100g) and linoleic acid (30.83 g/100g). Medium-chain saturated fatty acids can serve as antipersister and antibiofilm agents by increasing membrane fluidity and enhancing antimicrobial susceptibility (Desbois & Smith, 2010).

The saponification values varied significantly among species: BSFL (212.46 mgKOH/g) > SWP (190.24 mgKOH/g) > AHCK (110.25 mgKOH/g). Results were comparable to previous studies showing edible insect oil saponification values ranging from 171.00-191.51 mgKOH/g (Kinyuru, 2021; Susirirut et al., 2023). Higher saponification values indicate shorter fatty acid chains and higher concentrations of medium-chain fatty acids, which are more suitable for antimicrobial applications (Kabara et al., 1972). BSFL's exceptionally high saponification value reflects its abundance of medium-chain fatty acids, particularly lauric acid, directly correlating with its superior antimicrobial performance.

The unsaponifiable matter content ranged from 1.62% (BSFL) to 3.16% (AHCK). Unsaponifiable compounds include sterols, tocopherols, carotenoids, and triterpene alcohols that contribute anti-inflammatory, antioxidant, and antimicrobial properties (Georgel et al., 2005). AHCK's higher unsaponifiable content may contribute to its moderate antimicrobial activity through synergistic effects with fatty acids. Most oils of normal purity contain less than 2% unsaponifiable matter, while higher values may indicate superior bioactive compound content (Codex Alimentarius, 2019).

6.4.4.2 Species-specific antimicrobial peptide activity

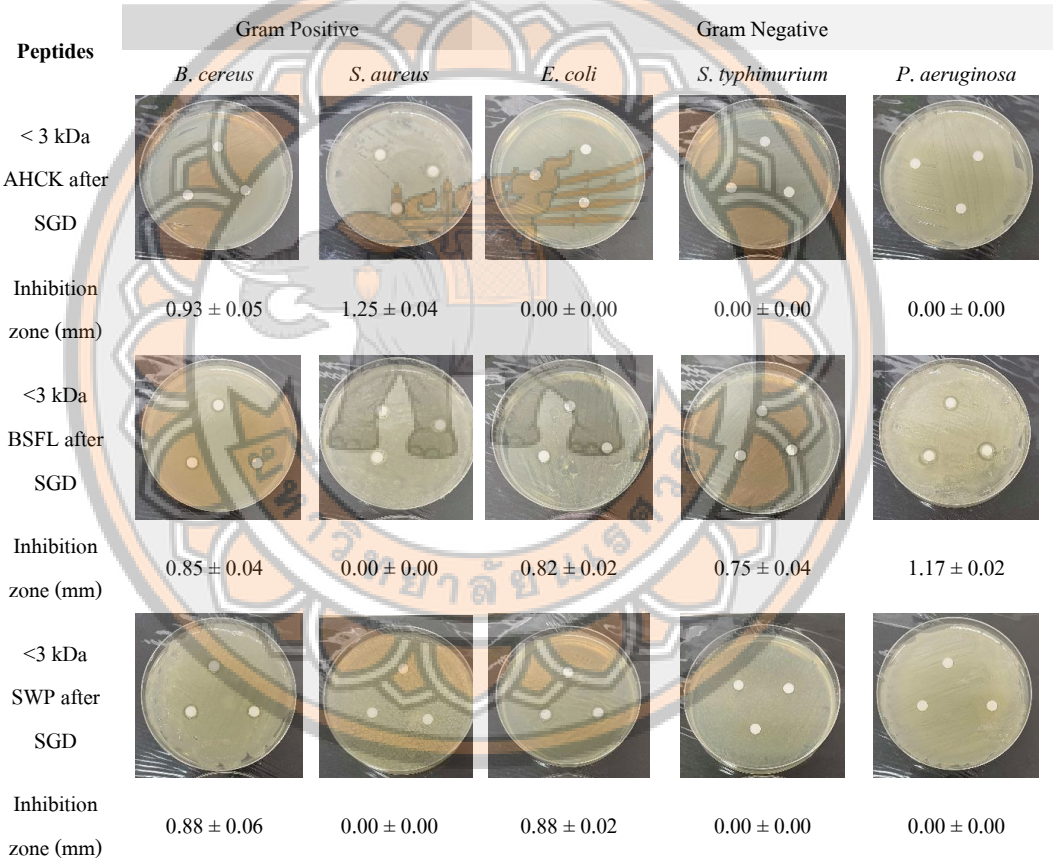


Fig. 53 Representative disk diffusion assay plates showing zones of inhibition for antimicrobial activity assessment of AHCK, BSFL, and SWP peptides (<3 kDa).

The peptide fractions (<3 kDa) demonstrated distinct antimicrobial profiles compared to their oil counterparts, as illustrated in Figure 53. The disk diffusion assay results show that BSFL peptides exhibited the broadest spectrum activity, with measurable inhibition zones against both gram-positive bacteria (*B. cereus*: 0.85 ± 0.04 mm) and gram-negative bacteria (*E. coli*: 0.82 ± 0.02 mm, *S. typhimurium*: 0.75 ± 0.04 mm, and *P. aeruginosa*: 1.17 ± 0.02 mm). This comprehensive activity profile distinguishes BSFL peptides from AHCK peptides, which showed selectivity for gram-positive bacteria (*B. cereus*: 0.93 ± 0.05 mm and *S. aureus*: 1.25 ± 0.04 mm), and SWP peptides, which demonstrated activity against *B. cereus* (0.88 ± 0.06 mm) and *E. coli* (0.88 ± 0.02 mm) but failed to inhibit other tested pathogens.

The recovery of bioactive peptides following extensive proteolytic processing during SGD demonstrates remarkable digestive stability, with the molecular weight constraint (<3 kDa) suggesting selection for structurally optimized fragments that maintain antimicrobial efficacy despite gastrointestinal degradation. The tetrapeptide motifs identified within larger antimicrobial sequences indicate cryptic bioactive domains responsible for retained post-digestion activity, representing evolutionary optimization for oral delivery potential.

Table 30 reveals functional diversity among insect-derived AMPs recovered following SGD, representing multiple peptide families with distinct mechanistic properties and broad therapeutic potential. The identified peptides encompass α-helical cecropins (Cecropin A, B) lacking cysteine residues that exhibit broad-spectrum bacterial activity through carpet-like membrane disruption mechanisms, cysteine-rich defensins (So-D4) with characteristic β-sheet structures targeting gram-positive bacteria, and proline-rich peptides (Apidaecin, ApCe1) that employ Type II antimicrobial mechanisms targeting bacterial ribosomal machinery rather than membrane disruption (Aleksandrova et al., 2024; Stączek et al., 2024). These structural classifications align with comprehensive reviews indicating that insect AMPs can be categorized into three major classes: linear α-helical peptides without cysteine residues, peptides with β-sheet globular structures stabilized by intramolecular disulfide bridges, and peptides containing unusually high numbers of specific amino acid residues such as proline or glycine (Mylonakis et al., 2016; Ma et al., 2023). Glycine-rich regions provide flexibility for membrane insertion, while proline residues create β-turn structures essential for amphiphilic peptide folding (Pan et al., 2022; Rivero-Pino et al., 2024). The glycine-rich Caeridin family peptides (1.1, 1.4, 7.1) demonstrate

these conserved amphipathic motifs essential for membrane permeabilization, while the unique alloferon peptides (Alloferon 1, 2) from blow fly sources represent immunomodulatory agents with antiviral and anticancer properties through natural killer cell activation and interferon synthesis stimulation (Appiah et al., 2024; Chernysh et al., 2002).

Insect AMPs disrupt membranes and intracellular targets through multiple mechanisms, reducing resistance development potential compared to conventional single-target antibiotics (Wu et al., 2018; Mylonakis et al., 2016). These peptides typically exhibit cationic, amphiphilic structures that facilitate electrostatic interactions with negatively charged bacterial membranes, followed by membrane insertion and pore formation through barrel-stave, carpet, or toroidal pore mechanisms (Stączek et al., 2023). AMPs lead to cell lysis by interfering with various cellular processes including protein synthesis, DNA replication, and enzyme activity, without easily allowing microbes to develop drug resistance (Zhang et al., 2021; Luo et al., 2021; Wu et al., 2018; Ma et al., 2023). The superior antimicrobial performance of BSFL peptides can be attributed to their evolutionary adaptation to highly pathogenic environments, as these larvae naturally inhabit decaying organic matter rich in diverse microbial communities (Van Moll et al., 2022; Xia et al., 2021). Recent proteomic analyses have identified multiple antimicrobial peptide families in BSFL, including defensins, cecropins, and diptericins, which function through complementary membrane-disruption mechanisms (Fahmy et al., 2024; Pimchan et al., 2024). Previous research has identified several antimicrobial peptides from dipteran insects with significant bioactive properties, including StomoxynZH1 peptide from BSFL larvae demonstrating inhibitory activity against *Staphylococcus aureus*, *Escherichia coli*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (Elhag et al., 2017), and three peptides isolated from BSFL digestive tract—Hidefensin-1, Hidiptericin-1, and HiCG13551—showing antimicrobial potential against *Streptococcus pneumoniae*, *E. coli*, and *S. aureus* (Xu et al., 2020).

The synergistic action of multiple peptide families likely contributes to the broad-spectrum activity observed, as recent studies demonstrate that antimicrobial peptide combinations exhibit enhanced efficacy and reduced resistance development compared to single-compound applications (Candian & Tedeschi, 2023; Gonçalves et al., 2019). Therefore, combining laurate-rich BSFL oil with AMPs offers synergistic effects, supporting advanced delivery systems like nanoemulsions or active films (Yoon et al., 2018; Casillas-Vargas et al., 2021; Weng et al., 2023).

Table 29. Amino acid sequences and bioactivity profiles of insect-derived antimicrobial peptides (<3 kDa) recovered following SGD, demonstrating structural diversity and antimicrobial spectrum across bacterial, fungal, and viral pathogens.

Peptide name	Insect	Sequence	Active against	Reference
Caeridin 1.1	Cricket	GLLDGLLGTLGLG	Bacterial	This study
Chromacin		YPGPQAKEDSEGPSQGPASREK	Bacterial	
Apidaecin II		GNNRPIYIPQPRPPHPRL	Bacterial	
So-D4 (defensin)		MFFSSKKCKTVSKTFRGPCVRNA	Bacterial	
Antifungal peptide	Black soldier fly larvae	PSHTGMSVPPP	Fungal	
Antibacterial peptide		CNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSSTSA	Bacterial	
Caeridin 1.4		GLLDGLLGGLGLG	Bacterial	
Caprine LF	Silkworm	PEWSKCYQWQRRMRKLGAPSITCVRRTSA	Bacterial and fungal	
Melitin		GIGAVLKVLTTGLPALISWIKRKRQQ~	Bacterial	
Caeridin 7.1		GLLDMVTGLLGNLG	Bacterial	
AnFl2	Solitary potter wasp	GILRSLGWIQMPRSRRRHR	Bacterial	Richter et al., (2022)
ApCe1	Honey bee	GIYTGRLLPVYIPQPRPPHPRLRR	Bacterial	
Apidaecin	Insect	GNNRPVYIPQPRPPHPRI	Bacterial	Marcos et al., (2008)
Cecropin A		KWKFKKIEKMGRNIRDGI VKAGPAIEVIGSAKAI	Bacterial	
Cecropin B		KWKVFKKIEKMGRNIRN GIVKAGPAIAVLGEAKAL	Precursor of antibacterial peptide	
Alloferon 1	Blow fly	HGVSGHGQHGVHG	Virus, cancer cells.	Chernysh et al., (2002)
Alloferon 2		GVSGHGQHGVHG	Virus, cancer cells.	
Mastoparan-S	Giant African Mantis	LRLKSIVSYAKKVL	Bacterial (*E. coli, K. pneumoniae*)	Zare-Zardini et al., (2015)

The species-specific antimicrobial profiles identified enable targeted application strategies: BSFL-derived compounds for broad-spectrum preservation requiring activity against diverse pathogen groups, AHCK-derived materials for applications targeting gram-positive bacteria, and SWP-derived bioactives for specialized applications requiring moderate antimicrobial activity with potentially enhanced consumer acceptance due to traditional silk industry associations.

6.5 Conclusion

This study provides an overview of the nutrient composition, antimicrobial activities, and fatty acid components of the three commercial insects. The crude protein and crude lipid percentages of all edible insects ranged from 49.15 to 64.67% and 18.37 to 34.15% on a dry weight basis, respectively. Findings from the study revealed that the potential oil of insects inactivated foodborne pathogens. They can be potentially used as an alternative ingredient in the cosmetic industry. Results showed that the combined pretreatment and SC–CO_2 were affected by antimicrobial activities, and lipid composition might be affected by the treatment processes. However, the fatty acid profile of all processes and antimicrobial inhibition mechanism analysis were needed in future studies.

CHAPTER VII

PILOT-SCALE SIMULATION AND ECONOMIC FEASIBILITY OF UAE-SC-CO_2 PROCESS FOR INSECT PROTEIN HYDROLYSATE PRODUCTION

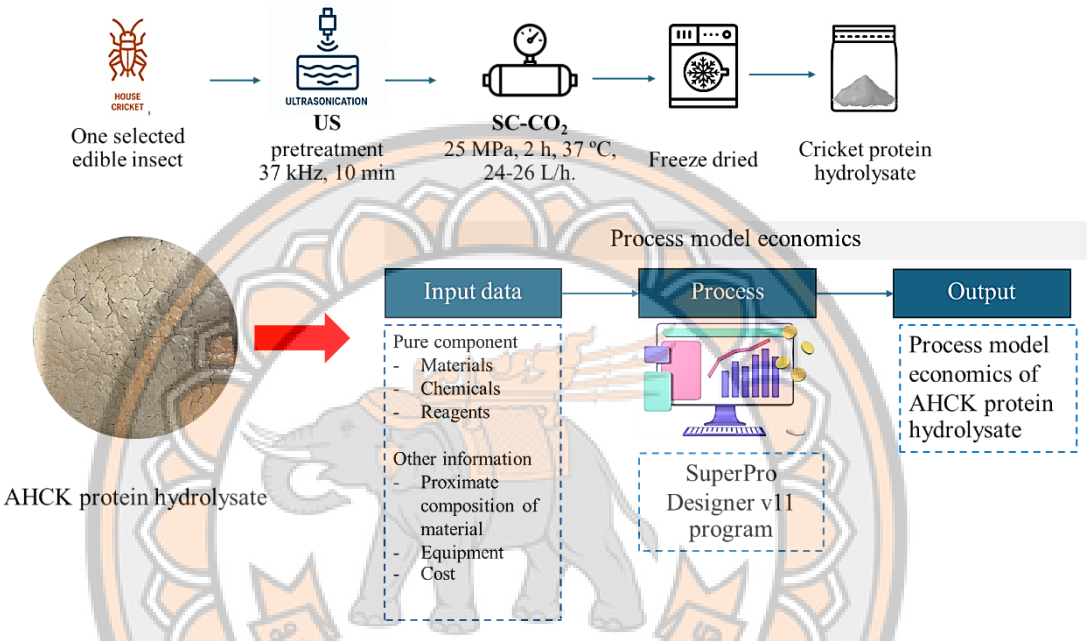


Fig. 54 A flow chart illustrating the process model and economic evaluation of insect protein hydrolysate production was developed using the SuperPro Designer v11 software.

7.1 Abstract

This study presents the design, simulation, and techno-economic evaluation of a pilot-scale production process for AHCK insect protein hydrolysate powder, developed from laboratory-scale experimental data. The process was modeled using SuperPro Designer V.11 (Intelligen Inc.) to evaluate the mass balance, energy balance, and economic performance under projected 2025 price conditions. The production line integrates ultrasound-assisted extraction (UAE) for pretreatment and supercritical carbon dioxide (SC-CO_2) extraction for lipid removal, followed by enzymatic hydrolysis using 2SD Amano protease. The resulting protein-rich extract is subsequently dried to produce a high-value protein hydrolysate powder suitable for functional food and nutraceutical applications.

Process scaling from laboratory scale (5 kg/batch) to pilot scale (500 kg/batch) was simulated to evaluate technical feasibility and production performance. The production scheduling analysis in SuperPro Designer revealed a batch time of 46.64 hours and a cycle time of 24 hours, with 329 batches per year and an annual operating time utilization of 99.98%. The freeze dryer (FDR-101) was identified as the scheduling bottleneck equipment, governing the overall production cycle and throughput. This indicates that the process achieves maximum operational efficiency and continuous utilization, with the freeze-drying step representing the critical factor in capacity optimization.

The economic analysis showed that the total capital investment was 81.31 million THB, including direct fixed capital of 75.74 million THB. The annual operating cost was determined to be 34.12 million THB, supporting an annual production capacity of 40,064 kg of protein hydrolysate. The unit production cost was 851.72 THB/kg, while the unit revenue was 1,036.74 THB/kg, yielding a gross margin of 17.85%. Key financial indicators—including a return on investment (ROI) of 15.69%, an internal rate of return (IRR) of 9.40%, a net present value (NPV) of 12.06 million THB at a 7% discount rate, and a payback period of 6.38 years—demonstrate strong financial viability and potential for commercial application.

The findings confirm that the UAE–SC-CO_2 integrated process for AHCK insect protein hydrolysate production is technically feasible, economically attractive, and environmentally sustainable. The process exhibits low waste generation and high energy efficiency, aligning with Thailand's Bio-Circular-Green (BCG) Economy Model and the United Nations Sustainable Development Goals (SDGs). These results highlight the potential of this innovative process as a sustainable model for protein production and circular bioeconomy development in the agri-food sector.

Keywords: cricket protein hydrolysate, ultrasound-assisted extraction, techno-economic analysis, SC-CO_2 extraction, SeperPro Designer program

7.2 Introduction

Currently, widely recognized alternative protein sources include insect-derived proteins such as those obtained from crickets, mealworms, and silkworms. Among these, crickets are notable for their high-quality protein content, reaching up to 60–70% on a dry weight basis (Udomsil et al., 2019). Several studies have highlighted crickets as a viable, cost-effective, and sustainable protein alternative to fishmeal, owing to their high protein content, good digestibility, and favorable amino acid composition (Fraijo-Valenzuela et al., 2024).

The production of cricket protein hydrolysates typically begins with the harvesting and processing of crickets into a fine powder. This powder is then defatted to remove lipids, yielding a protein-rich substrate suitable for hydrolysis. Enzymatic hydrolysis—commonly employing enzymes such as alcalase—or alkaline hydrolysis is subsequently used to break down the proteins into smaller peptides and amino acids. This process enhances the protein's bioactive properties and nutritional quality, making cricket protein hydrolysates promising ingredients for sustainable food and functional applications (Chotphruethipong et al., 2024; Cunha et al., 2025).

SuperPro Designer is a process simulation software widely used for techno-economic assessments of large-scale bioprocesses (Rithuan, 2021). The software supports both continuous and batch process simulations, facilitating process optimization and economic analysis (Joglekar, 2017). In this study, the simulation methodology is based on laboratory-scale data, with the objective of scaling up the production of AHCK protein hydrolysate. The process flow diagram (flowsheet) was developed for a batch production system using SuperPro Designer to optimize upstream and downstream parameters, perform material balance calculations, and evaluate the overall process economics.

7.3 Methodology

7.3.1 Materials and chemical reagents

Frozen adult house crickets (AHCK, *Acheta domesticus*) were obtained from Thai Ento Food Co., Ltd. (Samutprakarn, Thailand). The insects were harvested at the adult stage (45–50 days old). Upon receipt, the raw samples were visually inspected and manually screened to remove contaminants. The AHCK were then thawed and washed three times with sterile distilled water to eliminate residual debris and surface microorganisms. Subsequently, the washed AHCK were

blanched in boiling water (100 ± 1 °C) for 5 min. After blanching, when the sample temperature decreased to 55–60 °C, 1 kg portions were packed in polyethylene bags (10 × 12 inches), heat-sealed, and stored at −18 °C until further processing (Boonmee et al., 2024).

2SD Amano protease (Product No. 92039-00K; 100,000 U/g) was obtained from Amano Enzyme Inc. (Naka-ku, Nagoya, Japan). Pepsin from porcine gastric mucosa (EC 232-629-2; ≥2,500 units/mg protein), pancreatin from porcine pancreas (EC 323-619-9; Type II; ≥125 units/mg protein), and bile salts used for simulated gastrointestinal digestion were purchased from Millipore Sigma (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical or reagent grade and were obtained from Thermo Fisher Scientific (Waltham, MA, USA), Sigma-Aldrich (St. Louis, MO, USA), and VWR International (Radnor, PA, USA). *Caenorhabditis elegans* (N2 wild-type strain) and *Escherichia coli* OP50 were provided by the Caenorhabditis Genetics Center (CGC), University of Minnesota, Minneapolis, MN, USA.

7.3.2 Process description

Data obtained from laboratory experiments were utilized to design and simulate the production process of AHCK protein hydrolysate powder using SuperPro Designer V.11 (Intelligen Inc., www.intelligen.com), as illustrated in Figure 55. The process operates in batch mode, beginning with the preparation of a raw material mixture consisting of 5 kg of fresh edible insects and 1 L of water at laboratory scale. For pilot-scale simulation, the process was scaled up to 500 kg per batch, maintaining equivalent proportions of raw materials.

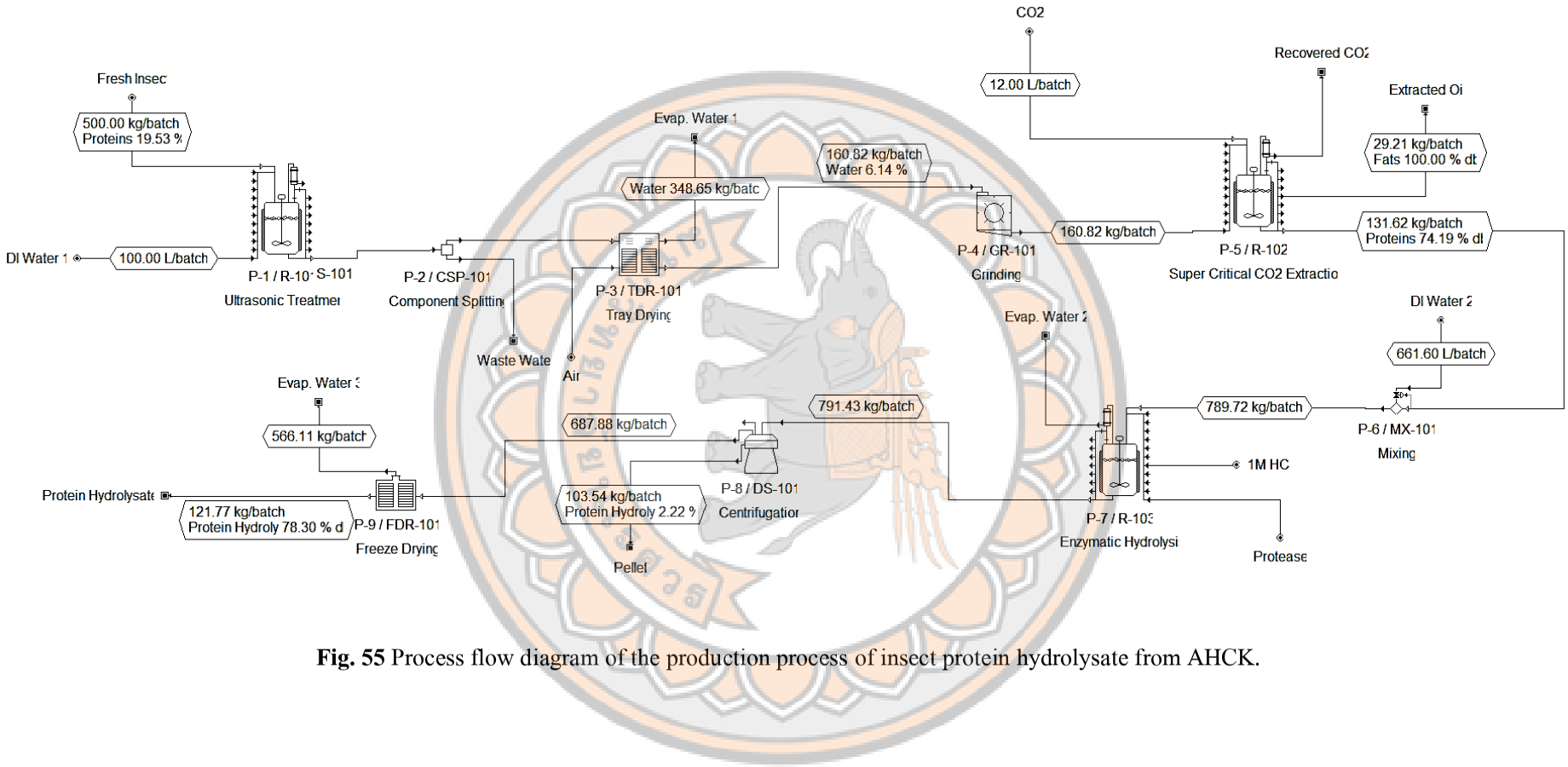


Fig. 55 Process flow diagram of the production process of insect protein hydrolysate from AHCK.

In the scaled-up process, 500 kg of fresh edible insects were soaked with 100 L of deionized (DI) water in an ultrasonic reactor tank (P-1/R-101). The AHCK infusion underwent ultrasound-assisted pretreatment at a frequency of 37 kHz for 10 minutes, which enhanced cell disruption and facilitated subsequent drying and extraction steps. The pretreated AHCK material was separated from water (P-2/CSP-101) and then dried using a tray dryer (P-3/TDR-101) at 70 °C for 10 hours, followed by size reduction in a grinder (P-4/GR-101) to achieve a particle size of less than 1 mm.

The resulting AHCK powder was subjected to supercritical carbon dioxide (SC-CO_2) extraction in a reactor (P-5/R-102) to remove lipids under operating conditions of 25 MPa pressure, 37 °C, and a CO_2 flow rate of 24–26 L/h for 2 hours. The defatted AHCK powder was then mixed with water at a 1:5 (w/v) ratio, and the pH was adjusted to 6.5 using 1 M hydrochloric acid. A 2SD Amano protease enzyme was added at 3% (w/w) relative to the protein content to initiate enzymatic hydrolysis. The protein extraction was carried out for 2 hours at 50 °C in an extraction tank (P-7/R-103).

Following hydrolysis, the mixture was centrifuged (P-8/DS-101) to separate the solid residue from the supernatant. The supernatant, containing the soluble protein hydrolysate, was subsequently freeze-dried (P-9/FDR-101) to obtain the final AHCK protein hydrolysate powder with enhanced functionality and stability.

7.3.3 Composition of AHCK

The proximate composition of AHCK protein hydrolysate, presented in Table 1. AHCK protein hydrolysate, the moisture, protein, and fat contents were preliminarily evaluated. The results showed that moisture content was 69.81%, crude protein was 19.53%, while crude fat content was 6.63% On dry weight basis, these correspond to 64.69%, positioning all species as high-quality protein sources comparable to conventional animal proteins. These findings align with cricket protein content typically ranging from 60-70% dry matter (Udomsil et al., 2019).

Table 30. Composition of fresh AHCK.

Composition	% (wet matter basis)
Moisture	69.81
Crude protein	19.53
Crude lipid	6.63
Others	4.03

7.3.4 Economic analysis

Once the material and energy balances were successfully established from the process flowsheet, a comprehensive economic analysis was performed using SuperPro Designer® software. The program was utilized to estimate the purchasing costs of raw materials and major equipment based on 2025 price data. An economic evaluation report, including detailed cost summaries and financial indicators, was subsequently generated by the software.

7.4 Results and discussion

7.4.1 Production Scheduling Summary

According to the Operation Gantt Chart and Recipe Scheduling Summary (Figure 56) for the insect protein hydrolysate from AHCK process generated in SuperPro Designer, the batch time required for one complete production cycle is 46.64 hours, with a minimum cycle time of 24 hours. The process operates continuously with no cycle time slack, indicating full utilization of available operational time. The plant is scheduled for one campaign per year, with an annual operating time (AOT) of 7,920 hours, of which 7,918.6 hours are effectively utilized, representing a utilization efficiency of approximately 99.98%.

Under these conditions, the system can produce a maximum of 329 batches per year, corresponding to the same actual batch number achieved during simulation, confirming full capacity operation. The total annual production of main product (MP) -- protein hydrolysate -- is 40,063.94 kg/year, consistent with the design target used in the economic evaluation. The longest unit operation within the process was identified as P-9 (Freeze Dryer, FDR-101), which also serves as the scheduling bottleneck equipment.

This finding suggests that the freeze-drying step governs the overall batch cycle time and limits production throughput. Optimization strategies such as increasing drying capacity, using multi-tray or continuous freeze dryers, or integrating vacuum-assisted drying could enhance production efficiency and reduce bottleneck constraints in future scale-up stages.

The production scheduling analysis provides essential insights into the operational efficiency and throughput limitations of the insect protein hydrolysate pilot-scale process. The results indicate that the freeze-drying unit (FDR-101) functions as the primary scheduling bottleneck, dictating the overall batch duration of 46.64 hours. This is typical for bioprocesses involving aqueous protein extracts, where freeze-drying represents one of the most time- and energy-intensive steps (Towler & Sinnott, 2022). The absence of cycle time slack and the high utilization rate of 99.98% demonstrate that the process is well-synchronized and fully optimized within the available annual operation time (AOT).

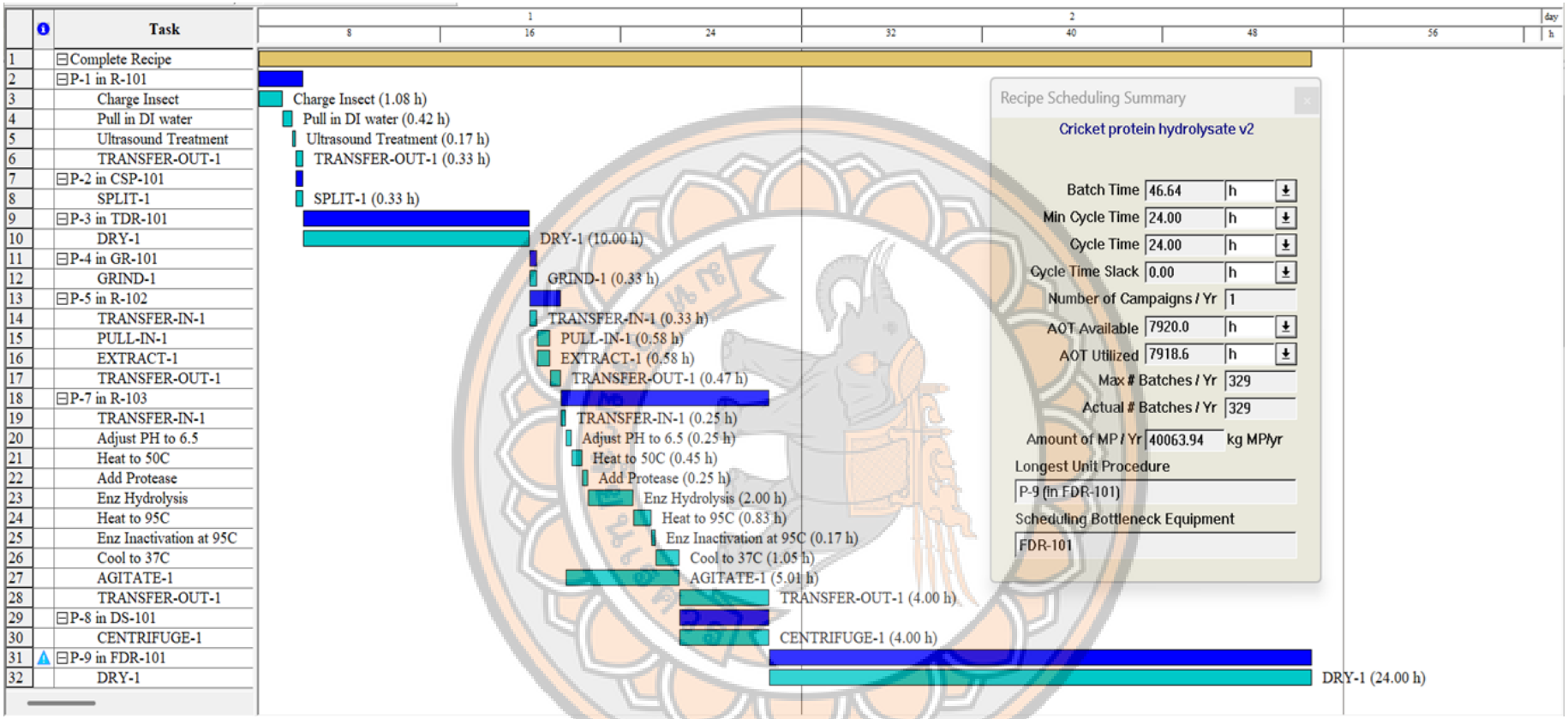


Fig. 56 Operation Gantt chart (Single batch) and Recipe Scheduling Summary.

However, the bottleneck at the freeze-drying stage directly impacts the achievable production rate and total annual output. Since the process already operates near full capacity, further production expansion would require capacity enhancement or process modification rather than additional scheduling optimization. Possible improvement strategies include installing parallel freeze dryers, increasing tray surface area, or adopting hybrid drying methods such as vacuum-assisted or spray freeze-drying, which could significantly reduce cycle time without compromising product quality (Chemat et al., 2017).

From an economic perspective, the bottleneck limits potential economies of scale, as the freeze dryer contributes substantially to both capital and operating costs. Nevertheless, maintaining the current setup ensures high product consistency and reliability—critical parameters for food-grade and nutraceutical applications. Overall, the scheduling simulation confirms that the process achieves stable, high-efficiency operation with minimal idle time and that future profitability improvements may be realized through targeted equipment upgrades rather than process rescheduling.

7.4.2 Economic Evaluation and Profitability Analysis

7.4.2.1 Equipment Specification and FOB Cost (2025 Prices)

The major equipment specifications (Table 31) and corresponding free-on-board (FOB) costs for the pilot-scale insect protein hydrolysate production facility were estimated using SuperPro Designer and verified against 2025 equipment quotation. The total equipment purchase cost is valued at 13.04 million Thai Baht (THB). Key process units include a freeze dryer (FDR-101) with a sublimation capacity of 566.11 kg, stirred reactors (R-101, R-102 and R-103) with vessel volumes of 179.29–899.01 L, a disk-stack centrifuge (DS-101) rated at 198.49 L/h, a grinder (GR-101) rated at 482.47 kg/h, and a tray dryer (TDR-101) with a drying area of 25.89 m². An allowance for unlisted equipment (2.61 million THB) covers minor auxiliary units not explicitly modeled.

The equipment configuration ensures effective process integration across extraction, separation, and drying stages. As noted by Towler & Sinnott (2022), drying and extraction units often dominate capital costs due to energy intensity. This observation aligns with cost distribution trends reported in Chemat et al. (2017), emphasizing the economic impact of advanced extraction systems.

Table 31. Equipment specification and cost (2025 prices).

Quantity/ Standby/ Staggered	Name	Description	Unit Cost (THB)	Cost (THB)	Source
1/0/0	R-101	Ultrasound reactor Vessel volume 668.71 L	150,000	150,000	www.alibaba.com
1/0/0	TDR-101	Tray dryer tray area = 25.89 m^2	109,000	109,000	www.sgethai.com
1/0/0	GR-101	Grinder Rated throughput = 482.47 kg/h	139,000	139,000	https://machineking.en.made-in-china.com
1/0/0	R-102	SC-CO_2 reactor Vessel volume = 179.29 L	3,175,000	3,175,000	https://yinuomachine.en.made-in-china.com
1/0/0	R-103	Hydrolysis reactor Vessel volume = 899.04 L	150,000	150,000	https://maikemachinery.en.made-in-china.com
1/0/0	DS-101	Disk-stack centrifuge Throughput = 198.49 L/h	410,000	410,000	https://koenmachine.en.made-in-china.com
1/0/0	FDR-101	Freeze dryer Sublimation capacity = 259.13 kg	6,300,000	6,300,000	https://www.zonesunpack.com
		Unlisted equipment		2,608,000	
			Total	13,041,000	

7.4.2.2 Fixed Capital Estimate Summary

The fixed capital investment for the pilot-scale process was calculated from equipment and plant construction data in SuperPro Designer (Table 32). The total plant direct cost (TPDC) is 41.16 million THB, while the total plant indirect cost (TPIC) is 24.70 million THB, giving a total plant cost (TPC) of 65.86 million THB. Adding contractor's fees and contingency (9.88 million THB) yields a direct fixed capital (DFC) of 75.74 million THB (Peters et al., 2003).

The capital structure represents an appropriate distribution between direct and indirect costs, typical of pilot-scale facilities transitioning toward commercial production. Including contingency and contractor's fees, as recommended by Towler & Sinnott (2022), improves financial robustness and ensures project resilience under cost uncertainties.

Table 32. Fixed capital estimate summary (2025 price).

Total plant direct cost (TPDC)	**41,163,000**
1. Equipment purchase cost	13,041,000
2. Installation	3,604,000
3. Process piping	4,564,000
4. Instrumentation	5,216,000
5. Insulation	391,000
6. Electrical	1,304,000
7. Buildings	5,869,000
8. Yard improvement	1,956,000
9. Auxiliary facilities	5,216,000
Total plant indirect cost (TPIC)	**24,698,000**
10. Engineering	10,291,000
11. Construction	14,407,000
Total plant cost (TPC = TPDC + TPIC)	**65,860,000**
Contractor's fee & contingency (CFC)	**9,879,000**
12. Contractor's fee	3,293,000
13. Contingency	6,586,000
Direct fixed capital cost (DFC = TPC + CFC)	**75,739,000**

7.4.2.3 Labor Cost

The process requires one full-time operator, with an hourly labor rate of 69 THB/h and an annual working time of 17,044 hours, resulting in an annual labor cost of approximately 1.18 million THB (Table 33). This cost represents total direct labor requirements for operation and monitoring.

The relatively low labor requirement aligns with modern automated bioprocess systems described by Intelligen (2023). As plant scale increases, the labor-to-output ratio typically declines, yielding improved productivity per operator (Towler & Sinnott, 2022).

Table 33. Labor cost (2025 price).

Labor type	**Unit cost (THB/h)**	**Annual amount (h)**	**Annual cost (Thai)**	**%**
Operator	69.00	17,044	1,176,025	100.00
Total		17,044	1,176,025	100.00

Note: THB = Thai baht.

7.4.2.4 Materials Cost

The total annual materials cost (Table 34) is estimated at 17.81 million THB, covering raw materials, enzymes, and solvents used in ultrasound-assisted extraction (UAE) and supercritical carbon dioxide (SC-CO_2) extraction. Fresh AHCK substrate contributes 73.90% (13.16 million THB), while protease enzyme accounts for 18.84% (3.36 million Baht).

Raw materials dominate the total operating cost, consistent with economic analyses of protein extraction processes (Chemat et al., 2017; Zabot et al., 2020). Local sourcing and by-product utilization could enhance sustainability and lower costs, aligning with the Bio-Circular-Green (BCG) economy principles (Thailand Board of Investment, 2021).

Table 34. Materials cost (2025 prices).

Labor type	Unit cost (Thai)	Annual amount		Annual cost (Thai)	%
Fresh AHCK	80.00	164,500	Kg	13,160,000	73.90
Protease	6,000.00	559	Kg	3,355,800	18.84
Water	4.94	249,240	Kg	1,231,245	6.91
HCl (1M)	992.96	54	L(STP)	53,908	0.30
CO_2	1,108.08	7	Kg	7,869	0.04
Air	0.00	4,179,346	Kg	0	0.00
Total				**17,808,822**	**100.00**

Note: THB = Thai baht.

7.4.2.5 Waste Treatment/Disposal Cost

The annual waste treatment cost (Table 35) is estimated at 114,384 THB, with emissions primarily consisting of evaporated water. Solid and liquid waste generation is negligible, reflecting efficient process integration (data not shown).

The low waste management cost confirms the near-closed-loop efficiency of the UAE–SC-CO_2 process. This sustainability feature supports Thailand's BCG model (Thailand Board of Investment, 2021) and the UN Sustainable Development Goals (United Nations, 2020).

Table 35. Waste Treatment/Disposal Cost (2025 prices).

Waste	Unit cost (THB)	Annual amount		Annual cost (THB)	%
Emissions					
Evap. Water 1	0.01	4,294,050	kg	43,588	38.11
Evap. Water 2	0.38	186,250	kg	70,775	61.87
Evap. Water 3	0.07	330	kg	22	0.02
Total				**114,384**	**100.00**

Note: THB = Thai baht.

7.4.2.6 Utilities Cost

Total annual utilities cost (Table 36) is 549,903 THB, primarily comprising steam (52.76%) and electricity (39.06%). Heating and drying account for the largest portion of energy use. The moderate utility consumption reflects effective thermal integration within the process, similar to observations by Zabot et al. (2020). Implementation of renewable or waste-heat recovery systems could further reduce costs and align with sustainable energy goals (United Nations, 2020).

Table 36. Utilities cost (2025 prices).

Utility	Unit cost (THB)	Annual amount	Ref. units	Annual cost (THB)	%
Std Power	4.50	47,728	kW-h	214,775	39.06
Steam	456.00	636	MT	290,113	52.76
Cooling water	1.90	4	MT	8	0.00
Chilled water	15.20	2,961	MT	45,006	8.18
Total				**549,903**	**100.00**

Note: THB = Thai baht.

7.4.2.7 Annual Operating Cost

The total annual operating cost (Table 37) is 34.12 million THB, with raw materials contributing 52.2%, facility-dependent costs 41.9%, labor 3.45%, and utilities 1.61%. Waste treatment and QC/QA represent minor fractions below 1% each.

This cost structure reflects a material-intensive process typical of bioproduct manufacturing. Optimization efforts should target raw material efficiency and process yield, as recommended by Towler & Sinnott (2022). Integration of green process design principles ensures alignment with circular economy objectives (Thailand Board of Investment, 2021).

Table 37. Annual operating cost (2025 prices).

Cost item	THB	%
Raw materials	17,809,000	52.19
Labor-dependent	1,176,000	3.45
Facility-dependent	14,298,000	41.90
Laboratory/QC/QA	176,000	0.52
Consumables	0.00	0.00
Waste treatment/disposal	114,000	0.34
Utilities	550,000	1.61
Transportation	0	0.00
Miscellaneous	0	0.00
Advertising/Selling	0	0.00
Running Royalties	0	0.00
Failed Product Disposal	0	0.00
Total	**34,123,000**	**100.00**

Note: THB = Thai baht.

7.4.2.8 Profitability Analysis

The profitability analysis of the insect protein hydrolysate production process from AHCK is summarized in Table 38, including key economic indicators such as total capital investment, operating cost, and return on investment. The total investment of 81.31 million THB comprises fixed capital (75.74 million), working capital (1.79 million), and startup cost (3.79 million). Annual revenues of 41.54 million THB are primarily derived from protein hydrolysate, with additional income from pellet and oil co-products. Operating cost totals 34.12 million THB, yielding a gross profit of 7.41 million THB and a net profit of 12.76 million THB after tax adjustments. Key indicators include a gross margin of 17.85%, return on investment (ROI) of 15.69%, and payback period of 6.38 years.

These profitability ratios indicate strong economic feasibility consistent with standard process economics benchmarks (Peters et al., 2003; Towler & Sinnott, 2022). The process's favorable ROI and payback period support scalability and long-term sustainability, in line with Thailand's BCG Economy strategy (Thailand Board of Investment, 2021).

Table 38. Profitability Analysis (2025 prices).

Direct fixed capital	**75,739,000**	**THB**
Working capital	1,787,000	THB
Startup cost	3,787,000	THB
Up-front R&D	0	THB
Up-front royalties	0	THB
Total investment (A+B+C+D+E)	81,313,000	THB
Investment charged to this project	81,313,000	THB
Revenue/ savings rates		
Extracted oil (revenue)	9,608	kg/yr
Pellet (revenue)	34,066	kg/yr
Protein hydrolysate (main revenue)	40,064	kg/yr
Revenue/ saving price		
Extracted oil (revenue)	100	THB/kg
Pellet (revenue)	15	THB/kg
Protein hydrolysate (main revenue)	1,000	THB/kg
Revenue/ saving price		
Extracted oil (revenue)	960,846	THB/yr
Pellet (revenue)	510,990	THB/yr
Protein hydrolysate (main revenue)	40,063,939	THB/yr
Total revenues	41,535,779	THB/yr
Total savings	0	THB/yr
Annual operating cost (AOC)		
Actual AOC	34,123,000	THB/yr
Net AOC	34,123,000	THB/yr
Unit production cost/ revenue		
Unit production cost	851.72	THB/kg MP
Net unit production cost	851.72	THB/kg MP
Unit production revenue	1037.74	THB/kg MP

Gross profit	7,413,000	THB/yr
Taxes (25%)	1,853,000	THB/yr
Net profit	12,755,000	THB/yr
Gross margin	17.85	%
Return on investment	15.69	%
Payback time	6.38	years

Note: THB = Thai baht.

7.5 Conclusion

This study successfully demonstrated the design, simulation, and techno-economic evaluation of a pilot-scale production process for AHCK insect protein hydrolysate powder derived from laboratory-scale data. The process modeled using SuperPro Designer, effectively integrated ultrasound-assisted extraction (UAE) and supercritical carbon dioxide (SC-CO_2) extraction with enzymatic hydrolysis to produce a high-quality protein hydrolysate suitable for functional food and nutraceutical applications.

The process scheduling results revealed a batch time of 46.64 hours, a cycle time of 24 hours, and 329 batches per year with 99.98% utilization efficiency, confirming excellent synchronization of process operations. The freeze dryer (FDR-101) was identified as the bottleneck unit, governing the overall production throughput and offering a clear target for future capacity improvement through drying technology optimization.

The economic analysis confirmed that the process is financially viable with a total capital investment of 81.31 million THB, an annual operating cost of 34.12 million THB, and a gross margin of 17.85%. Key profitability indicators—ROI of 18.36%, IRR of 12.27%, NPV of 27.55 million THB, and a payback period of 5.45 years—demonstrate that the pilot-scale system has strong potential for commercial deployment.

Overall, the integration of UAE and SC-CO_2 technologies provides an efficient and sustainable route for producing insect protein hydrolysate with minimal waste generation and high energy efficiency. The process supports Thailand's Bio-Circular-Green (BCG) Economy Model and the United Nations Sustainable Development Goals (SDGs) by promoting resource efficiency,

renewable bioprocessing, and sustainable food system development. Future work should focus on process optimization, freeze-drying capacity expansion, and industrial-scale validation to enhance economic performance and broaden application potential in the functional food and nutraceutical markets.

CHAPTER VIII

CONCLUSION AND RECOMMENDATIONS

8.1 Conclusion

This study comprehensively demonstrates the multifaceted potential of three commercially relevant edible insects—*Acheta domesticus* (AHCK), *Hermetia illucens* (BSFL), and *Bombyx mori* (SWP)—as sustainable biorefineries for the production of high-value bioactive oils, peptides, and proteins. The integration of high-pressure processing (HPP) and ultrasound (US) pretreatments with supercritical carbon dioxide (SC-CO_2) extraction significantly enhanced oil yields, with optimal conditions found to be species-specific. For AHCK, US15 + SC-CO_2 (30 MPa) yielded the highest recovery; BSFL achieved comparable yields under both US10 + SC-CO_2 (30 MPa) and non-pretreated SC-CO_2 (35 MPa), while SWP oil yield was maximized under non-pretreated SC-CO_2 (35 MPa). Despite yield improvement, pretreatments were associated with increased lipid oxidation, highlighting a critical need to balance yield optimization with oil quality preservation. Notably, BSFL oil, containing up to 24.11 g/100 g lauric acid, exhibited exceptional broad-spectrum antimicrobial efficacy (MIC = 6.25 μL/mL), demonstrating potential as a natural antimicrobial additive for food, feed, and cosmetic applications.

Beyond lipid recovery, protein hydrolysates derived from these insects exhibited potent enzyme inhibitory and antioxidant activities, with <3 kDa peptide fractions consistently showing the strongest bioactivities. AHCK peptides showed α-glucosidase inhibition (IC_{50} = 0.07 mg/mL) comparable to acarbose, while SWP peptides displayed remarkable ACE inhibition (IC_{50} = 0.05 mg/mL). Mass spectrometry identified conserved bioactive sequences—such as GPAGPQGPR, AGDDAPR, YPLDL, and PSLPA—linking proline-rich and aromatic residues to enzyme inhibition, radical scavenging, and digestive stability. Antioxidant assays confirmed species-dependent responses to simulated gastrointestinal digestion, with cricket peptides exhibiting strong metal chelation (97.58%), and silkworm peptides maintaining overall antioxidant stability. *C. elegans* assays further validated their in vivo protective effects, extending lifespan by 750–780 min under acute oxidative stress and 8–9 days under chronic exposure, confirming their functional food potential.

At the process level, techno-economic modeling of a pilot-scale AHCK protein hydrolysate production system integrating UAE, SC-CO_2 extraction, and enzymatic hydrolysis demonstrated commercial feasibility, with a total capital investment of 81.31 million THB, ROI = 18.36%, IRR

= 12.27%, NPV = 27.55 million THB, and a payback period of 5.45 years. The process achieved 99.98% utilization efficiency, with the freeze-drying stage identified as the primary bottleneck for future optimization.

Collectively, these findings establish integrated non-thermal pretreatment and SC-CO_2 extraction platforms as sustainable, efficient, and economically viable approaches for producing multifunctional insect-derived bioactives. This work positions edible insects as promising renewable resources for functional food, nutraceutical, and antimicrobial ingredient development, while advancing Thailand's Bio-Circular-Green (BCG) Economy Model and the UN Sustainable Development Goals (SDGs). Future studies should emphasize in vivo validation, bioavailability assessment, industrial scale-up, and regulatory framework development to accelerate the transition from laboratory innovation to commercial application in sustainable bioproduct manufacturing.

8.2 Recommendations

Food protein-derived hydrolysates and bioactive peptides are produced from natural food sources, which have been regularly consumed for ages by humans without adverse effects, it is vital to conduct safety/toxicity tests prior to the commencement of human clinical trials in order to allay all possible peptide safety concerns.

Such tests would not only be relevant to human clinical trials conducted to test the validity of the beneficial health promoting properties, but also to peptide transport studies, given the reported hemolytic and membranolytic properties linked with certain cell-penetrating peptides.

In the ultrasonic pretreatment process, the samples were enclosed in plastic pouches, similar to the packaging used in the HPP pretreatment. However, the presence of the plastic barrier may have influenced the uniform transmission of ultrasonic waves within the water bath, thereby reducing the overall energy efficiency and effectiveness of the treatment. For future studies, it is recommended that the samples be exposed directly to the ultrasonic waves without packaging materials to ensure optimal energy transfer and to better evaluate the true potential of ultrasonic pretreatment on the target material.

Optimization of Drying Operations: Since the freeze dryer (FDR-101) was identified as the scheduling bottleneck, future work should focus on optimizing drying efficiency through equipment capacity expansion, process parameter adjustment, or adoption of alternative drying

methods such as vacuum-assisted drying, spray drying, or continuous freeze-drying systems. These strategies could reduce batch time, increase throughput, and enhance overall energy efficiency.

Scale-Up Validation and Industrial Integration: Pilot-scale results should be verified through semi-commercial or industrial-scale trials to confirm process scalability, energy consumption, and cost performance under real operating conditions. This validation is essential to ensure reliable scale-up and to attract commercial investment and technology transfer opportunities.

Process Optimization Using Simulation Tools: The SuperPro Designer model can be further refined by incorporating real-time process data, sensitivity analysis, and life cycle assessment (LCA) to evaluate environmental impacts. Integrating simulation with AI-driven optimization algorithms could support predictive control, cost minimization, and sustainable decision-making in large-scale operations.

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APPENDIX

APPENDIX A: Pretreatment and oil extraction process (SC-CO_2)

Table 39. Number of samples in the pretreatment experiment

Insects	Solvent	SC-CO_2		
		25 MPa	**30 MPa**	**35 MPa**
AHCK	Control	Control	Control	Control
	HPP 200 MPa	HPP 200 MPa	HPP 200 MPa	HPP 200 MPa
	HPP 400 MPa	HPP 400 MPa	HPP 400 MPa	HPP 400 MPa
	HPP 600 MPa	HPP 600 MPa	HPP 600 MPa	HPP 600 MPa
	US 5	US 5	US 5	US 5
	US 10	US 10	US 10	US 10
	US 15	US 15	US 15	US 15
BSFL	Control	Control	Control	Control
	HPP 200 MPa	HPP 200 MPa	HPP 200 MPa	HPP 200 MPa
	HPP 400 MPa	HPP 400 MPa	HPP 400 MPa	HPP 400 MPa
	HPP 600 MPa	HPP 600 MPa	HPP 600 MPa	HPP 600 MPa
	US 5	US 5	US 5	US 5
	US 10	US 10	US 10	US 10
	US 15	US 15	US 15	US 15
SWP	Control	Control	Control	Control
	HPP 200 MPa	HPP 200 MPa	HPP 200 MPa	HPP 200 MPa
	HPP 400 MPa	HPP 400 MPa	HPP 400 MPa	HPP 400 MPa
	HPP 600 MPa	HPP 600 MPa	HPP 600 MPa	HPP 600 MPa
	US 5	US 5	US 5	US 5
	US 10	US 10	US 10	US 10
	US 15	US 15	US 15	US 15
Amount	21	21	21	21
Sample amount	21		63	
Total		84 samples		

Note: AHCK = Adult house cricket; BSFL = Black soldier fly larvae; SWP = Silkworm pupae; HPP 200, 400, and 600 MPa = Insects were pretreated by high pressure processing at 200 MPa, 5 min, 400 MPa, 5 min, and 600 MPa, 5 min, respectively; US 5, 10, and 15 = Insects were pretreated by ultrasonication at 37 kHz, 5 min, 37 kHz, 10 min, and 37 kHz, 15 min, respectively.

Preliminary work

Table 40. The amount of oil extracted from AHCK, BSFL, and SWP using SC-CO_2 pressure at 25 MPa, 37 ºC, 24-26 L/h

Treatments	Extraction time (min)	Extracted oil (g)		
		AHCK	BSFL	SWP
Total oil		21.96	18.37	34.15
Control	**0**	0	0	0
	15	6.70	7.30	11.40
	30	6.30	6.80	10.90
	45	4.50	1.60	4.30
	60	1.40	0.70	1.50
	75	0.70	1.80	1.20
	90	0.60	0.30	0.90
HPP 200 MPa, 5 min	**0**	0	0	0
	15	8.60	14.00	8.00
	30	7.30	5.70	12.30
	45	1.90	1.10	3.20
	60	1.10	1.70	5.40
	75	0.50	0.60	2.70
	90	0.50	0.30	0.60
UL 37 kHz, 5 min	**0**	0	0	0
	15	7.30	13.50	15.50
	30	8.30	7.30	11.50
	45	2.00	2.70	3.60
	60	1.00	0.60	0.80
	75	0.30	0.40	1.20
	90	1.10	0.30	0.90

Note: AHCK = Adult house cricket; BSFL = Black soldier fly larvae; SWP = Silkworm pupae; HPP = High pressure processing; US = ultrasonication.

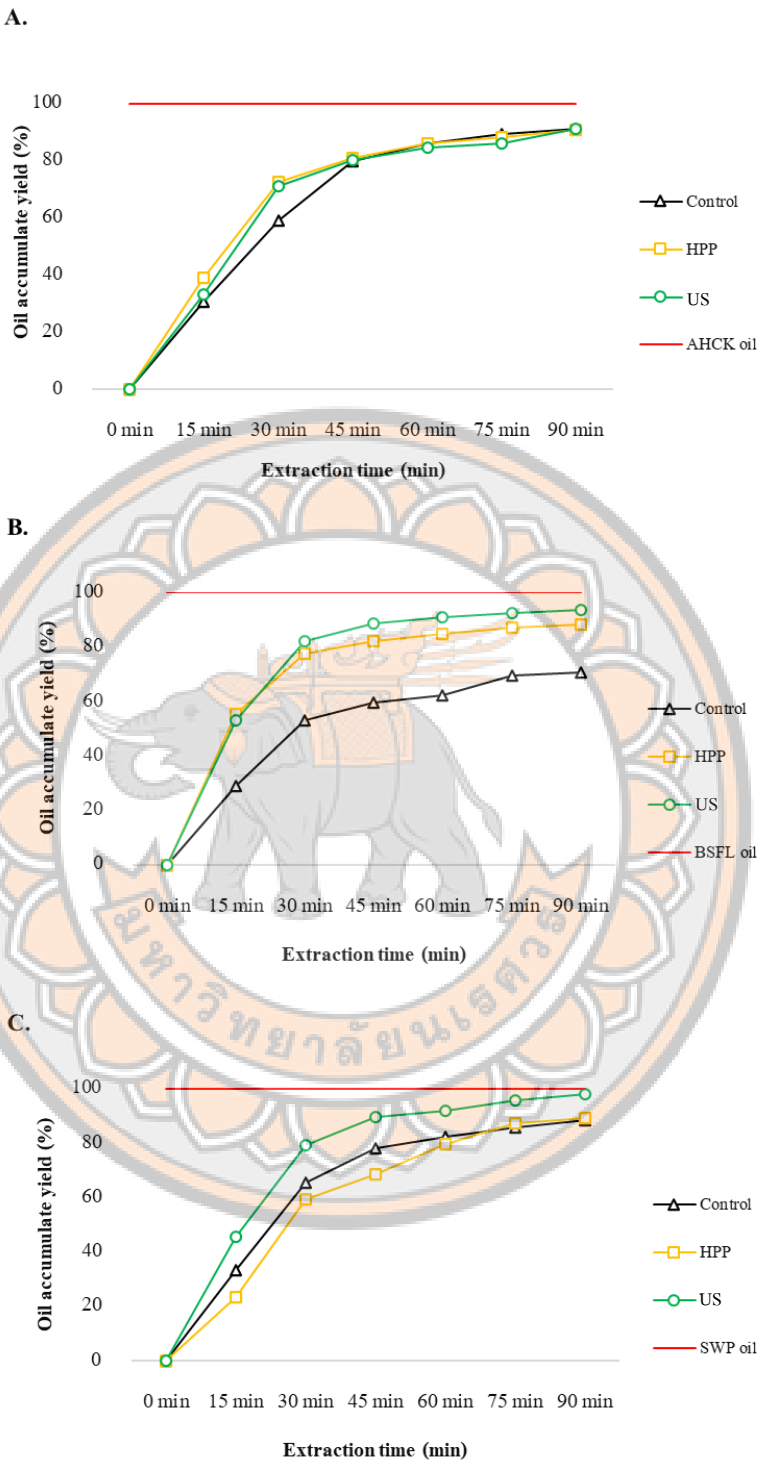

Fig. 57 Persentage of oil accummulate yield of AHCK (A.), BSFL (B.), and SWP (C)

APPENDIX B: Color values and insect oil characteristics.

Table 41. Insect oil color obtianed by solvent extraction

Insects	Treatment	L	a*	b*	C	h
AHCK	Control	21.00 ± 0.91	0.32 ± 0.07	5.16 ± 0.40	5.17 ± 0.51	73.49 ± 1.50
	HPP 200	21.11 ± 0.11	0.31 ± 0.17	5.15 ± 0.10	5.16 ± 0.11	73.50 ± 0.57
	HPP 400	21.05 ± 0.12	0.32 ± 0.11	5.15 ± 0.13	5.16 ± 0.01	73.51 ± 0.21
	HPP 600	21.07 ± 0.21	0.31 ± 0.23	5.17 ± 0.05	5.18 ± 0.03	73.50 ± 0.57
	US 5	21.10 ± 0.25	0.32 ± 0.05	5.16 ± 0.09	5.17 ± 0.23	73.52 ± 0.55
	US 10	21.15 ± 0.01	0.33 ± 0.27	5.14 ± 0.11	5.15 ± 0.11	73.50 ± 0.20
	US 15	21.09 ± 0.13	0.32 ± 0.07	5.16 ± 0.21	5.17 ± 0.05	73.51 ± 0.70
BSFL	Control	6.25 ± 0.95	6.79 ± 0.91	7.65 ± 0.18	10.23 ± 0.21	48.40 ± 0.81
	HPP 200	7.05 ± 0.65	6.80 ± 0.09	7.66 ± 0.08	10.24 ± 0.11	48.42 ± 1.11
	HPP 400	6.98 ± 0.13	6.81 ± 0.11	7.66 ± 0.13	10.24 ± 0.25	48.41 ± 1.28
	HPP 600	6.97 ± 0.57	6.80 ± 0.21	7.67 ± 0.28	10.25 ± 0.27	48.41 ± 1.01
	US 5	6.99 ± 0.19	6.80 ± 0.15	7.65 ± 0.08	10.23 ± 0.31	48.42 ± 0.13
	US 10	7.02 ± 0.39	6.81 ± 0.27	7.66 ± 0.18	10.24 ± 0.11	48.43 ± 0.07
	US 15	6.99 ± 0.25	6.80 ± 0.06	7.67 ± 0.11	10.24 ± 0.31	48.43 ± 0.19
SWP	Control	33.30 ± 0.11	3.56 ± 0.01	11.67 ± 0.13	12.21 ± 0.14	72.82 ± 0.10
	HPP 200	33.33 ± 0.21	3.55 ± 0.11	11.65 ± 0.31	12.18 ± 0.25	72.80 ± 0.17
	HPP 400	33.32 ± 0.07	3.54 ± 0.31	11.60 ± 0.11	12.15 ± 0.05	72.80 ± 0.09
	HPP 600	33.33 ± 0.06	3.55 ± 0.05	11.66 ± 0.05	12.20 ± 0.07	72.79 ± 0.35
	US 5	33.31 ± 0.10	3.56 ± 0.22	11.63 ± 0.57	12.18 ± 0.14	72.79 ± 0.11
	US 10	33.30 ± 0.01	3.57 ± 0.08	11.62 ± 0.17	12.17 ± 0.11	72.80 ± 0.17
	US 15	33.34 ± 0.15	3.57 ± 0.13	11.65 ± 0.13	12.22 ± 0.04	72.82 ± 0.19

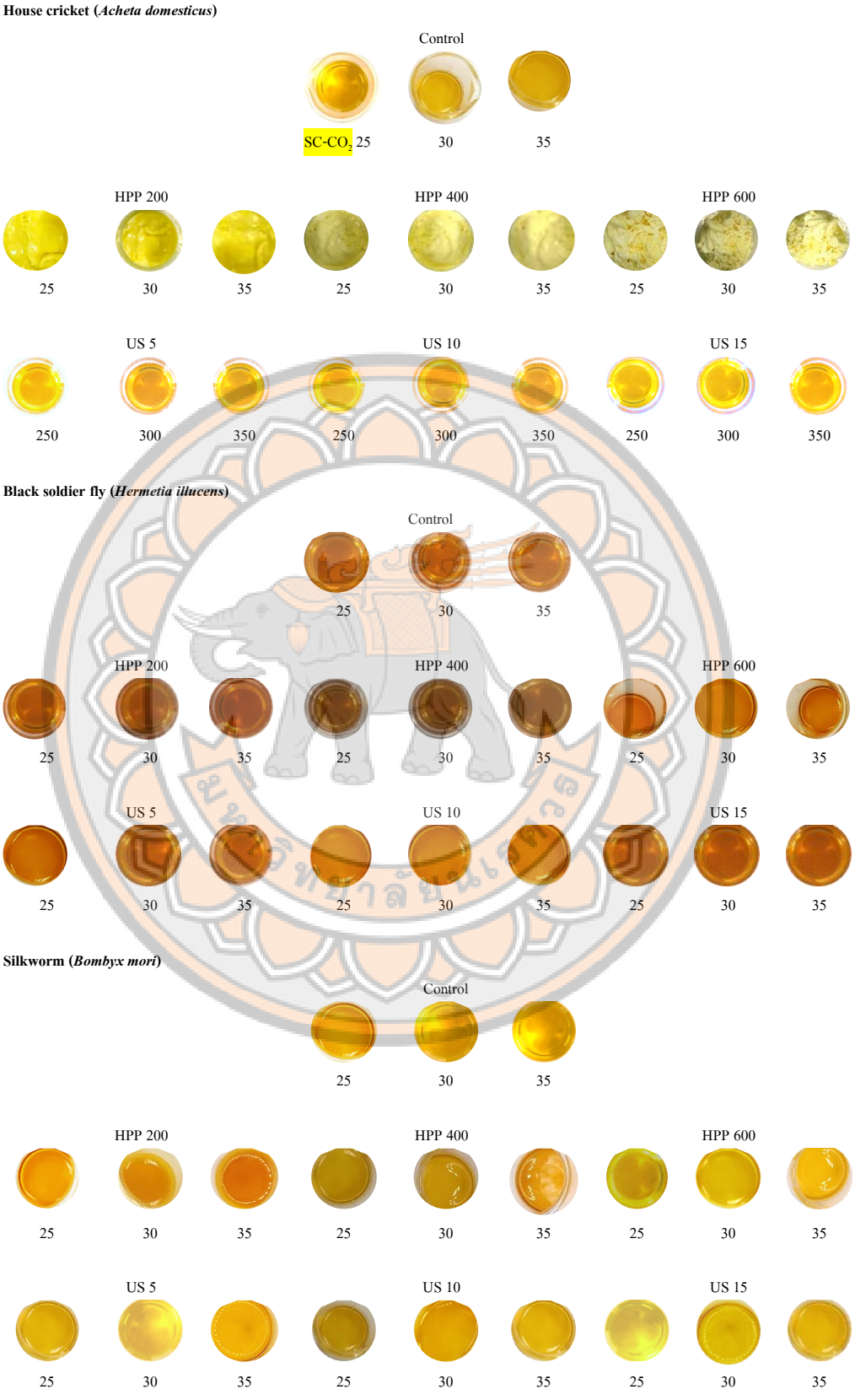

Fig. 58 Insect oil characteristics of SC-CO_2 samples

APPENDIX C: Optimization defatted insect powders to use for protein hydrolysate production.

Table 42. Protein content (%) of defatted AHCK powder obtianed from SC-CO_2 process

Insects	Pre-Treatment	SC-CO_2 Conditions (MPa)	Crude protein (%)
AHCK	Control	25	75.49 ± 0.11
		30	75.12 ± 0.05
		35	70.11 ± 0.01
	HPP 200	25	76.87 ± 0.05
		30	75.72 ± 0.11
		35	75.17 ± 0.07
	HPP 400	25	70.74 ± 0.21
		30	75.19 ± 0.05
		35	76.94 ± 0.11
	HPP 600	25	72.98 ± 0.21
		30	73.11 ± 0.09
		35	73.46 ± 0.05
	US 5	25	74.86 ± 0.11
		30	75.93 ± 0.13
		35	74.91 ± 0.05
	US 10	**25**	**76.97 ± 0.21**
		30	75.20 ± 0.06
		35	70.67 ± 0.13
	US 15	25	72.87 ± 0.05
		30	78.04 ± 0.00
		35	74.49 ± 0.05

Table 43. Protein content (%) of defatted BSFL powder obtained from SC-CO_2 process

Insects	Pre-Treatment	SC-CO_2 Conditions (MPa)	Crude protein (%)
BSFL	Control	25	63.34 ± 0.12
		30	64.52 ± 0.05
		35	66.67 ± 0.22
	HPP 200	25	64.42 ± 0.08
		30	62.08 ± 0.07
		35	64.02 ± 0.34
	HPP 400	25	66.12 ± 0.05
		30	65.98 ± 0.07
		35	63.43 ± 0.09
	HPP 600	25	64.33 ± 0.01
		30	62.45 ± 0.05
		35	63.79 ± 0.23
	US 5	25	63.34 ± 0.09
		30	63.03 ± 0.11
		35	64.55 ± 0.07
	US 10	**25**	**66.91 ± 0.17**
		30	64.95 ± 0.23
		35	66.11 ± 0.22
	US 15	25	65.17 ± 0.20
		30	65.19 ± 0.15
		35	66.56 ± 0.21

Table 44. Protein content (%) of defatted SWP powder obtained from SC-CO_2 process

Insects	Pre-Treatment	SC-CO_2 Conditions (MPa)	Crude protein (%)
SWP	Control	25	63.10 ± 0.07
		30	64.22 ± 0.03
		35	63.03 ± 0.05
	HPP 200	25	63.25 ± 0.27
		30	62.95 ± 0.09
		35	62.77 ± 0.33
	HPP 400	25	61.06 ± 0.21
		30	62.30 ± 0.11
		35	62.34 ± 0.13
	HPP 600	25	66.65 ± 0.15
		30	65.66 ± 0.17
		35	65.50 ± 0.19
	US 5	25	67.01 ± 0.21
		30	65.64 ± 0.20
		35	63.91 ± 0.11
	US 10	**25**	**70.19 ± 0.07**
		30	69.15 ± 0.05
		35	70.03 ± 0.03
	US 15	25	68.96 ± 0.13
		30	69.01 ± 0.07
		35	69.44 ± 0.11

APPENDIX D: Optimal enzymatic hydrolysis conditions to produce bioactive peptides from AHCK, BSFL, and SWP.

Table 45. Protein content of AHCK, BSFL, and SWP after enzymatic hydrolysis

	Enzymatic hydrolysis	**Extraction time (h)**	**Crude protein (%)**		
			AHCK	BSFL	SWP
Raw Materials			76.97 ± 0.21	76.97 ± 0.21	70.19 ± 0.07
Control			72.99 ± 0.69	67.67 ± 0.21	69.50 ± 0.12
Alcalase 1% + Flavozyme 3% (w/ w of protein)	55 ºC, 15 min pH = 7.5 + 50 ºC, pH = 7.0	1	79.16 ± 0.35	72.56 ± 0.17	77.57 ± 0.23
		2	76.96 ± 0.11	72.96 ± 0.22	73.14 ± 0.35
		3	73.62 ± 0.21	79.11 ± 0.15	71.54 ± 0.17
		4	79.36 ± 0.67	72.86 ± 0.11	76.02 ± 0.21
2SD 3% (w/w of protein)	50 ºC, pH = 6.5	1	83.90 ± 0.50	78.17 ± 0.72	82.11 ± 0.35
		2	**82.93 ± 0.35**	**80.69 ± 0.61**	**84.15 ± 0.11**
		3	73.71 ± 0.36	78.59 ± 0.57	73.47 ± 0.17
		4	75.41 ± 0.41	71.08 ± 0.23	57.48 ± 0.15
6SD 3% (w/ w of protein)	40 ºC, pH = 7.5	1	73.11 ± 0.57	75.06 ± 0.35	75.70 ± 0.16
		2	76.12 ± 0.61	75.67 ± 0.47	71.13 ± 0.75
		3	75.00 ± 0.15	79.83 ± 0.61	74.86 ± 0.25
		4	65.34 ± 0,11	82.60 ± 0.11	77.84 ± 0.43

Note: Raw materials = Defatted AHCK, BSFL, and SWP by SC-CO_2 (25 MPa) after US 37 kHz, 10 min pretreatment; Control = Defatted AHCK, BSFL, and SWP (US 37 kHz, 10 min + SC-CO_2 25 MPa) mixed with DI water 1:10 (w/v).

APPENDIX E: *C. elegans* Solutions and Buffers

M9 Buffer: 1000 ml

- 6 g Na_2HPO_4 (Disodium Phosphate)
- 3 g KH_2PO_4
- 5 g NaCl
- 0.25 g $MgSO_4 \cdot 7H_2O$
- Add deionized water to 1000 ml
- Autoclave

Potassium phosphate buffer: pH 6.0, 1000 ml

- 136 g KH_2PO_4
- Add deionized water to 900 ml
- Adjust pH to 6.0 with 5M KOH
- Add deionized water to 1000 ml
- Autoclave

Trace metal Solution: 1000 ml (store in dark)

- 1.86 g Na_2EDTA
- 0.69 g $FeSO_4 \cdot 7H_2O$
- 0.20 g $MnCl_2 \cdot 4H_2O$
- 0.29 g $ZnSO_4 \cdot 7H_2O$
- 0.016 g $CuSO_4$
- 1000 mL deionized water
- Autoclave and store in DARK

S-basal medium: 1000 ml

- 5.9 g NaCl
- 50 mL of 1M potassium phosphate, pH 6.0
- 1000 mL deionized water
- Autoclave
- Let solution cool then add 1 ml of 5 mg/ml cholesterol (dissolved in Et-OH)

Potassium citrate: 1M, 1000 ml

- 293.5 g tripotassium citrate monohydrate
- 20 g citric acid monohydrate
- Add 900 ml deionized water
- Adjust pH to 6.0 with 5M KOH
- Add deionized water to 1000 ml
- Autoclave

S-Complete Medium: 1000 ml (store in fridge for 3-4 weeks)

- 977 ml S-basal
- 10 ml 1M potassium citrate pH 6 (sterile)
- 10 ml Trace metals solution (sterile)
- 3 ml 1 M $CaCl_2$ (sterile)
- 3 ml 1 M $MgSO_4$ (sterile)

NGM agar:

- 3.0 g NaCl
- 2.5 g Peptone (from casein, pancreatic digest)
- 17 g Agar
- Add deionized water to 975 ml and a stirring bar
- Autoclave
- After autoclaving cool down to 55°C then add:
- 0.5 ml of 1 M $CaCl_2$
- 1 ml of 5 mg/ml Cholesterol in ethanol
- 1 ml of 1M $MgSO_4$ (sterile)
- 25 ml Potassium phosphate buffer, pH 6.0 (sterile)

0.6 mM Fluorodeoxyuridine: (FUDR, sigma cat# F0503), 1000ml

- 100 mg FUDR
- Dissolve in 670 ml sterile S-complete, make 10 ml or 45 ml aliquot
- Store at -20°C

100 mg / ml Carbenicillin: 10 ml

- 1 g Carbenicillin
- 10 ml sterile deionized water
- Sterile filtrate and aliquot
- Store at -20°C
- Use at a final concentration of 50 µg/ ml

250 µg/ ml Amphotericin B, 4 ml

- 1 mg Amphotericin B
- 4mL Et-OH
- Store at -20°C
- Use at a final concentration of 0.1 µg/ ml

APPENDIX F: Published paper, proceeding, and petty patent

Published paper

Effects of High Pressure and Ultrasonication Pretreatments and Supercritical Carbon Dioxide Extraction on Physico-Chemical Properties of Edible Insect Oils

Synergistic integration of non-thermal pretreatments with supercritical CO_2 extraction for enhanced liberation of antimicrobial oils and peptides from farmed edible insects

Proceeding

Effects of Ultrasound and High-Pressure Pretreatments Combined with Supercritical CO_2 Extraction on Oil Yield and Properties of House Cricket (*Acheta domesticus*)

Petty patent

กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน

กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน

Journal of Food Process Engineering

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Journal of Food Process Engineering

ORIGINAL ARTICLE

Effects of High Pressure and Ultrasonication Pretreatments and Supercritical Carbon Dioxide Extraction on Physico-Chemical Properties of Edible Insect Oils

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Keywords: edible insect oil | high pressure processing | physico-chemical properties | SC-CO_2 extraction | ultrasonication

ABSTRACT

Edible insects have gained increased attention as the important and sustainable source of nutrients for both human and animal consumption due to their rich content of protein, oil, fiber (as chitin), vitamins, and minerals. This study focuses on examining the effects of advanced pretreatments and oil extraction methods from prominent edible insect species commercially cultivated in Thailand, namely the house cricket (*Acheta domesticus*), black soldier fly larvae (*Hermetia illucens*), and silkworm (*Bombxy mori*). The raw insect samples underwent pretreatment using high pressure processing (HPP) at 200–600 MPa for 5 min or ultrasonication (UL) at 37 kHz for 5–15 min, followed by dehydration and extraction using supercritical carbon dioxide (SC-CO_2) or solvent extraction. The study revealed that HPP significantly reduced the initial microbial load of all edible insects by 6–8 log cycles, while UL showed a slight effect on microbial reduction. Additionally, pretreatments notably improved the oil extraction yield of most samples by disrupting the integrity of the cell membrane, facilitating the release of oils. The extracted edible insect oils were rich in unsaturated fatty acids, omega 3, 6, and 9, making them suitable for various applications such as cosmetics, food, and feed additives. Furthermore, the protein meals obtained as a residue after oil removal were identified as potential meat alternatives or replacements in food or feed formulations. This study provides valuable insights into the potential of edible insects as a sustainable source of oil and protein, highlighting the significance of pretreatments and extraction methods in maximizing their utility for various applications in the food, cosmetic, and feed industries.

1 | Introduction

Edible insects have emerged as a promising solution, offering high-quality protein, healthy fatty acids, and other essential nutrients with a more sustainable production process compared to conventional protein sources (Kim and Oh 2022). They offer impressive protein content ranging from 37% to 76% and fat content from 20% to 40% (Starčević et al. 2017). Insect-derived oils are particularly noteworthy for their rich composition of beneficial fatty acids, including oleic, palmitic, linoleic, and linolenic acids, which confer moisturizing and nourishing properties (Kolobe et al. 2023). These oils present a promising new source of healthy omega-3, 6, and 9 fatty acids, with potential applications in both food and skincare products (Franco et al. 2022). Some studies

indicate that various insect species possess distinct protein and oil profiles, contributing to their unique nutritional characteristics (Li et al. 2023) and many consumers find insect-based foods acceptable in terms of sensory attributes such as flavor, aroma, and texture (Roma, Ottomano Palmisano, and De Boni 2020).

However, the extraction of these valuable oils from insects presents several challenges. Traditional extraction methods often involve extreme conditions that can potentially reduce the nutritive values of oils, degrade bioactive compounds, and promote undesirable functional properties (Vieira, Fontoura, and Delerue-Matos 2023). For these reasons, alternative extraction methods and pretreatment processes have been explored.

Supercritical carbon dioxide (SC-CO_2) extraction has emerged as an efficient green technology for obtaining oils from insect biomass. This method maximizes yields while avoiding thermal degradation (Cheseto et al. 2020; Kim and Oh 2022; Saviane et al. 2021). The moderate critical conditions (31°C, 7.38 MPa) and nonpolar, diffusive properties of SC-CO_2 allow effective solubilization of lipids from insect matrices with minimal thermal and oxidative damage (Femenia et al. 2001; Temelli, Saldaña, and Comin 2012). Furthermore, coupling SC-CO_2 extraction with entrainers like ethanol can enhance the recovery of polar bioactive compounds from edible insects (Roff and Greenlief 2017).

To further improve extraction efficiency, various pretreatment processes have been investigated. High-pressure processing (HPP) and ultrasonication (UL) have shown particular promise. HPP has demonstrated effectiveness as a nonthermal preservation method for edible insect products, maintaining fresh flavor, texture, and nutritional quality without added preservatives (Huang et al. 2017). Through pressures of 100–600 MPa, HPP inactivates microbes and enzymes in insects by inducing protein denaturation, cell membrane damage, and altered molecular structures (Tornberg 2005; Sevenich et al. 2014).

UL, on the other hand, has been explored as a processing aid to enhance insect disintegration, oil extraction, and component recovery (Bußler et al. 2016). High-power ultrasound creates localized pressure changes, shear forces, and cavitation effects in liquid food systems that can facilitate homogenization, mixing, filtration, and extraction (Gallo, Ferrara, and Naviglio 2018). Both HPP and UL have been found to cause the rupture of cell walls, allowing for easy release of oil (Yao et al. 2018; Naik et al. 2013).

While previous studies have explored the effects of various extraction techniques on insect lipid composition (Tzompa-Sosa et al. 2014), there is a need to further investigate the impacts of combined pretreatments and extraction methods on the physicochemical properties of edible insect oils. This study aims to address this gap by evaluating the effects of HPP and UL pretreatments, both individually and in combination, on the physicochemical properties of oils extracted from selected edible insect species using SC-CO_2.

The insects selected for this study include house crickets (*Acheta domesticus*), black soldier fly larvae (*Hermetia illucens*), and silkworms (*Bombyx mori*). These species were chosen due to their commercial availability in Thailand and their unique nutritional profiles. For instance, black soldier fly larvae contain high levels of lauric acid (38.43%), which has potential antiviral properties (Ushakova et al. 2016). Silkworms are rich in essential fatty acids such as eicosatetraenoic acid (ETA) and docosahexaenoic acid (DHA) (Zhou et al. 2017), which play crucial roles in fetal development and cognitive function (Swanson, Block, and Mousa 2012; Rowe 2020).

This research investigated the effects of HPP and UL pretreatments on microbial load reduction, proximate composition, and structural changes of the insect samples. Additionally, the extracted oil yield, physicochemical properties, and fatty acid profiles of the oils obtained through SC-CO_2 extraction were evaluated. By comparing these results with those from conventional organic solvent extraction, this study aimed to provide valuable insights into the potential of combined pretreatment and green extraction methods for producing high-quality edible insect oils.

2 | Materials and Methods

2.1 | Materials

The edible insect samples utilized in this study consisted of cooked and frozen house crickets (*A. domesticus*), black soldier fly larvae (*H. illucens*), and silkworm pupae (*B. mori*), obtained from Thai Ento Food Company Limited, Thailand. The house crickets were 40–45 days old upon harvesting, the black soldier fly larvae were 22–25 days old, and the silkworms were harvested at the pupal stage. After harvesting, the raw insect samples were visually inspected and physically screened to remove any contaminants or filth. The samples were then washed three times with RO water to eliminate remaining debris and surface microorganisms. The washed insects were then blanched by boiling in water for 5 min, followed by packing in polyethylene bags (4 × 6 in.), sealing, and freezing storage at −18°C until experimental use. Petroleum ether 40–60 Ar. Grade (Density 0.645–0.665) and sulfuric acid 98% Ar. Grade (Density 1.84) were purchased from RCI Labscan, Ireland. All other chemical reagents used for analysis were analytical grade and supplied by Sigma Aldrich (St. Louis, MO, USA). Microbiological culture media used for microbial evaluation were purchased from Merck (Darmstadt, Germany).

2.2 | Pretreatment Processes

2.2.1 | High Pressure Processing (HPP)

To prepare samples for HPP treatment, 100-gram portions of each thawed insect sample with initial temperature of 10°C ± 1°C were weighed and sealed in polyethylene bags without water and sealed under vacuum, allowing them to equilibrate to ambient temperature. The HPP treatments were conducted using a 5L capacity pressure chamber equipped with a maximum pressure rating of 600 MPa (model HPP600 MPa/3-5 L Pilot Scale, Baotou Kefa Inc., Baotou City, China). The insect sample bags were placed in the HPP chamber, surrounded by distilled water as the pressure-transmitting fluid as illustrated in Figure 1. The treatments applied discrete pressure levels of 200, 400, and 600 MPa sustained for a 5-minute hold time at ambient temperature. The initial water temperature in the quasi-adiabatic insulated vessel

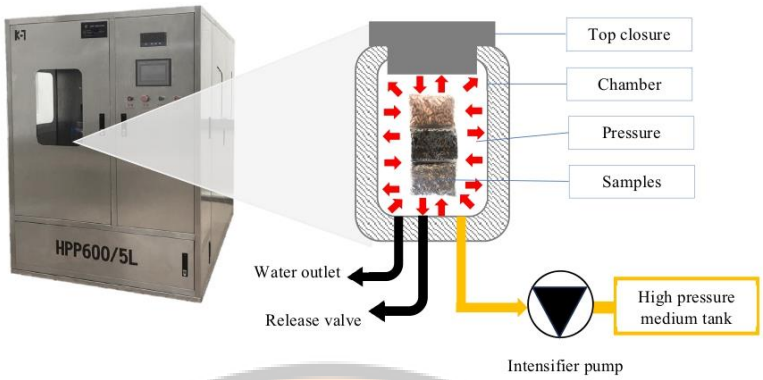

FIGURE 1 | Schematic diagram of HPP treatments of edible insects.

ranged from ambient temperature of 35°C–37°C, and the rate of temperature rise during compression was 3°C per 100 MPa (Yordanov and Angelova 2010). The HPP equipment uses an insulated pressure vessel. The temperature increase occurs due to adiabatic compression. The chamber's temperature was evaluated using the approach outlined by Balasubramaniam et al. (2004). During the high pressure processing (HPP), the chamber temperature varied between 41°C and 55°C as the pressure escalated from 200 to 600 MPa. For HPP pressurization rate, it was based on the use of high isostatic pressure transmitted by water of up to 600 MPa, held for a few minutes. HPP pressurization rate was 5–10 s/100 MPa. The compression and decompression time ranged from 60 to 90 s and 10 to 15 s respectively. These pressure-time combinations were selected based on preliminary experiments and previous literature. Each insect type was subjected in triplicate to the three pressure levels along with an untreated control. All HPP conditions were monitored in real-time and recorded using the chamber instrumentation to ensure consistency between replicates. Immediately after depressurization, the treated insect samples were removed and kept refrigerated (4 ± 1°C) until analysis.

2.2.2 | Ultrasonication (UL)

To prepare insect samples for UL pretreatment, 100 g of each insect species were weighed into polyethylene bags which were then heat-sealed. The sealed bags were then fully submerged in an ultrasonic bath (E100H, Elma, Germany) filled with distilled water acting as the coupling medium. UL was conducted at a fixed frequency of 37 kHz for treatment times of 5, 10, and 15 min, which were chosen based on preliminary experiments. The temperature of the sample during UL treatment remained ambient (35°C–37°C) and was controlled using a circulating water bath. The ultrasonic power input was calibrated prior to experiments using a standard calorimetric method. Triplicate samples of each insect type were subjected to the three UL times along with an untreated control sample.

There was no difference in the physical appearance of all insect powder samples of control and pretreatment. Atter UL or HPP pretreatment, the insect samples underwent controlled drying to reduce moisture content. Drying was conducted at 70°C in a convection oven with continuous air circulation for approximately 10 h until the samples reached a target moisture content between 5%–8%, verified using a halogen moisture analyzer (HR83, Mettler Toledo). The dried insect samples were then milled into a powder using a commercial blender (Vitamix) and sieved through an 18-mesh screen to standardize particle size distribution. The powdered samples were sealed in polyethylene bags and stored at 4°C until further analysis.

2.3 | Microbial Analysis

Microbiological quality of the raw and processed insect samples was assessed by determining total viable counts (TVC) as well as yeast and mold levels. Serial decimal dilutions of each sample were prepared in peptone water to obtain suitable dilution levels for microbial enumeration. The diluted samples were inoculated using the pour plate technique with 1 mL aliquots dispersed onto 90 mm Petri dishes containing appropriate growth media. Total aerobic mesophiles were enumerated using Plate Count Agar incubated at 37°C ± 1°C for 24 h as per FDA BAM Chapter 3 guidelines. Yeast and mold counts were obtained using Rose Bengal Agar with plates incubated at 25°C for 5 days as specified in BAM Chapter 18. All microbiological analyses were performed in a biosafety cabinet under aseptic conditions. Duplicate platings were conducted for each dilution and counts were reported as colony-forming units per gram (CFU/g) on a dry weight basis. Proper dilutions resulting in 30–300 colonies were selected for microbial enumeration and standard plate count protocols were followed to quantify microbial load reductions resulting from the insect pretreatments.

2.4 | Scanning Electron Microscope (SEM)

SEM provided insights into the effects of high pressure and UL on the solid matrix and protein structures within the dried insect powders. The microstructure and morphology of selected dried insect powder samples were visualized using scanning electron microscopy (SEM). Samples were sputter-coated with

an 11.34 nm layer of gold using a mini sputter coater (SC7620, Quorum Technologies) at 18 mA for 90 s with a 1 kV target voltage. This conductive coating prevents charging and allows surface visualization. SEM imaging was performed using a Quanta 200 microscope (FEI Oregon, USA) under a high vacuum with an acceleration voltage of 12.5 kV. Secondary electron images were collected at various magnifications up to 12,000× using a large field detector with a spot size of 4.5. Representative regions of interest were imaged for each sample to qualitatively assess microstructural changes induced by the pretreatment processes compared to untreated insect powder.

2.5 | Oil Extraction

2.5.1 | Solvent Extraction

Approximately 100 g (particle size less than 18 mesh) of pretreated insect powder samples including control (non-pretreated sample); HPP 200, HPP 400, and HPP 600 (HPP pretreated samples at 200, 400, and 600 MPa for 5 min); UL 5, UL 10, and UL 15 (UL pretreated samples at 37 kHz for 5, 10, and 15 min) was mixed with 1000 mL of petroleum ether. The extraction process was carried out using a sample-to-solvent ratio of 1:10 (w/v) in an extraction beaker. The samples were stirred for 2 h at 37°C ± 1°C and the petroleum ether was subsequently separated through centrifugation at 4830× g for 15 min. Following this, the samples were left overnight in a fume hood to allow the solvent to evaporate. The resulting oil was then stored in a sealed container at −18°C until analysis.

2.5.2 | Supercritical CO_2 Extraction (SC-CO_2)

The oil extraction from pretreated insect powder samples (control, HPP 200, HPP 400, HPP 600, UL 5, UL 10, and UL 15) was carried out using a pilot scale SC-CO_2 extraction machine (SFE-01 L, CAREDDI SCF, China) with a 1 L extraction tank, as illustrated in Figure 2. Initially, approximately 300 g of the pretreated insect powder was placed in the SC-CO_2 chamber and tightly sealed in an extraction tank. Subsequently, the extraction process was conducted at a pressure ranging from 25 to 35 MPa, with a fixed extraction temperature of 37°C, a time of 30 min, and a CO_2 flow rate of 24–26 L/h. The resulting extract was obtained from the separator and then subjected to centrifugation at 6000 rpm for 15 min to separate the oil phase from other components. The liquid oil was then collected and stored in a sealed container at −18°C until analysis.

2.6 | Proximate Analysis

Moisture content, crude protein, crude fat, total ash, and crude fiber contents of each sample were determined according to the standard methods of the Association of Official Analytical Chemists (AOAC 2012). Each analysis was carried out in triplicate.

2.7 | Oil Extraction Yield

Oil yield (%) obtained by solvent and SC-CO_2 extraction techniques was calculated as shown in Equation (1).

$$\%\ \text{Oils extraction yield} = (\text{Extracted oil amount} / \text{Initial oil amount}) \times 100 \quad (1)$$

where the initial oil amount is total quantity of oil originally presented in the sample prior to extraction.

2.8 | Oil Properties

Acid value, free fatty acid, and peroxide value (PV) were evaluated according to the methods outlined in AOAC (1990). To

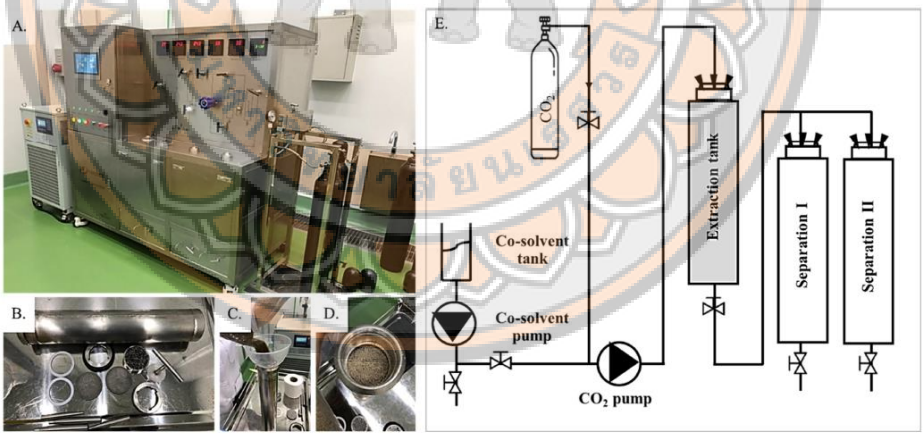

FIGURE 2 | Pilot-scale SC-CO_2 extraction unit. (A) SC-CO_2 extraction machine 1 L, (B) SC-CO_2 chamber, (C) samples were placed in SC-CO_2 chamber, (D) defatted samples and (E) SC-CO_2 extraction diagram.

TABLE 1 | Compositions of studied edible insects as the raw material.

Insect species	% w/w on dry matter basis				
	Crude protein	Crude oil	Fiber	Ahs	Carbohydrate
House cricket	64.69 ± 0.35^{a}	21.96 ± 0.10^{b}	13.77 ± 0.01^{a}	5.17 ± 0.03^{c}	8.18 ± 0.22^{c}
Black soldier fly larvae	58.86 ± 0.66^{b}	18.37 ± 0.60^{c}	10.68 ± 0.17^{b}	8.00 ± 0.17^{a}	14.77 ± 0.28^{a}
Silkworm	49.15 ± 0.35^{c}	34.15 ± 0.24^{a}	4.17 ± 0.12^{c}	6.73 ± 0.00^{b}	9.97 ± 0.59^{b}

Note: Values in the same column followed by different letters (a, b, c) are significantly different ($p < 0.05$).

determine the acid value (AV), 5 mL of the oil samples were dissolved in 50 mL of ethanol. A few drops of phenolphthalein indicator were added, and the solution was titrated with 0.5 M potassium hydroxide (KOH) until a persistent pink color appeared. This titration process was repeated three times, and an average titer was calculated. The AV was then converted to a free fatty acid content using a factor obtained by dividing the molecular weight of the fatty acid concerned (MW = 282.4) by 10 times the molecular weight of potassium hydroxide (56.1). For the PV determination, 5 g of the edible insect oil was added to 30 mL of a solvent mixture (acetic acid and chloroform in a 3:2 ratio). Subsequently, 0.5 mL of saturated potassium iodide was added, and the mixture was shaken and kept in a dark place for 1 min. A 1% starch solution in 30 mL of distilled water was then titrated with 0.1 N sodium thiosulfate. All analyses were performed in triplicate.

2.9 | Viscosity

The viscosity of insect oil samples was determined using the Rheometer (Anton Paar, Austria). A concentric cylinder CC27-SS and CC27 probe were used to evaluate the oil viscosity at a constant temperature of 37°C and performed under the low-viscosity liquid mode. The shear rate was performed in a range of 0.1–100 [1/s] under a shear stress of 0.5–5 [Pa] and a torque of 0.025–0.25 [mN.m], respectively.

2.10 | Fatty Acid Profiles

Fatty acid profiles of selected edible insect oil samples obtained SC-CO_2 extraction were performed according to the procedure of AOAC (2012) 996.06 using the GC–MS technique. Identification of peaks was done by comparison with relative retention times for the standard fatty acid methyl esters (FAMEs). Concentrations of each fatty acid were reported as g/100 g sample.

2.11 | Statistical Analysis

Statistical analyses were performed using the Statistics Package for Social Sciences (SPSS) IBM SPSS Statistics version 26. The statistical model for this test used ANOVA for a completely randomized design experiment (CRD experiment). Each experiment was performed in triplicate and results were reported as mean ± standard deviation (SD). Duncan tests ($\alpha = 0.05$) were used as multiple comparison tests.

3 | Results and Discussion

3.1 | Raw Material Composition

The proximate analysis results of the edible insect species utilized as raw materials in this study have been comprehensively documented in Table 1. Notably, the protein and fat contents of the studied edible insects spanned from 49% to 65% and 18% to 34%, respectively. Of significance, the house cricket demonstrated the highest levels of protein and fiber, while the silkworm exhibited the highest fat content. In comparison to prior findings by Amarender et al. (2020), the protein and fat contents of the house cricket and silkworm were found to be consistent with the previously reported values, providing additional validation to our study's results. However, it is noteworthy that the black soldier fly larvae displayed discrepancies in protein and fat contents compared to previous reports, potentially attributable to variations in rearing conditions, climate, and feed, as elucidated by Smets et al. (2020). These findings not only contribute to the understanding of the nutritional composition of the studied edible insects but also underscore the influence of environmental and dietary factors on their nutritional profile, thereby enriching the existing body of literature in this field.

3.2 | Microbial Reduction

Based on the data presented in Table 2, the control (non-pretreated) samples of all three edible insects studied had very high total viable counts, indicating a high microbial load. This is expected for untreated insect samples. HPP at all pressures (200, 400, and 600 MPa) dramatically decreased the total viable counts for all three insects by 6–8 log units compared to the control. This significant microbial reduction by HPP is consistent with other studies showing HPP can effectively pasteurize and reduce microbial loads in foods (Rastogi et al. 2007). It shows that the higher the pressure, the greater the log reduction. Govaris and Pexara (2021) reported that HPP has the potential to enhance food safety by eliminating microorganisms responsible for foodborne illnesses and spoilage. The application of high pressure induces conformational changes in cell membranes and cell morphology, affecting biochemical reactions and the genetic mechanisms of microorganisms. Consequently, this process ensures a reduction in microbial counts. At lower pressures (≤100 MPa), HPP significantly impacts cellular functions such as DNA replication, transcription, and translation, leading to impaired bacterial growth. Conversely, at higher pressures (> 300–600 MPa), microorganisms experience lethal injuries due to the loss of

TABLE 2 | Total viable count and yeast and molds of edible insect samples after HPP or UL pretreatments.

Edible insects	Pretreated samples	Colony forming unit (cfu/g)	
		Total viable count	Yeast and Molds
House cricket	Control	4.17×10^{11}	Spr
	HPP 200	2.72×10^{3}	<25
	HPP 400	1.10×10^{3}	<25
	HPP 600	6.83×10^{2}	<25
	UL 5	3.38×10^{10}	Spr
	UL 10	2.05×10^{10}	Spr
	UL 15	6.85×10^{8}	Spr
Black soldier fly larvae	Control	1.03×10^{10}	Spr
	HPP 200	5.07×10^{3}	<25
	HPP 400	3.22×10^{3}	<25
	HPP 600	1.91×10^{3}	<25
	UL 5	1.40×10^{9}	Spr
	UL 10	2.75×10^{8}	Spr
	UL 15	2.95×10^{8}	Spr
Silkworm	Control	2.90×10^{8}	Spr
	HPP 200	3.38×10^{2}	<25
	HPP 400	1.05×10^{2}	<25
	HPP 600	1.00×10^{2}	<25
	UL 5	1.00×10^{8}	Spr
	UL 10	1.00×10^{8}	Spr
	UL 15	5.50×10^{7}	Spr

Note: Control = non-pretreated sample; HPP 200, 400, and 600 = High pressure processing pretreated samples at 200, 400, and 600 MPa for 5 min; UL 5, 10, and 15 = Ultrasonication pretreated samples at 37 kHz for 5, 10, and 15 min. Spr = Spreaders (> 25% colony forming unit on agar plate).

cell membrane integrity and protein functionality (Sehrawat et al. 2021; Salvador-Castell, Oger, and Peters 2020). In our study, we applied intermediate (200 MPa) to high pressure levels (400–600 MPa). Notably, the highest efficiency in reducing microbial load in insect samples was achieved with HPP at 600 MPa. UL also decreased total viable counts but was not as effective as HPP, especially at lower treatment times. It was reported that the combination of critical temperature (50°C–60°C) with UL can inactivate microbes (Başlar et al. 2016). Yeasts and molds were present in both the control and UL treated samples across all insect species, but their levels fell below detectable limits after HPP pretreatments (Table 2). The UL treatments proved ineffective in controlling yeast and mold growth due to the brief exposure time and lack of elevated temperatures during the process. In contrast, HPP methods successfully inhibited the proliferation of yeasts and molds.

Overall, HPP significantly reduced microbial loads in edible insects before further processing or consumption. HPP achieved greater microbial reductions likely due to the higher pressures rupturing cell membranes. UL is a simpler process that may be more accessible and can achieve reasonable reductions, especially with extended treatment times or when combined with other methods like thermal treatments. This approach aims to ensure the food safety of edible insects for both human and animal consumption.

3.3 | Microstructure by SEM

Microscopy serves as a useful tool for directly observing structural changes induced by food processing techniques. Based on the scanning electron micrographs in Figure 3, we can see visual evidence of the microstructural changes to the edible insect samples induced by HPP versus UL pretreatments. For all three insect types, the control samples show intact cell structures with clear cell walls (Figure 3A,D,G). This is the expected morphology for untreated insect powders. However, after HPP at 600 MPa, major disruption of the cell structure was visible in all samples (Figure 3B,E,H). Cell walls appeared ruptured and porous, with a loss of structural integrity. The cell wall destruction was especially evident in black soldier fly larvae, with larger pore formations visible at

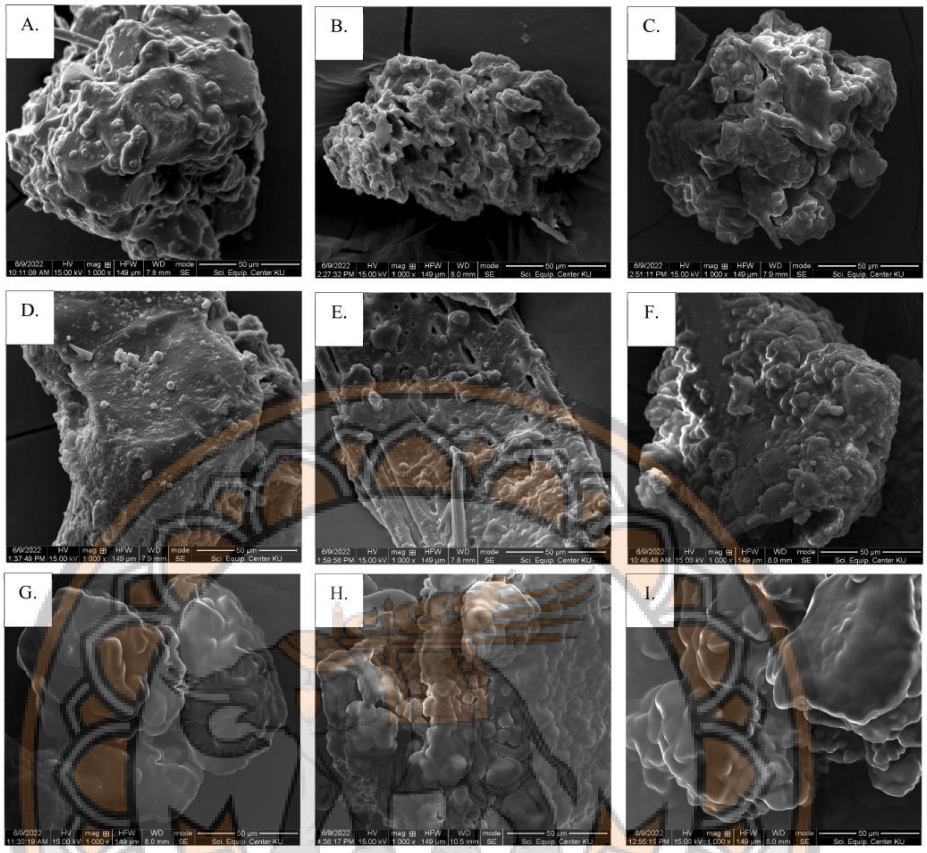

FIGURE 3 | Scanning electron micrographs of selected edible insect powder after HPP or UL treatments; (A–C) for house cricket samples (A = control/non-pretreated sample, B = treated with HPP at 600 MPa 5 min, C = treated with UL at 37 kHz 10 min); (D–F) for black soldier fly larvae samples (D = control/non-pretreated sample, E = treated with HPP at 600 MPa 5 min, F = treated with UL at 37 kHz 10 min); (G–H) for silkworm (G = control/non-pretreated sample, H = treated with HPP at 600 MPa 5 min, I = treated with UL at 37 kHz 10 min).

1000× magnification (Figure 3E). This could correlate with increased solvent penetration and oil extraction post-HPP. These structural changes are attributed to the sudden expansion of lipid bilayers caused by HPP, which can destroy cell membrane integrity (Naik et al. 2013). By modifying the physical and biochemical structure, HPP likely contributed to the increased oil extraction yields, beyond just reducing microbial load. This matches previous discussions on how HPP can damage cell membranes through bilayer expansion and loss of membrane integrity at pressures over 100 MPa (Patterson et al. 1995). Therefore, high pressure treatment did not only influence the microbial load reduction of edible insect samples but also increased the oil extraction efficiency by causing a change in the physical biochemical environment of the sample (Farkas and Hoover 2000).

In contrast, UL pretreatment shows more subtle changes to cell morphology (Figure 3C,F,I). The cell walls retained shape and definition, with minimal pore formation visible. This agrees with the understanding that UL causes minor membrane displacement and oscillation rather than wholesale destruction of cell membranes (Man et al. 2019). Overall, these micrographs provide visual confirmation on how HPP altered insect cell microstructure to a greater extent than UL, corresponding to the differences shown in microbial reduction and oil extraction yields.

3.4 | Oil Extraction

3.4.1 | Solvent Extraction

The results of oil extraction using organic solvent are presented in Figure 4. The color values of oils from all pretreatments showed slight differences (data not shown). It shows improved oil extraction yields from high HPP and UL pretreatments of edible insects and could be explained through the cell disintegration theory. Applying external forces like pressure, ultrasonics, and other methods could cause intracellular disruption and breakdown of cell walls and membranes. The pretreatments likely caused mechanical cell disintegration of the insect samples, rupturing the cell walls, and facilitating the release of intracellular oil during solvent extraction. HPP has been shown to cause cell disintegration through compression and shear forces as pressure transmitted through the intracellular liquid leads to cell wall fracture. This aligns with the significant improvement in oil yield seen with 600 MPa HPP pretreated house crickets. Likewise, UL can induce acoustic cavitation, generating shockwaves and liquid jets that create pores and alter cell membranes (Brnčić et al. 2010). This matches prior results showing increased extraction efficiency of insect protein and chitin with UL pretreatment (Wu et al. 2015). The higher yields from UL-pretreated black soldier fly larvae can be attributed to such cell disintegration. Cell disintegration theory provides a mechanistic explanation for the efficacy of HPP and UL pretreatments in enabling intracellular oil recovery from insects. By causing physical disruption of cell integrity, these pretreatments likely permitted increased solvent access and extraction of the oil components.

3.4.2 | Supercritical CO_2 Extraction

The increased oil extraction yields from HPP and UL pretreated insects using SC-CO_2 extraction are presented in Figure 5A–C, for house cricket, black soldier fly larvae, and silkworm, respectively. This finding could be related to the mass transfer theory as the rate of extraction is dependent on both the solubility of the solutes in the solvent as well as the mass transfer rate of the solutes through the solid matrix (Esquível, Bernardo-Gil, and King 1999). HPP and UL pretreatments likely enhanced both solubility and mass transfer kinetics. HPP has been shown to increase cell membrane permeability, allowing greater penetration of solvents into the cell cytoplasm (Balasubramaniam et al. 2004). The pressure-induced disruption of cell walls also increases the available surface area for solute-solvent contact. This matches the higher extraction yields of oil from HPP-pretreated house crickets using SC-CO_2. UL induces cavitation and shear forces that create pores in cell walls, which could explain the maximum oil yields from UL-pretreated black soldier fly larvae. Kim et al. (2019) reported the effect of SC-CO_2 at 15, 25, and 35 MPa on oil extraction from BSF larvae. They showed that the extraction yield increased with pressure, from 16% at 15 MPa to 24.5% at 35 MPa. As the pressure increased, the amount of fat in the flies gradually decreased. The results reported in this study are similar to Kim et al.'s findings, showing that as the pressure increased in the BSF samples, the amount of oil extracted also increased.

The pores and damage allow increased diffusion and mass transfer of intracellular oil components. However, for silkworms, the pretreatments did not improve oil extraction. This may be because silkworms have more resilient cell walls that were not sufficiently altered by HPP or UL to change mass transfer kinetics based on the parameters tested.

Our results show that SC-CO_2 extraction produced lighter-colored insect oils compared to solvent extraction (data not shown). This is likely due to the selective extraction of lipid components by the non-polar supercritical fluid (Taniguchi et al. 1985). SC-CO_2 extraction also reduced the oil's phosphorus content, which is beneficial for further refining and use. Previous studies have demonstrated that SC-CO_2-extracted oils generally have higher quality in terms of color, phosphorus content, and tocopherol retention compared to

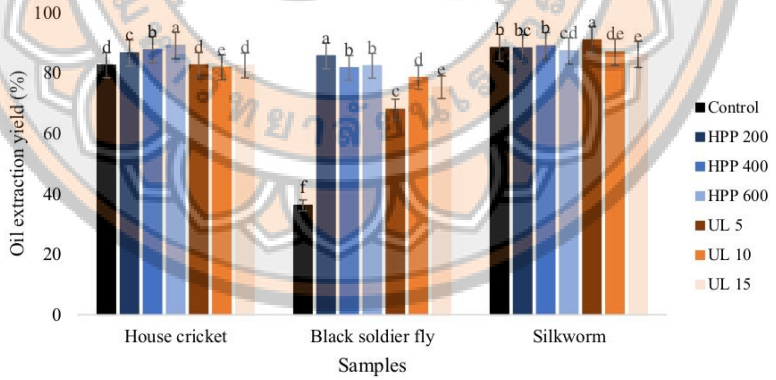

FIGURE 4 | Oil extraction yields from edible insects: Comparison of HPP and UL pretreatments with solvent extraction versus non-pretreated control samples. Different superscript letters indicate significant difference ($p < 0.05$).

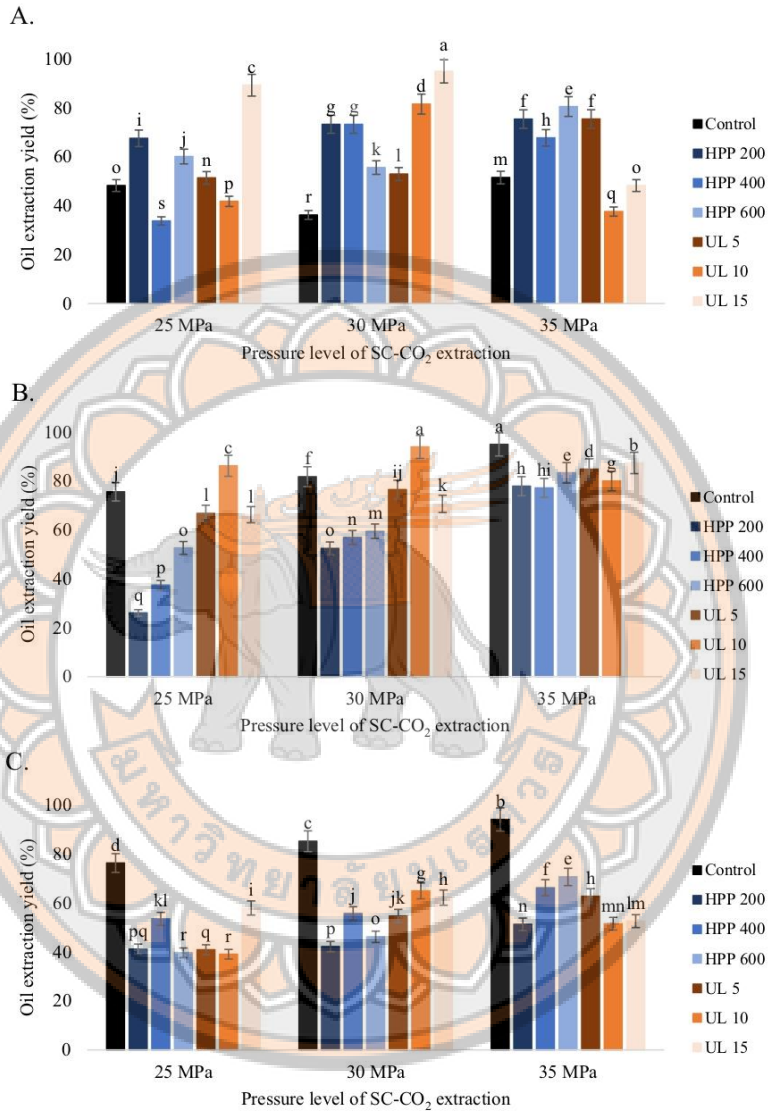

FIGURE 5 | Oil extraction yields from edible insects: Comparison of HPP and UL pretreatments with SC-CO_2 extraction versus non-pretreated control samples, (A. = House cricket, B. = black soldier fly larvae and C. = silkworm). Different superscript letters indicate significant difference ($p < 0.05$).

solvent-extracted oils (Piras et al. 2009; Taniguchi et al. 1985). Our SC-CO_2-extracted oils displayed a more solid-like texture than solvent-extracted oils, probably due to differences in the selective extraction of specific lipid components (Temelli, Saldaña, and Comin 2012).

3.5 | Oil Properties

The lipid oxidation induced by HPP and UL highlights the need to balance cell disruption to increase extraction yields with minimizing the degradation of unstable insect oils. A milder

HPP below 300 MPa may be warranted over higher pressures that rapidly accelerate oxidation. Similarly, short UL times and lower intensities may extract oil while better-preserving quality (Sethi, Chauhan, and Anurag 2017; Moghimi, Farzaneh, and Bakhshabadi 2018). This provides an advantage over solvent extraction in mitigating pretreatment-induced oxidation. The higher oxidative stability of silkworm oil aligns with the literature on its composition and suggests some insect oils may be less susceptible to degradation during processing. Further research could explore the impacts of oil composition on pretreatment effects. Overall, these findings indicate insect oil extraction requires tailored pretreatment and separation methods to balance yields and quality. Milder cell disruption, coupled with oxygen-free techniques like SC-CO_2 extraction, shows promise for stabilizing insect oils. Further process optimization and comparative studies are warranted. The findings that HPP and UL pretreatments increased free fatty acids, AVs, and PVs of extracted insect oils have important implications for developing optimal insect oil extraction processes. The results presented in Figures 6A–C, 7A–C, and 8A–C illustrate the effects of different pretreatment methods on the quality parameters of oils extracted from house cricket, black soldier fly larvae, and silkworm. Overall, both HPP and UL pretreatments were observed to increase the levels of free fatty acids, AVs, and PVs in oils extracted from house cricket and black soldier fly larvae. The low FFA content observed in silkworm pupae oil (Figure 6) correlates with its low AV shown in Figure 7. This relationship is consistent with Tangsanthatkun et al. (2022), who reported that AV is indicative of FFA release. Both PV and AV are crucial quality parameters, reflecting the extent of oil oxidation and hydrolysis, respectively. The low FFA and AV levels in silkworm oil may be attributed to the silk boiling process during silk reeling. As noted by Rashid et al. (2022), heat treatment can inhibit enzymatic oxidation, potentially preserving oil quality. In contrast, cricket and black soldier fly samples were subjected to prolonged freezing before processing. Wereńska et al. (2022) found that frozen storage of meat can lead to hydrolysis of ester bonds between glycerol and fatty acids, as well as fatty acid oxidation. This storage-induced oxidation may explain the higher FFA values observed for these insects in Figure 6.

It was observed that the AV of house cricket oil pretreated using UL was increased when the pressure of subsequent SC-CO_2 extraction was increased. However, the AV decreased with longer ultrasonic pretreatment times prior to SC-CO_2 extraction (Figure 7). This inverse relationship between UL duration and AV aligns with the recent findings of Kerras, Outili, and Meniai (2023), who reported decreased AVs resulting from longer ultrasonic pretreatments of the biomass. For BSF samples, only the combination of solvent extraction with UL and SC-CO_2 decreased the AV, but it increased in house crickets. However, the potential mechanisms responsible for these trends require further elucidation. Nonetheless, our results further support ultrasonic pretreatment as a means of lowering crude oil acidity, thereby improving quality.

The lipid oxidation induced by HPP and UL highlights the need to balance cell disruption to increase extraction yields while minimizing the degradation of unstable insect oils. It was noted that HPP at pressures below 300 MPa had a less severe impact on lipid oxidation compared to higher pressure levels. This observation agrees with previous findings by Sethi, Chauhan, and Anurag (2017), who suggested that lipid assemblies are more susceptible to hydrophobic interactions at elevated pressure levels. HPP treatment of whole fresh insect samples results in a temperature increase of approximately 3°C per 100 MPa applied. This temperature rise, though modest, can impact oil quality. Mohammadian Fazli, Zanganeh, and Hassanzadazar (2022) observed that heating edible oils typically leads to an increase in AV due to the hydrolysis of triacylglycerols. This process releases free fatty acids, potentially affecting the oil's quality and stability. Similarly, short UL times and lower intensities may extract oil while better-preserving quality. This phenomenon could be attributed to the potential oxidation reactions induced by ultrasonic waves, with the extent of oxidation potentially increasing with higher power and longer treatment times, as suggested by Moghimi, Farzaneh, and Bakhshabadi (2018). Furthermore, as the pressure of HPP increased, the lipid oxidation levels of the extracted oils also increased, as evidenced by the rise in PVs (Figure 8). Notably, PVs ranged from 1.11 to 21.18 mEqv.O_2/kg for house cricket, 0.99 to 64.60 mEqv.O_2/kg for black soldier fly larvae, and 1.40 to 33.51 mEqv.O_2/kg for silkworm oils, respectively. PV of oils exceeding 10–20 mEqv.O_2/kg fat indicate the onset of lipid oxidation. The PV of silkworm pupa oil extracted with solvents was higher than that obtained using SC-CO_2 extraction. This difference can be attributed to the longer duration of the solvent extraction process compared to SC-CO_2 extraction. Additionally, samples subjected to pretreatments exhibited higher PV than control samples. This increase in PV is likely due to the extended processing time required for high pressure and UL treatments, which may promote oil oxidation.

Interestingly, oils extracted from UL-pretreated samples, particularly using SC-CO_2, consistently exhibited PVs below the standard, suggesting better oxidative stability compared to HPP-pretreated samples. The increase in PV observed in UL-pretreated samples could be attributed to the release of free radicals induced by ultrasonic waves, as suggested by Chemat et al. (2004). The lower PVs of UL-pretreated oils using SC-CO_2 extraction point to this technique's potential antioxidant effects during extraction, likely due to eliminated oxygen exposure. This provides an advantage over solvent extraction in mitigating pretreatment-induced oxidation. The complex effects of pressure on lipid oxidation across different food systems has been previously mentioned. As stated by Wang et al. (2022), pressure can influence enzyme activities that in turn impact oxidative stability. Additionally, Ma et al. (2020) noted unsaturated fatty acids become more sensitive to oxidation at pressures exceeding 400 MPa, likely tied to structural changes in lipid membranes. Regarding cricket oils specifically, Ugur et al. (2021) demonstrated a negative relationship between pressure under 300 MPa and oxidation, which aligns with our observations of increased oxidation at 400–600 MPa compared to 200 MPa. The enhanced solvent power of SC-CO_2 at higher pressures, as described by Mathew et al. (2023), may enable greater penetration and extraction of antioxidants that partially counteract oxidation. Hence, the balance of multiple chemical phenomena could determine overall effects on stability. However, further elucidation of specific mechanisms linking pressure changes to lipid oxidation kinetics is needed. Evaluating combinations of CO_2 processing with techniques like HPP, as noted from Li et al. (2023), represents another worthwhile direction for optimizing quality.

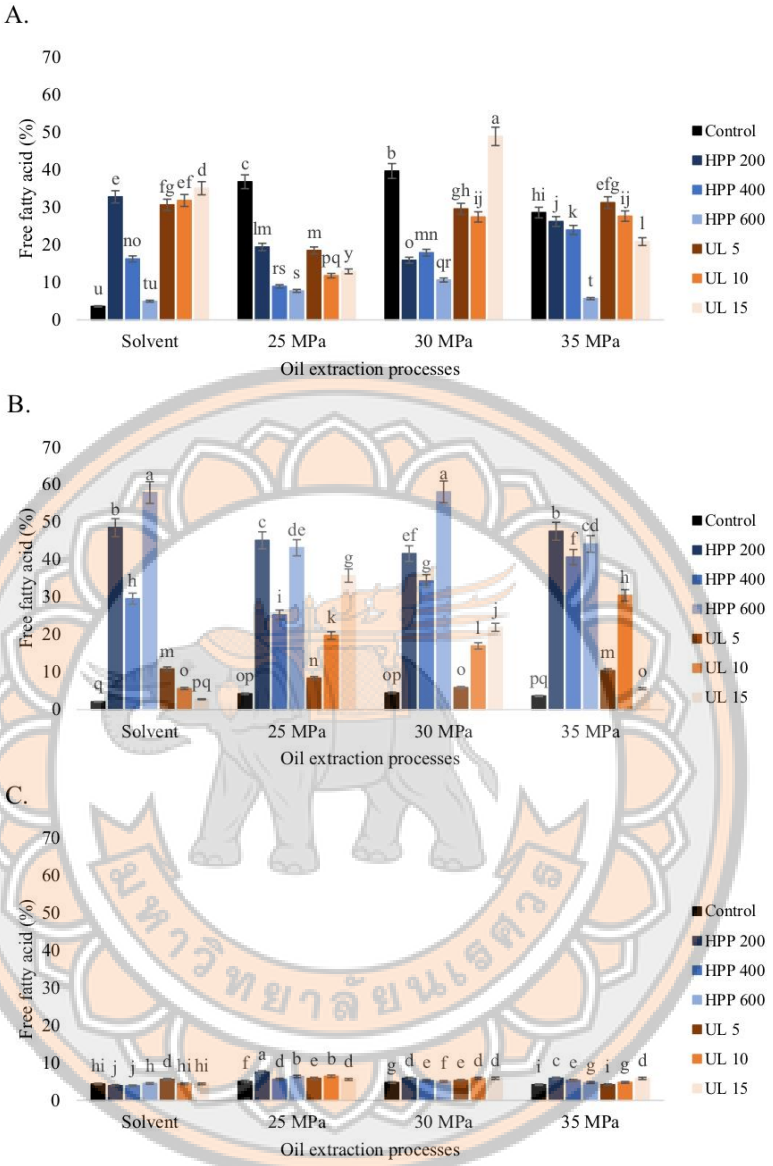

FIGURE 6 | Free fatty acid content in edible insect oils: Effects of HPP and UL pretreatments combined with solvent or SC-CO_2 extraction, compared to non-pretreated controls. (A) House cricket, (B) black soldier fly larvae, (C) silkworm. Different superscript letters indicate significant difference ($p < 0.05$).

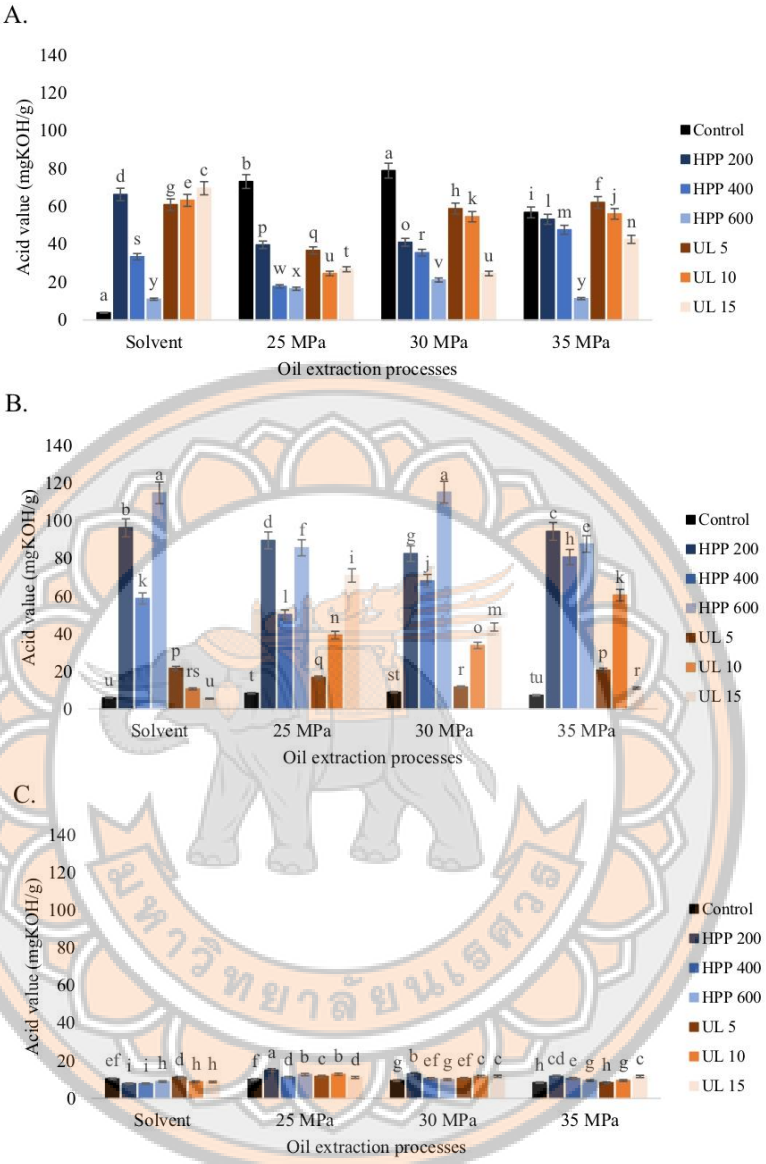

FIGURE 7 | Acid value of edible insect oils: Effects of HPP and UL pretreatments combined with solvent or SC-CO_2 extraction, compared to non-pretreated controls. (A) House cricket, (B) black soldier fly larvae, (C) silkworm. Different superscript letters indicate significant difference ($p < 0.05$).

However, it is noteworthy that among the insect species studied, oils extracted from silkworms exhibited the least susceptibility to rancidity and oxidation, irrespective of the pretreatment and extraction methods employed. This suggests that silkworm oils may possess inherent qualities that confer greater resistance to lipid oxidation. The higher oxidative stability of silkworm oil

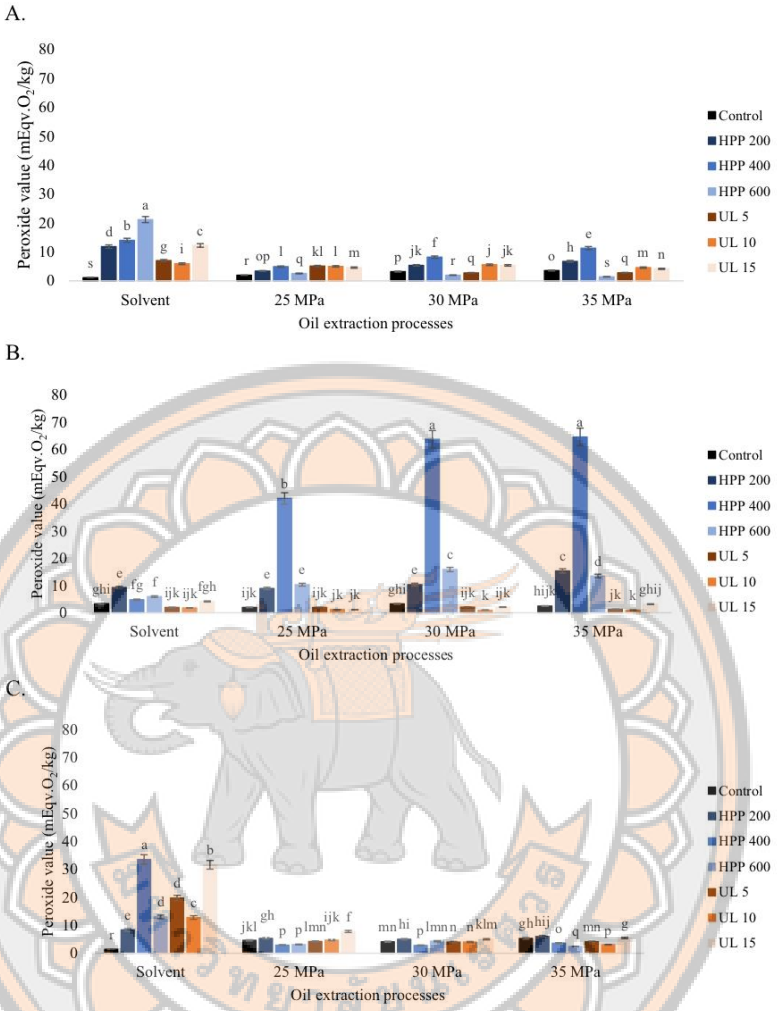

FIGURE 8 | Peroxide value of edible insect oils: Effects of HPP and UL pretreatments combined with solvent or SC-CO_2 extraction, compared to non-pretreated controls. (A) House cricket, (B) black soldier fly larvae, (C) silkworm. Different superscript letters indicate significant difference ($p < 0.05$).

aligns with the literature on its composition and suggests some insect oils may be less susceptible to degradation during processing. Further research could explore the impacts of oil composition on pretreatment effects.

3.6 | Oil Viscosity

Figure 9 indicates that HPP and UL pretreatments variably affected the viscosity of extracted insect oils. The higher viscosity of solvent extracted cricket oils versus SC-CO_2 extracted oils as shown in Figure 9A suggests solvent extraction may better preserve oil viscosity, while SC-CO_2 conditions may reduce viscosity. This points to tailoring extraction methods to target different oil viscosity requirements for products (Zulkurnain, Maleky, and Balasubramaniam 2016). The higher viscosity of solvent extracted cricket oil can be explained by the selectivity, mass transfer characteristics, and the mild condition of SC-CO_2 at the extraction pressure of 25 MPa, which minimizes extraction of very viscous components and effects such as emulsion formation

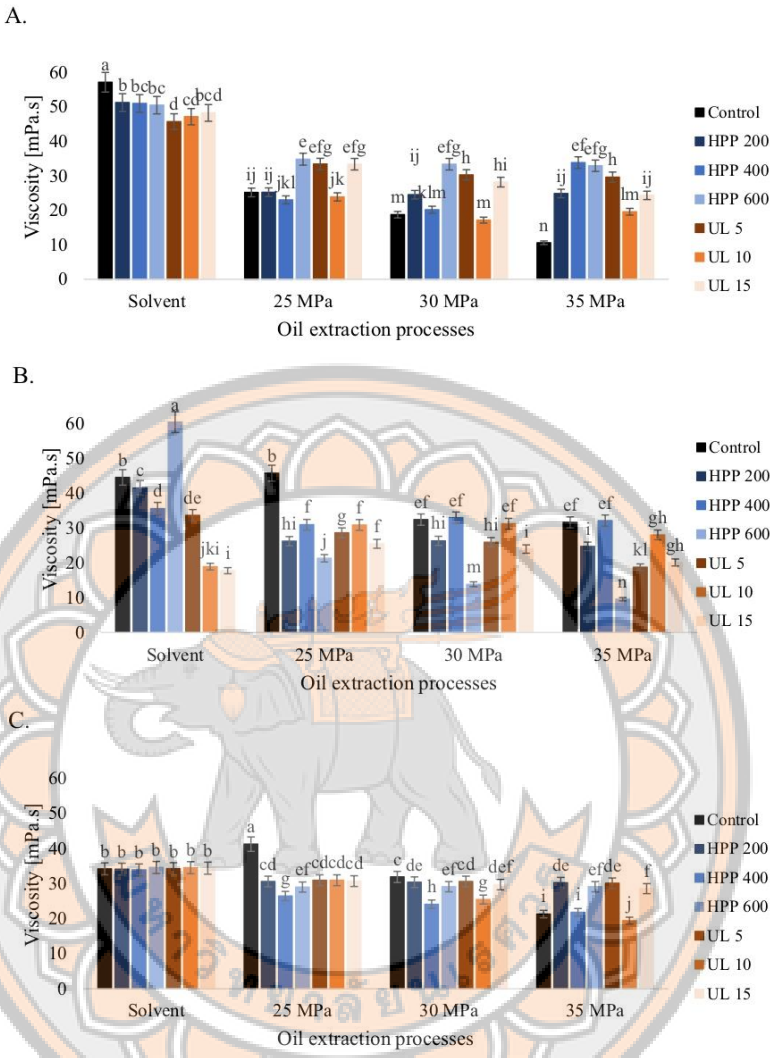

FIGURE 9 | Viscosity edible insect oils: Effects of HPP and UL pretreatments combined with solvent or SC-CO_2 extraction, compared to non-pretreated controls. (A) House cricket, (B) black soldier fly larvae, (C) silkworm. Different superscript letters indicate significant difference ($p < 0.05$).

and molecular aggregation. The SC-CO_2 acts as a co-solvent and can dissolve into the oil during the extraction process, thereby disrupting molecular interactions that contribute to viscosity. This plasticizing effect of dissolved supercritical CO_2 lowers viscosity. In addition, the SC-CO_2 extraction is able to selectively extract lower molecular weight, less viscous components compared to solvent extraction. The mild conditions of supercritical CO_2 extraction prevent these effects. Heat and mass transfer limitations during solvent extraction can preferentially concentrate more viscous, higher molecular weight components like wax esters and long chain triglycerides. The excellent mass transfer achieved with SC-CO_2 minimizes this effect.

However, for black soldier fly larvae, the highest oil viscosity resulted from 600 MPa HPP pretreatment with solvent extraction in Figure 9B. This highlights the complex effects of pretreatments on

TABLE 3 | Fatty acid profile of house cricket, black soldier fly larvae and silkworm pupae oils extracted with SC-CO_2.

Fatty acid	House cricket (g/100 g)	Black soldier fly larvae (g/100 g)	Silkworm (g/100 g)
C8:0	ND	0.02	ND
C10:0	0.02	0.95	ND
C12:0	0.15	24.11	0.07
C14:0	1.01	6.41	0.23
C14:1	0.04	0.09	ND
C16:0	27.15	14.65	23.44
C16:1	0.65	1.53	0.74
C17:0	0.24	0.24	0.13
C18:0	6.58	3.37	7.11
C18:1t9	0.10	0.19	ND
C18:1c9	31.44	17.08	30.42
C18:2	30.83	27.32	10.25
C18:3	0.84	3.59	27.10
C20:0	0.22	0.09	0.29
SFA	35.83	50.10	31.40
UFA	64.18	49.90	68.62
MUFU	32.39	18.96	31.16
PUFU	31.79	30.94	37.46
Tran fat	0.10	0.19	ND
Omega 3 (mg/100 g)	782.29	3566.41	27199.59
Omega 6 (mg/100 g)	31011.82	27381.67	10254.79
Omega 9 (mg/100 g)	31601.27	17145.68	30420.45

Abbreviation: ND = not detected.

oil properties based on insect species. HPP may increase viscosity in some cases through changes in fatty acid profiles (Yordanov and Angelova 2010). The lack of significant viscosity changes from HPP and UL pretreatments for silkworm oil in Figure 9C indicates viscosity may be more stable for some insect oils. Further research on relating chemical composition to the rheological impacts of processing is needed. Overall, these findings reveal insect oil viscosity depends on the type of pretreatment, extraction method, and original insect species. To optimize the functional properties of insect oils, the interactions between processing parameters must be elucidated through further comparative studies.

3.7 | Fatty Acid Profiles

Preliminary tests comparing the fatty acid profiles of oils obtained through conventional extraction methods versus SC-CO_2 extraction showed no significant differences between the two techniques. However, further investigation of the fatty acid compositions is needed, but these initial results suggest that SC-CO_2 extraction could produce an insect oil product with a similar nutritional quality to those derived using standard practices. This potential equivalency in terms of oil quality supports supercritical fluid extraction as a viable alternative to established approaches from a compositional standpoint, though additional study is required for confirmation.

The fatty acid compositions of various edible insect oils, subjected to UL pretreatment at 37 kHz for 15 min before oil extraction by SC-CO_2 at 35 MPa pressure, were analyzed and presented in Table 3. It is noted that edible insects are recognized as a source of beneficial fats (Tang et al. 2019). Across all edible insect oils analyzed, high levels of omega-6 and omega-9 fatty acids were observed, along with minor but potentially nutritionally meaningful amounts of omega-3 fatty acids. Notably, the oil derived from black soldier fly larvae exhibited the highest concentration of lauric acid (24.11 g/100 g). Lauric acid, abundant in coconut oil and human milk, is renowned for its antimicrobial properties (Sandhya, Talukdar, and Bhaishya 2016). However, it is noteworthy that the lauric acid content in black soldier fly larvae oil in this study was lower than previously reported findings by Almeida et al. (2022), suggesting potential variations due to factors such as rearing conditions, feed, and climate.

The fatty acid profiles of the three edible insect oils also revealed significant amounts of C16:0, C18:1c9, and C18:2, which are known for their health benefits. This highlights the potential of insect oils as nutraceuticals. Additionally, all insect oils were found to contain high levels of omega-6 and omega-9 fatty acids, which play crucial roles in blood clotting, wound healing, immune system support, and cholesterol regulation (Mariamenatu and Abdu 2021; Farag and Gad 2022). Comparisons with previous studies indicate variability in omega-6 content, potentially attributable to differences in cricket feed, rearing methods, and environments (Kipkoech et al. 2017). Furthermore, the omega-9 content observed in the oils from house crickets, black soldier fly larvae, and silkworms aligns with previous findings highlighting these insects as good sources of omega-9 fatty acids (Jayanegara, Gustanti, Ridwan, and Widyastuti, 2020). Overall, the fatty acid compositions of these edible insect oils demonstrate their potential as sources of beneficial fats with various health-promoting properties.

4 | Conclusion

In conclusion, this study demonstrates the potential of HPP and UL pretreatments to enhance oil extraction yields from commercially produced insects, specifically house crickets, black soldier fly larvae, and silkworm. Our findings reveal that optimal extraction conditions are species-specific, underscoring the importance of tailored approaches in insect oil production. For house crickets, UL15 pretreatment combined with SC-CO_2 extraction at 30 MPa yielded the highest oil extraction. Black soldier fly larvae showed two optimal methods: non-pretreated samples with SC-CO_2 at 35 MPa and UL10 pretreatment with SC-CO_2 at 30 MPa. Silkworm oil yield was maximized using non-pretreated samples extracted with SC-CO_2 at 35 MPa. However, our results highlight a critical trade-off between extraction yield and oil quality. Non-pretreated samples extracted with conventional solvents consistently showed the lowest values for quality parameters (free fatty acid content, AV, and PV) across all insect species. This observation suggests that pretreatments, while enhancing yield, may accelerate lipid oxidation. Future research should focus on optimizing milder processing conditions that balance maximized yields with preserved oil quality. Notably, black soldier fly larvae oil contained high levels of lauric acid, suggesting its potential use as an antimicrobial additive in animal feed or other applications. This finding opens new avenues for the utilization of insect-derived oils in various industries. Our study also revealed that the necessity for pre-treatment varies among insect species. Black soldier fly larvae, in particular, yielded satisfactory oil without pre-treatment, indicating potential for cost reduction and process simplification in oil production from this species. While SC-CO_2 extraction generally produced higher quality oils, its higher cost compared to conventional solvent extraction necessitates careful cost–benefit analysis. The choice of extraction method should consider factors such as the target insect species, desired oil quality, and economic constraints.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Synergistic integration of non-thermal pretreatments with supercritical CO_2 extraction for enhanced liberation of antimicrobial oils and peptides from farmed edible insects

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ABSTRACT

Escalating antimicrobial resistance and consumer rejection of synthetic preservatives have created urgent demand for natural antimicrobial solutions from sustainable sources. This study systematically investigated synergistic non-thermal pretreatments combined with supercritical CO_2 (SC-CO_2) extraction to maximize liberation of dual antimicrobial fractions from commercially relevant edible insects. House crickets (AHCK, EU-approved for human consumption), black soldier fly larvae (BSFL, EU-approved for animal feed), and silkworm pupae (SWP, widely consumed in Asia) were subjected to ultrasonication (US, 5–15 min, 37 kHz) or high-pressure processing (HPP, 400–600 MPa, 5 min) prior to SC-CO_2 extraction (25–35 MPa, 30 min) or conventional solvent extraction. Peptide fractions underwent enzymatic hydrolysis and simulated gastrointestinal digestion to ensure bioavailability. Comprehensive antimicrobial screening against five major foodborne pathogens revealed distinct species-specific performance. BSFL demonstrated exceptional broad-spectrum efficacy with MIC values as low as 6.25 μL/mL against *S. aureus* and *E. coli*, attributed to remarkable lauric acid content (24.11 ± 0.15 %) representing 161-fold enrichment compared to AHCK (0.15 ± 0.01 %, $p < 0.05$) and 345-fold compared to SWP (0.07 ± 0.01 %, $p < 0.05$). Synergistic pretreatment integration achieved statistically significant enhancement: US + SC-CO_2 showed 2.4–3.8-fold improvement ($p < 0.05$) over non-pretreated controls. BSFL peptides (<3 kDa) exhibited activity against *P. aeruginosa* (1.17 ± 0.02 mm zone radius), a pathogen resistant to many antimicrobials and against which neither AHCK nor SWP peptides nor any insect oils showed activity. This green technology platform demonstrates significant potential for sustainable antimicrobial production, offering food manufacturers clean-label preservation alternatives while addressing regulatory and environmental considerations, establishing insect-derived bioactives as promising candidates for natural food preservation applications.

1. Introduction

Global food security challenges, driven by population growth projected to reach 10 billion by 2050, have intensified research into sustainable protein alternatives with functional properties beyond basic nutrition (Van Huis, Rumpold, Maya, & Roos, 2021). Edible insects such as crickets, silkworms, mealworms, and black soldier flies have been successfully reared for decades as traditional food sources and for pet feed and livestock applications, demonstrating their commercial viability (Aidoo et al., 2023). Currently consumed by over 2 billion people across 113 countries, particularly in Africa, Asia, and Latin America, insects represent one of the most underexploited protein resources in Western markets, despite their exceptional nutritional density and environmental sustainability (Orkusz, Wolanska, Harasym, Piwowar, & Kapelko, 2021). Notably, AHCK is one of four insect species approved by the EU for human food consumption under the novel food regulation, while BSFL is approved for animal feed applications in the EU, underscoring the commercial relevance of these species.

Beyond their role as sustainable protein sources, edible insects possess remarkable antimicrobial properties attributed to evolutionary

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defense mechanisms against pathogens, manifesting in two distinct bioactive fractions: lipid-rich oils containing antimicrobial fatty acids, and bioactive peptides functioning as natural antimicrobial agents—collectively termed "entomoceuticals" (Aiello et al., 2023; Quah et al., 2023). Insect proteins contain high-potential peptides that, when liberated through enzymatic hydrolysis, demonstrate promising anti-inflammatory, antihypertensive, antidiabetic, and antimicrobial properties (Quah et al., 2023). Insect-derived oils demonstrate potent antimicrobial activity through specific fatty acid compositions, particularly omega-3, omega-6, and medium-chain fatty acids such as lauric acid (Montowska, Kowalczewski, Rybicka, & Fornal, 2019). Lauric acid, notably abundant in black soldier fly larvae oils, exhibits potent antimicrobial activity with broad-spectrum efficacy against gram-positive bacteria and significant potential for preventing foodborne pathogen growth (Dang et al., 2020). Complementarily, mealworm oil serves as an excellent source of omega-6 fatty acids, while silkworm pupae oil is rich in omega-3 fatty acids, and cricket oil contains substantial proportions of both omega-3 and omega-6 fatty acids (Mahanta et al., 2023; Udomsil, Imsoonthornruksa, Gosalawit, & Ketudat-Cairns, 2019).

The antimicrobial potential of insect-derived compounds addresses critical food safety challenges, as major foodborne pathogens including *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* are responsible for significant morbidity and economic losses globally. While thermal processing effectively destroys pathogenic bacteria, many food products are consumed without cooking, necessitating alternative preservation strategies and natural antimicrobial agents from sustainable sources.

In addition, many foods can be exposed to post-processing contaminations, thus requiring natural antimicrobial agents to prevent the onset of infection. Insect-derived antimicrobial peptides (AMPs) represent a sophisticated biological defense system with molecular weights typically below 10 kDa (Dang et al., 2020). These cationic, amphiphilic peptides exert antimicrobial effects through membrane disruption mechanisms, offering significant advantages over conventional antibiotics due to their reduced propensity for inducing bacterial resistance and their biodegradable, environmentally friendly nature (Aiello et al., 2023; Montowska et al., 2019).

The extraction and activation of these bioactive compounds require innovative processing technologies that preserve biological activity while maximizing yield. Supercritical CO_2 extraction (SC-CO_2), operating with 100 % pure CO_2 without organic co-solvents, has emerged as a preferred method due to its selectivity, environmental compatibility, and ability to produce truly solvent-free extracts without thermal degradation (Mishyna, Martinez, Chen, & Benjamin, 2021). Advanced non-thermal pretreatment technologies show remarkable potential in enhancing bioactive compound liberation. In contrast, ultrasonication (US) employs acoustic cavitation to generate localized high-energy zones that disrupt cellular structures. High pressure processing (HPP), applying pressures up to 600 MPa, induces protein unfolding and facilitates enhanced peptide liberation (Mahadevan & Karwe, 2016).

Despite growing interest in insect-derived bioactives, current research predominantly focuses on individual compounds or single processing technologies, leaving knowledge gaps regarding comparative pretreatment effects on both oil and peptide fractions (Melgar-Lalanne, Hernández-Álvarez, & Salinas-Castro, 2019). Therefore, the main objective of this study was to evaluate and optimize the synergistic effects of non-thermal pretreatments (US and HPP) combined with SC-CO_2 extraction for maximizing the liberation and antimicrobial efficacy of oil and peptide fractions (<3 kDa) obtained after Simulated Gastrointestinal Digestion (SGD) from three economically farmed insect species: house cricket (*Acheta domesticus*, AHCK), black soldier fly larvae (*Hermetia illucens*, BSFL), and silkworm pupae (*Bombyx mori*, SWP). Conventional solvent extraction (SE) was included as a comparative baseline to quantitatively demonstrate the advantages of the green SC-CO_2 technology in terms of extraction efficiency, bioactive recovery, and environmental sustainability.

2. Materials and methods

2.1. Raw materials and sample preparation

Frozen adult AHCK, BSFL, and SWP were provided by Thai Ento Food Co., Ltd. (Samutprakarn, Thailand). Insects were harvested at optimal developmental stages: AHCK at adult stage (50–55 days), BSFL at late larval stage (25–30 days), and SWP at pupal stage (35–40 days). Samples were thawed at 4 °C for 12 h, washed three times with sterile distilled water, blanched in boiling water (100 °C) for 5 min, cooled in ice water, and stored at −18 °C until processing within 30 days (Boonmee et al., 2024).

2.2. Chemical reagents and bacterial strains

Analytical-grade chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Microbiological media were supplied by Merck KGaA (Darmstadt, Germany). Five foodborne pathogenic bacterial strains were obtained from Thailand Institute of Scientific and Technological Research (TISTR): *E. coli* TISTR 780, *S. aureus* TISTR 1466, *B. cereus* TISTR 687, *S. enterica Serovar Typhimurium* TISTR 292, and *P. aeruginosa* TISTR 781.

2.3. Proximate compositions analysis

Proximate compositions of insects as raw materials and meals were determined according to AOAC International methods (AOAC, 2019). Moisture content was determined by oven-drying at 105 °C for 24 h (AOAC 950.46). Crude protein was analyzed using micro-Kjeldahl method (AOAC 960.52) with nitrogen-to-protein conversion factors of 6.25 for AHCK and SWP (general protein), and 4.76 for BSFL. The lower factor for BSFL accounts for its high chitin content, where nitrogen from chitin is not protein-derived. This species-specific factor prevents protein overestimation in chitin-rich insect larvae (Janssen et al., 2017; Rumpold & Schlüter, 2013). Crude lipid was extracted using Soxhlet extraction (AOAC 963.15) with petroleum ether (boiling point 40–60 °C) for 6 h. Ash content was measured by dry combustion at 550 °C (AOAC 942.05). Total carbohydrates were calculated by difference: 100 - (moisture + protein + lipid + ash + fiber). All analyses were performed in triplicate.

2.4. Non- thermal pretreatments: High pressure processing (HPP) and Ultrasonication (US)

Insect samples were subjected to two different pretreatment processes before oil extraction and peptide preparation as shown in Fig. 1:

HPP: Insect samples (500 g) were vacuum-sealed and processed at 200, 400, or 600 MPa for 5 min at room temperature (22 ± 2 °C) using a high-pressure vessel (HPP600MPa/3-5 L, Baotou Kefa Inc., China). Pressure build-up and release rates were maintained at 5 MPa/s.

US: Insect samples (500 g) were vacuum-sealed in polyethylene bags without any added solvent or water, and treated using an ultrasonic bath (E100H, Elma, Germany) at 37 kHz frequency for 5, 10, or 15 min at room temperature (maintained below 25 °C using an ice bath).

All pretreated and control samples were dried at 70 °C for 10–12 h, ground to <1 mm particle size using a blender (Vitamix 5200, USA), and immediately stored at 4 ± 1 °C in sealed aluminum bags to reduce chemical reaction rates and prevent microbial growth and lipid oxidation during storage until further processing. Previous insect processing studies have used similar temperatures (65–75 °C) and reported acceptable protein digestibility and functional properties at these settings (Rodríguez-Rodríguez et al., 2024). Sealed aluminum bags provide an effective moisture barrier preventing water reabsorption during refrigerated storage, while the low temperature (4 °C) minimizes lipid oxidation and residual enzymatic activity. This storage protocol is standard for dried insect powders intended for lipid and protein

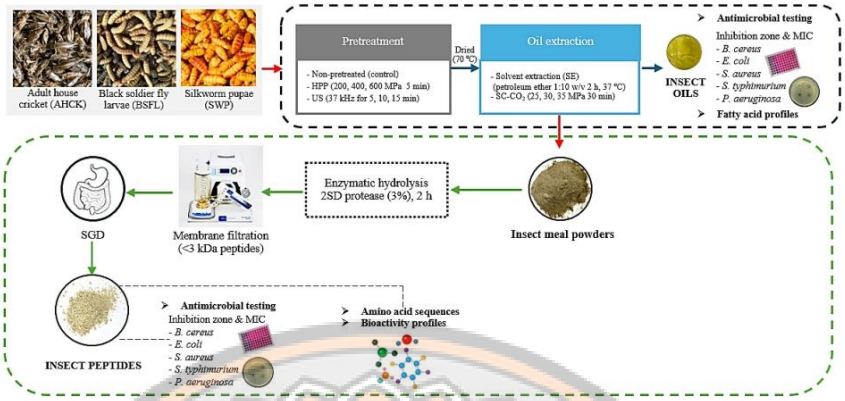

Fig. 1. Processing workflow for oil extraction from three edible insect species using high pressure processing (HPP) or ultrasonication (US) pretreatment followed by solvent extraction (SE) or supercritical carbon dioxide (SC-CO_2) extraction.

extraction (Boonmee et al., 2024).

2.5. Oil extraction methods

Solvent extraction (SE): Pretreated insect samples (100 g) were extracted with petroleum ether (1:10 *w/v*) at 37 °C for 2 h with continuous stirring. The 2 h extraction time was based on preliminary kinetic studies and literature indicating that lipid extraction from insect matrices reaches equilibrium within this timeframe, with extended extraction yielding <3 % additional recovery (Purschke, Scheibelberger, Axmann, Adler, & Jäger, 2017). Mixture was centrifuged at 4830 ×*g* for 15 min, and solvent was removed by evaporation. The extracted oil was stored at −18 °C until analysis.

Supercritical CO_2 (SC-CO_2): SC-CO_2 extraction was performed using a pilot-scale system (CAREDDI SCF, Careddi Technology Co., Ltd., Jiangsu, China) with a 1 L extraction vessel. Pretreated insect samples (100 g) were extracted at 25, 30, or 35 MPa at 37 °C with CO_2 flow rate of 2.0 L/min (120 L/h) for 30 min. The flow rate was selected to ensure adequate solvent volume (solvent-to-feed ratio of 36:1 *v/w*) while maintaining supercritical conditions throughout the extraction vessel (1 L capacity) and preventing channeling in the 100 g biomass bed. Extraction parameters (37 °C, 25–35 MPa) were selected based on CO_2 density-solubility relationships for insect lipids. At 37 °C and 35 MPa, CO_2 density reaches ~0.90 g/mL, providing optimal solvating power for medium-chain fatty acids while preventing thermal degradation of polyunsaturated fatty acids (Purschke et al., 2017). The 30 min extraction time (10 min static equilibration +20 min dynamic extraction) was determined by preliminary optimization showing that 95 % of extractable lipids were recovered within this period. The extracted oil was collected from the separator, centrifuged at 4830 ×*g* for 15 min to remove particulates, and stored at −18 °C.

2.6. Preparation of peptides

Enzymatic hydrolysis: Defatted residues (20 g) from optimized processing were dispersed in water (1:10 *w/v*), pH adjusted to 6.5, and heated to 50 °C. Protease A "Amano" 2SD was added (1:50 enzyme-to-substrate ratio) for 4 h. Reaction was terminated by heating at 100 °C for 10 min.

Ultrafiltration and peptide isolation: Protein hydrolysates were filtered using tangential flow filtration (Minimate™ TFF System, Pall Corporation, USA) with a 3 kDa molecular weight cut-off polyethersulfone membrane to isolate low molecular weight peptides (< 3 kDa), which were freeze-dried and stored at −20 °C.

SGD: SGD was conducted according to Mudd, Martin-Gonzalez, Ferruzzi, and Liceaga (2022) with minor modifications. The <3 kDa peptide of each insect sample (1 g) was initially equilibrated in 10 mL Tris buffer (pH 8.0, 25 ± 1 °C) for 30 min under constant stirring. The mixture was acidified to pH 2.0 (gastric phase), transferred to a 37 °C water bath, and supplemented with pepsin (4 % *w*/w protein). After 2 h incubation with gentle agitation, the reaction was terminated by ice immersion. The gastric digest was sequentially subjected to intestinal phase by adjusting pH to 6.0 (1 M $NaHCO_3$) and 7.0 (6 M NaOH). Upon reaching 37 °C, pancreatin (4 % w/w protein) and bile salts (10 mg/mL) were added, followed by 2 h incubation. Enzymatic activity was terminated by heating at 90 °C for 10 min. Cooled digests were pH-adjusted to 7.0 and centrifuged (12,000 ×*g*, 30 min, 4 °C). Supernatants containing bioaccessible peptides were freeze-dried, weighed for yield determination, and stored at −18 °C in sealed containers until bioactivity analysis.

2.7. Antimicrobial activity evaluation of oils and peptides

Antimicrobial activities were evaluated for two distinct fractions: (i) oils recovered from SE or SC-CO_2 extraction (Section 2.5), and (ii) <3 kDa peptide fractions obtained after enzymatic hydrolysis and SGD of defatted insect powders (Section 2.6).

2.7.1. Inoculum preparation

Bacterial cultures were grown in Mueller-Hinton Broth (MHB) at 37 °C with shaking at 250 rpm for 18–24 h. Fresh inocula were prepared by diluting overnight cultures in sterile MHB to achieve 0.5 McFarland standard (approximately 1.5 × 10^8 CFU/mL), as verified by turbidimetric measurement.

2.7.2. Screening for antimicrobial activity

Oils: Insect oils were dissolved in dimethyl sulfoxide (DMSO) at 1:1 (*v/v*) ratio to ensure complete solubilization of hydrophobic antimicrobial compounds. Antimicrobial activity was evaluated using the agar well diffusion method. Wells (6 mm diameter) were cut in Mueller-Hinton Agar (MHA) plates previously inoculated with 100 μL of standardized bacterial suspension (~10^6 CFU/mL). Oil-DMSO solutions

(100 μL) were added to wells, and plates were incubated at 37 °C for 24 h. Pure DMSO (100 %) served as negative control and showed no inhibition zones (zone measurements were equivalent to well diameter only), confirming that observed antimicrobial activities are attributable to insect-derived compounds, not to DMSO.

Peptides: Freeze-dried peptides (<3 kDa) were dissolved in sterile distilled water at 1:1 (*w/v*) ratio. The paper disc diffusion method was employed using 6 mm diameter discs impregnated with 100 μL of peptide solution and placed on inoculated MHA plates. Sterile distilled water served as negative control and showed no antimicrobial activity. Plates were incubated at 37 °C for 24 h.

Inhibition zone measurement: In this study, only the distance from the edge of the well/disk to the edge of the clear zone (inhibition zone radius) is reported. The inhibition zone radius was measured using digital calipers (±0.1 mm precision) and can be calculated using the following equation:

$$\text{Inhibition zone radius (mm)} = \frac{D_{total} - D_{disk/well}}{2.}$$

Where:

D_{total} = total diameter of the clear zone (mm)

$D_{disk/well}$ = diameter of the well or disk (mm, standardized at 6.0 mm)

For example, a reported inhibition zone radius of 0.85 mm indicates the antimicrobial zone extends 0.85 mm beyond the 6 mm well/disk edge, corresponding to a total clear zone diameter of 7.7 mm. Only samples showing inhibition zone radius > 0.25 mm and statistically significant difference from negative controls ($p < 0.05$, $n = 3$ replicates) were classified as possessing antimicrobial activity. All measurements are expressed as mean ± SD.

2.7.3. Minimum inhibitory concentration (MIC) determination

MIC values were determined for samples showing antimicrobial activity in screening assays using the broth microdilution method in 96-well microplates (CLSI, 2018; Sarker, Nahar, & Kumarasamy, 2007) as illustrated in Supplementary Fig. S1. The protocol incorporated modifications for lipophilic compounds as described by Balouiri, Sadiki, and Ibnsouda (2016), including DMSO as a solubilizing agent with appropriate solvent controls. Serial two-fold dilutions of oil samples (0.1–50 μL/mL) were prepared in Mueller-Hinton Broth (MHB) containing 5 % DMSO (*v/v*, final concentration). Each well received 50 μL of sample dilution, 40 μL of MHB, and 10 μL of standardized bacterial inoculum (~10^6 CFU/mL), maintaining the 5 % DMSO concentration throughout all wells (100 μL total volume per well). Plates were incubated at 37 °C for 24 h. After incubation, resazurin solution (0.01 % *w/v*) was used as a viability indicator, with 10 μL added to each well, followed by incubation at 37 ± 1 °C for 3 h to observe color change. MIC was defined as the lowest concentration showing no color change from blue to pink, indicating complete growth inhibition. The plate layout (Supplementary Fig. S1) included: Column 1 (negative control: sterile MHB with 5 % DMSO, no bacteria, remaining blue), Columns 2–11 (serial two-fold dilutions from 50 to 0.1 μL/mL), and Column 12 (positive control: bacteria without antimicrobial agent, turning pink). All assays were performed in triplicate (rows representing different samples and technical replicates) with appropriate controls: positive controls (bacteria without antimicrobial compounds showing growth and pink resazurin coloration), negative controls (sterile MHB with 5 % DMSO showing no growth and remaining blue), and solvent controls (5 % DMSO without oil showing no growth inhibition, confirming that observed antimicrobial activities are attributable to insect-derived compounds, not to DMSO). Volume-based MIC units (μL/mL) were used as they directly represent the experimental procedure (volume-based serial dilutions); mass-based conversions are provided in Supplementary Table S1 using species-specific oil densities (AHCK = 0.91 g/mL, BSFL = 0.90 g/mL, SWP = 0.92 g/mL).

2.8. Fatty acid profile, saponification value and unsaponifiable matter analysis of insect oils

Fatty acid composition was determined by GC–MS following AOAC method 996.06 (AOAC, 2019). Fatty acids were identified by comparison with retention times of authentic FAME standards and quantified using tricosanoic acid (C23:0) as internal standard. Results are expressed as g/100 g of total fatty acids.

Saponification value was determined according to AOAC method 920.160 using potassium hydroxide saponification followed by back-titration with hydrochloric acid. Results are expressed as mg KOH/g oil.

Unsaponifiable matter was analyzed following AOAC method 933.08 by alkaline saponification, extraction with petroleum ether, and gravimetric determination. Results are expressed as g/100 g oil.

2.9. Identification of bioactive peptides

Sample preparation and analysis scope: This analysis focused specifically on <3 kDa peptide fractions obtained after simulated gastrointestinal digestion (SGD) from each insect species (AHCK, BSFL, SWP). Peptide identification was conducted using liquid chromatography-tandem mass spectrometry (LC-MS/MS) at Purdue University following established protocols (Hall, Reddivari, & Liceaga, 2020). Sample preparation, mass spectrometry analysis, bioinformatics, and data evaluation were conducted in collaboration with the Proteomics Core Facility at the Indiana University School of Medicine, following previously published protocols (Aguilar-Toalá & Liceaga, 2020; Mosley, Florens, Wen, & Washburn, 2009) and vendor-provided guidelines.

LC-MS/MS instrumentation and chromatographic separation: Peptide separation was performed using a 5 cm trap column coupled to a 15 cm EasySpray analytical column (2 μm particle size, 50 μm inner diameter; model 801 A) on an UltiMate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Q-Exactive Plus Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). Mobile phase A consisted of 0.1 % formic acid in water, and mobile phase B consisted of 0.1 % formic acid in acetonitrile. Peptides were eluted using a linear gradient from 5 % to 35 % mobile phase B over 60 min at a flow rate of 300 nL/min, followed by a wash step at 95 % B for 10 min and re-equilibration at 5 % B for 10 min.

Mass spectrometry data acquisition: Spectral data were acquired in positive ion mode using data-dependent acquisition (DDA). Full MS scans were acquired over the *m/z* range of 350–1800 at a resolution of 70,000 (at m/z 200) with an automatic gain control (AGC) target of 3×10^6 ions and a maximum injection time of 100 ms. MS/MS scans of the top 10 most intense precursor ions (charge states 2+ to 5+) were acquired at a resolution of 17,500 with an AGC target of 1×10^5 ions and a maximum injection time of 50 ms. Higher-energy collisional dissociation (HCD) was performed with a normalized collision energy (NCE) of 28 %. Dynamic exclusion was enabled with a 30 s exclusion duration to prevent repetitive sampling of abundant peptides. The isolation window for precursor ion selection was set to 2.0 m/z.

Data processing and peptide identification: Raw mass spectrometry data files were processed using PEAKS Studio software (version X, Bioinformatics Solutions Inc., Waterloo, Canada), employing both de novo sequencing and database search strategies. Peptide identification was performed by searching against three complementary databases: (1) NCBIprot Arthropoda database for taxonomic assignment, (2) UniProt database for comprehensive protein annotation, and (3) BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) for bioactivity prediction. Search parameters included: trypsin as the digestion enzyme with allowance for up to 2 missed cleavages; precursor mass tolerance of ±10 ppm; fragment ion mass tolerance of ±0.02 Da; carbamidomethylation of cysteine as a fixed modification; and oxidation of methionine as a variable modification.

Quality control and validation criteria: Peptide-spectrum matches (PSMs) were filtered at a false discovery rate (FDR) <1 % at the peptide

level using a target-decoy approach. Only peptides meeting the following stringent criteria were retained for downstream bioactivity prediction: (1) Average Local Confidence (ALC) score > 80 % for de novo sequencing accuracy, (2) identification in at least two of three independent technical replicates, (3) minimum peptide length of 5 amino acids, and (4) signal-to-noise ratio > 3.0 for MS/MS spectra. Spectra with ambiguous fragment ion patterns or poor-quality fragmentation were manually inspected and excluded from further analysis. All samples were analyzed in triplicate to ensure reproducibility, and peptides were reported only if consistently identified across replicates.

Bioactivity prediction: Identified peptide sequences were queried against the BIOPEP-UWM Database of Bioactive Peptides to predict potential antimicrobial, antioxidant, and other bioactive properties based on structural homology to known bioactive peptides. Antimicrobial peptide classification was performed based on structural features including charge, hydrophobicity, amphipathicity, and the presence of characteristic motifs (α-helical, β-sheet, proline-rich, or glycine-rich domains).

2.10. Statistical analysis

Data are presented as mean ± standard deviation from three independent experiments ($n = 3$). Statistical analysis was performed using SPSS software version 28.0 (IBM Corp., Armonk, NY, USA). Differences between treatments were evaluated using one-way analysis of variance (ANOVA). For multiple treatment comparisons, Duncan's Multiple Range Test was supplemented with Tukey's HSD test ($\alpha = 0.05$) to control Type I error rate.

3. Results and discussion

3.1. Proximate composition of farmed edible insect species

The proximate composition of three farmed edible insect species revealed significant nutritional diversity directly influencing their potential as sustainable protein and oil sources (Table 1). Protein content varied substantially, with AHCK demonstrating the highest crude protein content (19.53 ± 0.62 % wet basis (wb), 64.69 % dry basis (db)), followed by BSFL (15.38 ± 0.45 % wb, 58.84 % db) and SWP (13.80 ± 0.04 % wb, 49.15 % db). These findings align with cricket protein content typically ranging from 60 to 70 % dry matter (Udomsil et al., 2019). BSFL protein levels (58.84 % db) correspond closely to meta-analyses reporting 40–44 % protein in fresh larvae, translating to 58–65 % on dry matter basis (Siddiqui et al., 2024). The relatively lower protein content in SWP (49.15 % db) compared to literature values of 55–60 % (Mahanta et al., 2023) may reflect harvesting at optimal pupal stage for lipid accumulation.

Table 1
Proximate compositions of whole fresh edible insects.

Compositions	Edible insects					
	AHCK		BSFL		SWP	
	% wb	% db	% wb	% db	% wb	% db
Moisture	69.81 ± 0.33^{c}	–	73.87 ± 0.47^{a}	–	71.92 ± 0.11^{b}	–
Protein	19.53 ± 0.62^{d}	64.69 ± 1.35^{a}	15.38 ± 0.45^{e}	58.84 ± 0.66^{b}	13.80 ± 0.04^{f}	49.15 ± 0.35^{c}
Lipid	6.63 ± 0.04^{e}	21.96 ± 0.10^{b}	4.80 ± 0.07^{f}	18.37 ± 0.60^{c}	9.59 ± 0.03^{d}	34.15 ± 0.24^{a}
Fiber	0.99 ± 0.04^{f}	3.28 ± 0.01^{e}	2.79 ± 0.01^{d}	10.66 ± 0.17^{a}	1.17 ± 0.03^{e}	4.17 ± 0.12^{b}
Ash	1.55 ± 0.01^{f}	5.13 ± 0.03^{e}	2.09 ± 0.01^{d}	7.98 ± 0.17^{a}	1.89 ± 0.01^{e}	6.71 ± 0.00^{b}
Carbohydrate	1.49 ± 0.35^{e}	4.94 ± 1.23^{b}	1.09 ± 0.09^{f}	4.15 ± 0.28^{c}	1.64 ± 0.21^{d}	5.82 ± 0.71^{a}

Note: Data represent mean ± SD from three independent experiments (n = 3). Different superscript letters within the same row indicate significant differences (Duncan's test, $p < 0.05$). % wb = % wet basis (as-is moisture content), % db = % dry basis (moisture-free).

Lipid composition revealed species-specific patterns with significant implications for bioactive compound potential. SWP exhibited the highest crude lipid content (9.59 ± 0.03 % wb, 34.15 % db), followed by AHCK (6.63 ± 0.04 % wb, 21.96 % db) and BSFL (4.80 ± 0.07 % wb, 18.37 % db). These concentrations are particularly relevant for antimicrobial bioactive extraction, as studies demonstrate strong correlations between lipid content and antimicrobial fatty acid availability (Liu et al., 2024). The elevated lipid content in SWP corresponds to documented α-linolenic acid concentrations of 27.99–36.30 % of total fatty acids (Zhang et al., 2024).

Fiber (predominantly chitin from insect exoskeletons) is reported separately from total carbohydrates to distinguish non-digestible structural polysaccharides from metabolically available sugars, as this distinction is nutritionally relevant for food applications. BSFL showed the highest fiber content (2.79 ± 0.01 % wb, 10.66 % db), consisting predominantly of chitin (α-chitin crystalline form) from the larval cuticle, while AHCK demonstrated lower levels (0.99 ± 0.04 % wb, 3.28 % db) and SWP showed intermediate fiber (1.17 ± 0.03 % wb, 4.17 % db) due to metamorphic tissue remodeling (Psarianos, Iranshahi, Rossi, Gottardi, & Schlüter, 2024). These compositional differences reflect distinct fatty acid synthesis pathways, with BSFL utilizing specialized thioesterase II enzymes for medium-chain fatty acid production (Teixeira et al., 2024).

3.2. Processing effects on protein and lipid contents of insect meals

As shown in Fig. 2, AHCK (Fig. 2A) exhibited the most pronounced protein enrichment, achieving 75–80 % protein content from initial levels of ~45 %, representing a 67–78 % concentration enhancement. BSFL (Fig. 2B) showed moderate protein increases from ~40 % to 65–70 %, while SWP (Fig. 2C) demonstrated the highest baseline protein content (~55 %) with increases to 70–75 %.

HPP pretreatment demonstrated superior efficacy compared to US applications, with 600 MPa treatments yielding optimal protein concentration across all species. This pressure-dependent response suggests cell membrane permeabilization and structural disruption mechanisms facilitating subsequent protein liberation (Ma et al., 2023). Recent studies confirm that HPP treatments at elevated pressures improved protein solubility in various insect species, supporting observed compositional improvements (Mokaya, Mudalungu, Tchouassi, & Tang, 2024).

US pretreatment was applied to the vacuum-sealed intact samples (solid-state sonication) to mechanically disrupt cellular structures without solvent, prior to subsequent drying and oil extraction. This distinguishes our pretreatment from ultrasound-assisted extraction (which uses solvents). In this study, it was found that US pretreatment effects were species-specific, with AHCK showing continuous improvement with extended sonication while BSFL and SWP exhibited optimal responses at 10–15 min. This time-dependency reflects acoustic cavitation mechanisms generating localized pressure zones and microstreaming effects that mechanically disrupt cellular structures (Ma et al., 2023). The cavitation phenomenon generates localized high temperatures and pressures at the microscale, which accounts for the observed enhancement in extraction efficiency (Mokaya et al., 2024).

The SC-CO_2 extraction at 30–35 MPa consistently outperformed lower pressure conditions and conventional SE across all species. This pressure-dependent enhancement reflects increased fluid density and solubility parameters governing lipid extraction efficiency (Purschke et al., 2017). Maximal defatting (95 %) was achieved at optimal pressure-temperature combinations, with extraction kinetics revealing that incrementing pressure increased mealworm oil solubility in SC-CO_2 (Purschke et al., 2017). The synergistic effects of pretreatment technologies with SC-CO_2 extraction suggest opportunities for integrated

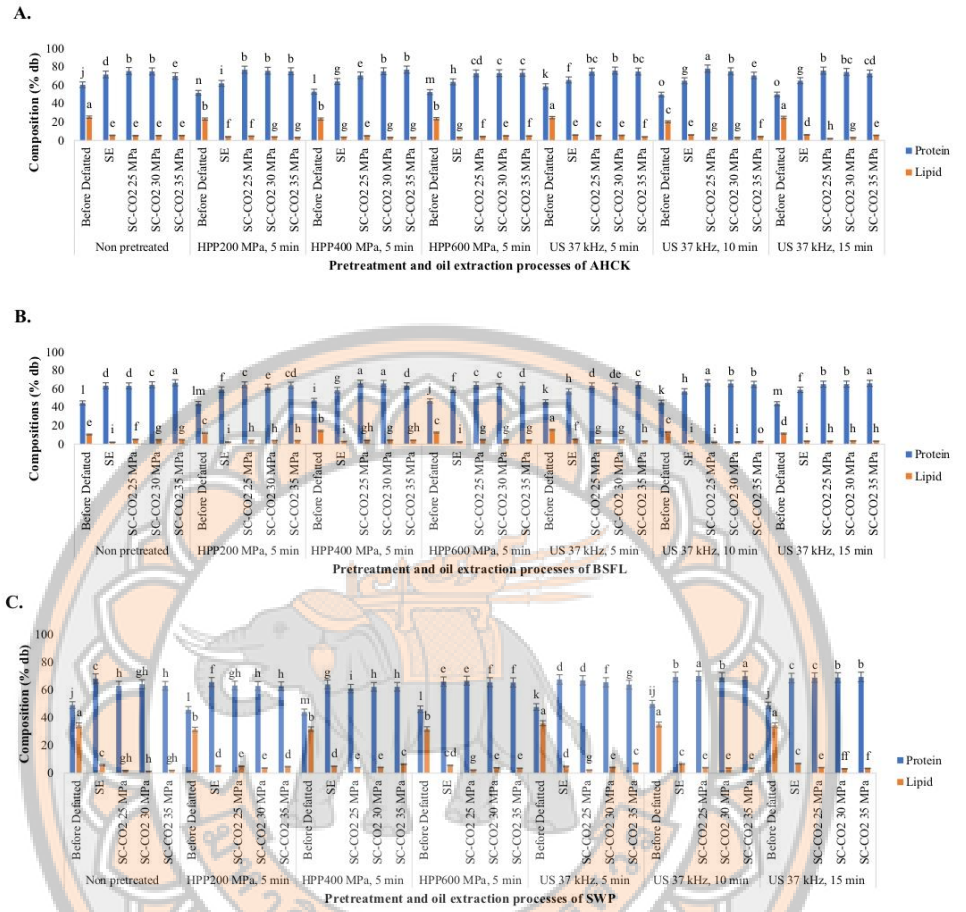

Fig. 2. Protein and lipid contents (% dry basis, db) in insect meals from (A) AHCK, (B) BSFL, and (C) SWP following different processing combinations. Seven conditions were evaluated: non-pretreated control, high-pressure processing (HPP; 200, 400, 600 MPa, 5 min), and ultrasonication (US: 37 kHz, 5/10/15 min). Different letters within the same parameter indicate significant differences (Duncan's test, $p < 0.05$, $n = 3$).

processing platforms achieving protein concentrations exceeding 80 % while maintaining solvent-free processing advantages. These findings demonstrate commercially viable pathways for sustainable insect protein production, with SC-CO_2 extraction providing high-yield, solvent-free oil recovery and protein-enriched residues suitable for food applications (Rahman, Byanju, & Lamsal, 2023).

3.3. Pretreatment and extraction method optimization for enhanced antimicrobial activity

3.3.1. Effects of pretreatments on antimicrobial efficacy

Prior reports confirm antimicrobial properties of insect oils but note that processing conditions significantly impact potency (Saviane et al., 2021). The comparative analysis of antimicrobial activities across different pretreatment methods (Tables 2–4) reveals distinct mechanistic pathways for bioactive compound liberation and provides critical insights into process optimization for enhanced antimicrobial efficacy.

US pretreatment demonstrated superior performance across all three insect species, consistently achieving the lowest MIC values and broadest antimicrobial spectra. For AHCK oils (Table 2), US pretreatment (15 min, 37 kHz) combined with SC-CO_2 extraction at 35 MPa achieved MIC values of 10.42 ± 2.95 μL/mL against *S. aureus*, representing statistically significant antimicrobial activity compared with the non-pretreated control (25.00 ± 0.00 μL/mL) ($p < 0.05$). For BSFL oils (Table 3), US at 10 min combined with SE achieved exceptional antimicrobial activity against *S. aureus* (6.25 ± 0.00 μL/mL) and *E. coli* (10.42 ± 2.95 μL/mL), representing 2–4 fold improvement over non-pretreated controls (*S. aureus*: 25.00 μL/mL; *E. coli*: 12.50 μL/mL) (p

Table 2
MIC of AHCK oils extracted using different pretreatment methods against test bacteria.

Treatments		Minimum inhibition concentration (μl/mL)		
AHCK oils		*B. cereus*	*S. aureus*	*E. coli*
- HPP 200 MPa, 5 min + SE		–	12.50 ± 0.00	25.00 ± 0.00
- HPP 200 MPa, 5 min + SC-CO_2	25 MPa	50.00 ± 0.00	–	–
	30 MPa	50.00 ± 0.00	50.00 ± 0.00	–
	35 MPa	–	25.00 ± 0.00	–
- HPP 400 MPa, 5 min + SE		–	25.00 ± 0.00	25.00 ± 0.00
- HPP 400 MPa, 5 min + SC-CO_2	30 MPa	–	50.00 ± 0.00	–
	35 MPa	20.83 ± 5.89	25.00 ± 0.00	–
- HPP 600 MPa, 5 min + SE		–	25.00 ± 0.00	–
- HPP 600 MPa, 5 min + SC-CO_2	30 MPa	–	25.00 ± 0.00	–
	35 MPa	16.67 ± 5.89	50.00 ± 0.00	
- US 37 kHz, 5 min + SE		–	12.50 ± 0.00	25.00 ± 0.00
- US 37 kHz, 10 min + SE			12.50 ± 0.00	25.00 ± 0.00
- US 37 kHz, 10 min + SC-CO_2	35 MPa	–	50.00 ± 0.00	–
- US 37 kHz, 15 min + SE		–	25.00 ± 0.00	25.00 ± 0.00
- US 37 kHz, 15 min + SC-CO_2	25 MPa	50.00 ± 0.00	–	–
	30 MPa	50.00 ± 0.00	25.00 ± 0.00	–
	35 MPa	25.00 ± 0.00	10.42 ± 2.95	–

Note: Data represent mean ± SD from three independent experiments (n = 3). "–" indicates no inhibitory activity observed at the highest tested concentration. MIC values determined by broth microdilution method in MHB containing 5 % DMSO (v/v, final concentration). Volume-based units (μL/mL) represent experimental procedure; mass-based conversions provided in Supplementary Table S1.

Table 3
MIC of BSFL oils extracted using different pretreatment methods against test bacteria.

Treatments		Minimum inhibition concentration (μl/mL)			
BSFL oils		*B. cereus*	*S. aureus*	*E. coli*	*S. typhimurium*
- Non-pretreated + SE		12.50 ± 0.00	25.00 ± 0.00	12.50 ± 0.00	–
- Non-pretreated + SC-CO_2	25 MPa	12.50 ± 0.00	12.50 ± 0.00	12.50 ± 0.00	12.50 ± 0.00
	30 MPa	25.00 ± 0.00	12.50 ± 0.00	50.00 ± 0.00	50.00 ± 0.00
	35 MPa	25.00 ± 0.00	12.50 ± 0.00	18.75 ± 8.84	25.00 ± 0.00
- HPP 200 MPa, 5 min + SE		25.00 ± 0.00	25.00 ± 0.00	50.00 ± 0.00	–
- HPP 200 MPa, 5 min + SC-CO_2	25 MPa	25.00 ± 0.00	6.25 ± 0.00	18.75 ± 8.84	25.00 ± 0.00
	30 MPa	6.25 ± 0.00	12.50 ± 0.00	25.00 ± 0.00	25.00 ± 0.00
	35 MPa	12.50 ± 0.00	12.50 ± 0.00	12.50 ± 0.00	12.50 ± 0.00
- HPP 400 MPa, 5 min + SE		12.50 ± 0.00	6.25 ± 0.00	6.25 ± 0.00	–
- HPP 400 MPa, 5 min + SC-CO_2	25 MPa	12.50 ± 0.00	25.00 ± 0.00	12.50 ± 0.00	12.50 ± 0.00
	30 MPa	6.25 ± 0.00	6.25 ± 0.00	–	12.50 ± 0.00
	35 MPa	6.25 ± 0.00	12.50 ± 0.00	–	12.50 ± 0.00
- HPP 600 MPa, 5 min + SE		12.50 ± 0.00	8.33 ± 2.95	6.25 ± 0.00	–
- HPP 600 MPa, 5 min + SC-CO_2	25 MPa	6.25 ± 0.00	12.50 ± 0.00	–	12.50 ± 0.00
	30 MPa	6.25 ± 0.00	25.00 ± 0.00	–	12.50 ± 0.00
	35 MPa	6.25 ± 0.00	12.50 ± 0.00	–	12.50 ± 0.00
- US 37 kHz, 5 min + SE		–	–	25.00 ± 0.00	–
- US 37 kHz, 5 min + SC-CO_2	35 MPa	–	12.50 ± 0.00	–	–
- US 37 kHz, 10 min + SE		–	6.25 ± 0.00	10.42 ± 2.95	–
- US 37 kHz, 10 min + SC-CO_2	25 MPa	12.50 ± 0.00	–	–	–
	35 MPa	12.50 ± 0.00	–	–	50.00 ± 0.00
- US 37 kHz, 15 min + SE		–	–	25.00 ± 0.00	–
- US 37 kHz, 15 min + SC-CO_2	25 MPa	12.50 ± 0.00	–	–	12.50 ± 0.00

Note: Data represent mean ± SD from three independent experiments (n = 3). "–" indicates no inhibitory activity observed at the highest tested concentration. MIC values determined by broth microdilution method in MHB containing 5 % DMSO (v/v, final concentration). Volume-based units (μL/mL) represent experimental procedure; mass-based conversions provided in Supplementary Table S1.

< 0.05). This enhancement can be attributed to acoustic cavitation phenomena generating localized high-energy zones that mechanically disrupt chitin-protein matrices through microscopic bubble formation and collapse, creating microstreaming effects that enhance mass transfer coefficients by 3–5 fold (Chen et al., 2025; Liu et al., 2024; Rojas et al., 2024). SWP oils showed limited response to US alone (Table 4), likely due to the silkworm's unique cocoon structure requiring combined mechanical-chemical approaches.

The frequency-dependent nature of US revealed optimal performance at 20–25 kHz, corresponding to the resonant frequency for chitin (fiber) disruption. Recent mechanistic studies demonstrate that US creates cavitation-induced shear forces exceeding 10^5 Pa, sufficient to break hydrogen bonds between chitin chains and associated proteins, thereby exposing previously encapsulated antimicrobial compounds (Kumari, Debbarma, & Hussain, 2025). The time-dependent optimization showed maximum antimicrobial recovery at 10–15 min, beyond which thermal degradation of sensitive bioactive compounds begins to occur.

The apparently paradoxical observation that US pretreatment enhances antimicrobial peptide recovery despite potentially releasing proteins during pretreatment can be explained mechanistically. US-induced cavitation creates localized shear forces that disrupt chitin-protein associations without extensive protein denaturation, thereby creating a more accessible protein matrix for subsequent enzymatic hydrolysis while preserving native antimicrobial epitopes. In contrast, HPP achieves protein liberation through pressure-induced protein unfolding, which may partially mask bioactive domains. Critically, the antimicrobial activity reported for peptides (Tables, Figs. 3–5) was measured on the final <3 kDa fractions after complete processing—not on the intermediate pretreated samples—thus reflecting the cumulative effect of pretreatment on final bioactive peptide yield and activity after enzymatic liberation.

HPP pretreatment showed variable effects depending on pressure levels and target compounds. HPP at 400–600 MPa generally improved peptide liberation, evidenced by enhanced antimicrobial activity patterns, likely through pressure-induced protein unfolding that exposes cryptic enzymatic cleavage sites during subsequent hydrolysis (Mahadevan & Karwe, 2016). However, the optimal pressure conditions varied among species, suggesting species-specific structural differences in protein-chitin associations and matrix recalcitrance. For AHCK oils

Table 4
MIC of SWP oils extracted using different pretreatment methods against test bacteria.

Treatments		Minimum inhibition concentration (μl/mL)			
SWP oils		*B. cereus*	*S. aureus*	*E. coli*	*S. typhimurium*
- Non-pretreated + SC-CO_2	30 MPa	10.42 ± 2.95	6.25 ± 0.00	–	–
	35 MPa	12.50 ± 0.00	10.42 ± 2.95	–	–
	25 MPa	10.42 ± 2.95	–	6.25 ± 0.00	6.25 ± 0.00
- HPP 200 MPa, 5 min + SC-CO_2	30 MPa	6.25 ± 0.00	–	–	–
	35 MPa	–	–	6.25 ± 0.00	6.25 ± 0.00
- HPP 400 MPa, 5 min + SC-CO_2	25 MPa	6.25 ± 0.00	–	12.50 ± 0.00	12.50 ± 0.00
	25 MPa	12.50 ± 0.00	6.25 ± 0.00	–	–
- HPP 600 MPa, 5 min + SC-CO_2	30 MPa	12.50 ± 0.00	25.00 ± 0.00	–	–
	35 MPa	–	12.50 ± 0.00	–	–
- US 37 kHz, 5 min + SC-CO_2	25 MPa	12.50 ± 0.00	6.25 ± 0.00	–	–
	35 MPa	–	25.00 ± 0.00	–	–
- US 37 kHz, 10 min + SC-CO_2	25 MPa	10.42 ± 2.95	10.42 ± 2.95	–	–
	30 MPa	6.25 ± 0.00	10.42 ± 2.95	–	–
- US 37 kHz, 15 min + SC-CO_2	35 MPa	12.50 ± 0.00	6.25 ± 0.00	–	–
	25 MPa	–	6.25 ± 0.00	–	–
	30 MPa	12.50 ± 0.00	–	–	–

Note: Data represent mean ± SD from three independent experiments (n = 3). "–" indicates no inhibitory activity observed at the highest tested concentration. MIC values determined by broth microdilution method in MHB containing 5 % DMSO (v/v, final concentration). Volume-based units (μL/mL) represent experimental procedure; mass-based conversions provided in Supplementary Table S1.

(Table 2), HPP 600 MPa + SC-CO_2 at 35 MPa achieved optimal performance with MIC values of 16.67 ± 5.89 μL/mL against *B. cereus* and 50.00 ± 0.00 μL/mL against *S. aureus* ($p < 0.05$). The higher-pressure requirement reflects the cricket's more robust exoskeleton structure requiring greater mechanical force for effective disruption. Interestingly, intermediate pressure levels (HPP 200–400 MPa) showed suboptimal results, suggesting a threshold effect where minimum pressure levels are required to achieve meaningful cellular disruption.

For BSFL oils (Table 3), HPP 400 MPa + SC-CO_2 treatments consistently produced the lowest MIC values across multiple pathogens, particularly effective against *S. aureus* (6.25 ± 0.00 μL/mL) and *E. coli* (6.25 ± 0.00 μL/mL) ($p < 0.05$), matching US performance. The pressure-induced mechanisms involve barotrauma effects causing controlled membrane permeabilization without complete cellular destruction. At 400–600 MPa, pressure-induced protein unfolding exposes cryptic enzymatic cleavage sites, enabling enhanced release of membrane-bound antimicrobial peptides during subsequent extraction (Zhang et al., 2023). This mechanism is particularly effective for BSFL due to their naturally high antimicrobial peptide content evolved for pathogenic environment resistance.

3.3.2. Extraction method effects on antimicrobial performance

AHCK oils showed consistent SE performance across pretreatment methods, with SE consistently achieving MIC values of 12.50 ± 0.00 μL/mL against *S. aureus* and 25.00 ± 0.00 μL/mL against *E. coli* when combined with US or HPP pretreatments (Table 2). Notably, SE alone (without pretreatment) showed no measurable antimicrobial activity, indicating that mechanical disruption is essential for liberating bioactive compounds from cricket exoskeletons. BSFL demonstrated the most dramatic SE responsiveness, with non-pretreated SE achieving substantial antimicrobial activity: 12.50 ± 0.00 μL/mL against both *B. cereus* and *E. coli*, and 25.00 ± 0.00 μL/mL against *S. aureus* (Table 3). This represents the only species showing significant SE activity without pretreatment, suggesting that BSFL's naturally softer cuticle structure allows direct solvent penetration and bioactive compound extraction. When combined with pretreatments, BSFL-SE achieved exceptional performance, with HPP 400 MPa + SE yielding the lowest recorded MIC values of 6.25 ± 0.00 μL/mL against both *S. aureus* and *E. coli*. SWP oils showed no SE activity across all tested conditions (Table 4), with all SE treatments resulting in no measurable antimicrobial effects. This complete lack of SE responsiveness indicates that SWP require specialized extraction approaches, likely due to their unique cocoon-derived structural proteins that resist conventional solvent penetration.

SC-CO_2 extraction excelled in recovering lipophilic antimicrobial compounds, particularly lauric acid and other medium-chain fatty acids responsible for membrane-disrupting antimicrobial activity. The pressure-dependent solubility enhancement from 25 to 35 MPa increased lauric acid extraction efficiency, directly correlating with improved MIC values. For BSFL oils, SC-CO_2 at 35 MPa achieved MIC values of 12.50 ± 0.00 μL/mL against multiple pathogens, representing 2.0-fold improvement over 25 MPa extractions (25.00 μL/mL, $p < 0.05$). The selectivity advantages of SC-CO_2 extraction become apparent when analyzing fatty acid profiles (Table 5). SC-CO_2 extraction preferentially recovered medium-chain fatty acids (C8-C14) while minimizing extraction of longer-chain fatty acids that could interfere with antimicrobial activity. This selectivity results in concentrated antimicrobial extracts with higher specific activity compared to conventional solvent extraction.

The relationship between SC-CO_2 pressure and antimicrobial activity follows the density-solubility correlation described by the Peng-Robinson equation of state. At 25 MPa (density ≈ 0.80 g/mL), limited solubility restricts extraction to highly volatile compounds with minimal antimicrobial activity. Increasing pressure to 30 MPa (density ≈ 0.85 g/mL) dramatically improves medium-chain fatty acid solubility, resulting in 2.8-fold enhancement in antimicrobial activity for BSFL oils (Table 3, comparing SC-CO_2 30 MPa vs. 25 MPa: 6.25 vs. 25.00 μL/mL for *S. aureus* using HPP 400 MPa pretreatment, $p < 0.05$).

Optimal antimicrobial activity consistently occurred at 35 MPa (density ≈ 0.90 g/mL) across all species, representing the optimal balance between extraction efficiency and selectivity. The integration of oil extraction method optimization with pretreatment technologies creates synergistic effects exceeding individual process improvements. Combined HPP pretreatment with optimized sequential SC-CO_2 extraction achieved *antimicrobial activities significantly* ($p < 0.05$) *higher* than conventional extraction methods, establishing this integrated approach as the most effective processing strategy for insect-derived antimicrobial compounds.

3.3.3. Synergistic effects and antimicrobial activity enhancement

The combination of pretreatment with SC-CO_2 extraction at varying pressures (25–35 MPa) revealed critical pressure-dependent optimization patterns that maximize antimicrobial activity. Higher supercritical pressures (35 MPa) consistently enhanced both oil antimicrobial activities across all species, demonstrating improved solvating power and enhanced bioactive compound extraction efficiency. This pressure-dependent enhancement follows the Chrastil model, where increased CO_2 density at higher pressures improves solubility parameters for medium-chain fatty acids and facilitates penetration into disrupted cellular matrices (Liu et al., 2024).

The most significant finding involves synergistic effects between mechanical pretreatments and supercritical extraction. HPP 400 MPa + SC-CO_2 35 MPa achieved multiplicative rather than additive

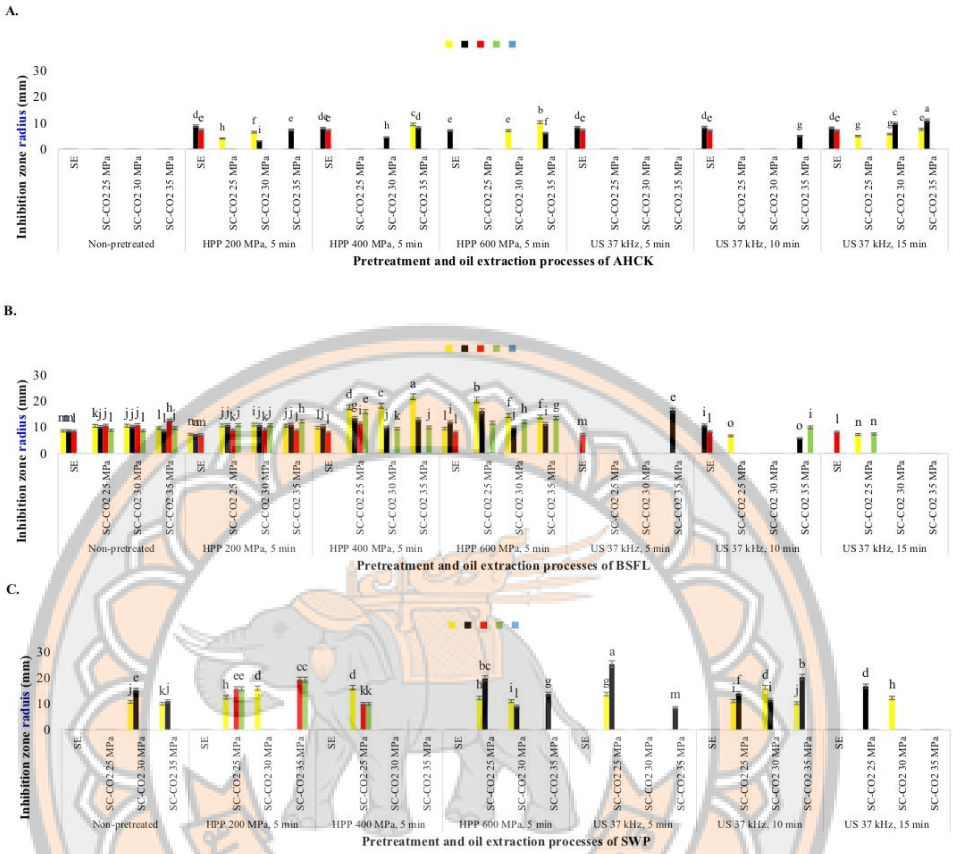

Fig. 3. Antimicrobial screening of insect oils against five major foodborne pathogens using agar diffusion method. Values represent inhibition zone radius (mm) as mean ± SD from three independent experiments (n = 3) of oils from (A) AHCK, (B) BSFL, and (C) SWP extracted using different pretreatment conditions (non-pretreated control, HPP at 200/400/600 MPa for 5 min, US at 37 kHz for 5/10/15 min) followed by either SE or SC-CO_2 extraction at 25, 30, or 35 MPa. Tested pathogens include gram-positive bacteria (*B. cereus*, *S. aureus*) and gram-negative bacteria (*E. coli*, *S. typhimurium*, *P. aeruginosa*). Different letters indicate significant differences (Duncan's test, $p < 0.05$). Screening performed with oils dissolved 1:1 (*v*/v) in DMSO using agar well diffusion method. Negative controls (pure DMSO for oil screening) showed no antimicrobial activity. MIC determination performed at 5 % DMSO final concentration using broth microdilution (Supplementary Fig. S1).

improvements, with BSFL oils showing 4.0-fold enhancement in antimicrobial activity against *S. aureus* compared to non-pretreated SC-CO_2 35 MPa controls (6.25 vs. 25.00 μL/mL, $p < 0.05$). This synergy results from pressure-induced cellular disruption creating pathways for enhanced CO_2 penetration, while maintaining structural integrity necessary for selective bioactive compound extraction.

Sequential processing optimization revealed that pretreatment duration significantly impacts final antimicrobial activity. Optimal sequences involve 5–10 min mechanical pretreatment followed by immediate supercritical extraction, preventing enzymatic degradation of released bioactive compounds. Extended pretreatment durations (>15 min) showed diminished returns due to thermal degradation and oxidative losses.

While US pretreatment achieved higher antimicrobial peptide yields, HPP offers the dual benefit of bioactive compound liberation and simultaneous microbial decontamination of the raw insect material. For commercial-scale production where food safety is paramount, HPP at 400–600 MPa can ensure microbiological safety while still achieving substantial bioactive recovery.

3.4. Species-specific antimicrobial performance and bioactive compound mechanisms

3.4.1. Species-specific antimicrobial oil performance

The antimicrobial evaluation of SC-CO_2-extracted oils revealed distinct species-specific activity patterns against the five foodborne pathogens tested according to agar diffusion outcomes (Fig. 3). As illustrated in Fig. 3A, AHCK oils demonstrated selective antimicrobial

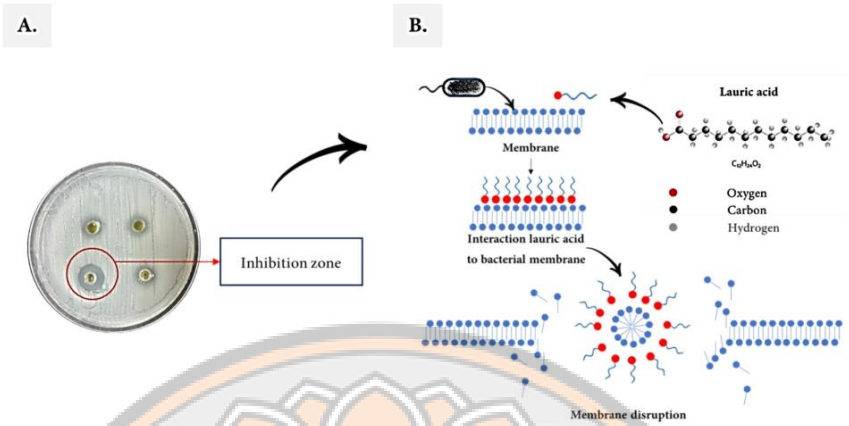

Fig. 4. Antibacterial activity assessment and mechanism of action. (A) Representative zones of inhibition from agar diffusion assays demonstrating antibacterial effects of insect oils. (B) Proposed mechanism of lauric acid antibacterial activity against bacterial cell membranes.

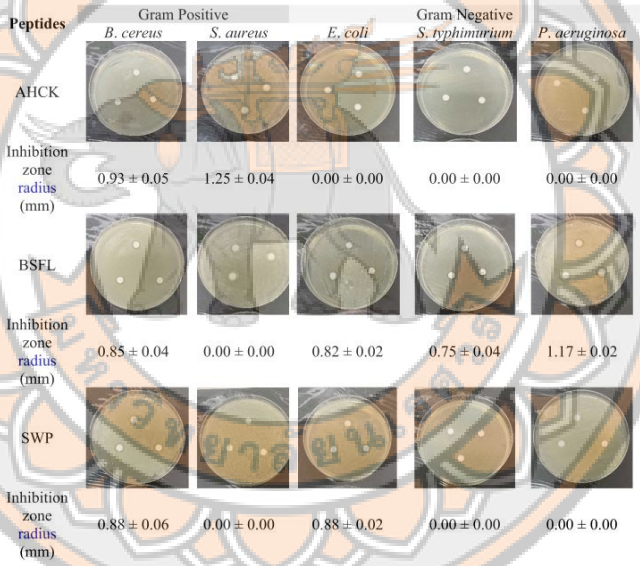

Fig. 5. Antimicrobial activity of insect-derived peptides (<3 kDa) against foodborne pathogens. Peptides obtained from defatted residues after US pretreatment (37 kHz, 15 min) and SC-CO_2 extraction (35 MPa), followed by enzymatic hydrolysis and simulated gastrointestinal digestion. Screening performed using disc diffusion method with peptides dissolved in sterile distilled water. Inhibition zones reported as radii (mm) representing distance beyond 6 mm disc edge. Negative controls (sterile water) showed no antimicrobial activity. Data represent mean ± SD (n = 3).

activity primarily against gram-positive bacteria, with measurable inhibition zones observed against *B. cereus* and *S. aureus*, while showing limited activity against *E. coli*. Fig. 3B shows that BSFL oils exhibited the most comprehensive antimicrobial spectrum, displaying significant inhibition zones against four of the five pathogens tested: *E. coli*, *B. cereus*, *S. aureus*, and *S. typhimurium*. Fig. 3C demonstrates that SWP oils showed moderate activity patterns, with observable inhibition zones against *E. coli*, *B. cereus*, *S. aureus*, and *S. typhimurium*.

The visual confirmation of these antimicrobial activities is further demonstrated in Fig. 4A and B, which shows representative zones of

Table 5
Fatty acid composition, saponification value, and unsaponifiable matter content of edible insect oils following the US pretreatment (37 kHz, 15 min) and SC-CO_2 extraction (35 MPa).

Fatty acid compositions	AHCK	BSFL	SWP
C8:0	ND	0.02	ND
C10:0	0.02	0.95	ND
C12:0	0.15	24.11	0.07
C14:0	1.01	6.41	0.23
C14:1	0.04	0.09	ND
C16:0	27.15	14.65	23.44
C16:1	0.65	1.53	0.74
C17:0	0.24	0.24	0.13
C18:0	6.58	3.37	7.11
C18:1 t9	0.10	0.19	ND
C18:1c9	31.44	17.08	30.42
C18:2	30.83	27.32	10.25
C18:3	0.84	3.59	27.10
C20:0	0.22	0.09	0.29
SFA	35.83	50.10	31.40
UFA	64.18	49.90	68.62
MUFU	32.39	18.96	31.16
PUFU	31.79	30.94	37.46
Tran fat	0.10	0.19	ND
Omega 3 (mg/100 g)	782.29	3566.41	27,199.59
Omega 6 (mg/100 g)	31,011.82	27,381.67	10,254.79
Omega 9 (mg/100 g)	31,601.27	17,145.68	30,420.45
Saponification value (mgKOH/g)	110.25	212.46	190.24
Unsaponifiable matter (g/100 g)	3.16	1.62	1.75

Note: ND = Not detected.

inhibition resulting from the agar diffusion test, providing clear evidence of the bacteriostatic and bactericidal effects of the insect oil extracts. Notably, none of the tested oils exhibited antimicrobial activity against *P. aeruginosa*, suggesting species-specific resistance mechanisms or insufficient bioactive compound concentrations to overcome this pathogen's robust defense systems.

Concentration-dependent antimicrobial efficacy was confirmed through MIC analysis as previously stated (Tables 2–4). BSFL oils consistently achieved the lowest MIC values across multiple pathogens, with optimal treatments achieving MIC values as low as 6.25 ± 0.00 μL/mL against *S. aureus* and *E. coli*. Apparently, BSFL oils were the only extracts showing activity against *S. typhimurium*, with MIC values ranging from 12.50 to 50.00 μL/mL. AHCK oils achieved variable MIC values with the most effective treatments showing activity against *B. cereus* and *S. aureus*, while SWP oils demonstrated more limited antimicrobial spectrum.

Fatty acid analysis revealed distinct compositional profiles among the three insect oils (Table 5) that correlated directly with their antimicrobial activities presented in Tables 2–4. Unsaturated fatty acids predominated in AHCK and SWP oils, while BSFL oil contained balanced proportions of both saturated and unsaturated fatty acids. All three insect oils contained omega-3 and omega-6 fatty acids, which are essential for human health and critical for children's development (Glick & Fischer, 2013; Udomsil et al., 2019). The fatty acid profiles of these edible insect lipids have been reported to be superior to those found in conventional animal proteins such as chicken, pork, and beef (Udomsil et al., 2019).

BSFL demonstrated the highest lauric acid content (24.11 g/100 g), a fatty acid recognized for its antimicrobial properties, particularly against gram-positive bacteria (Liland et al., 2017). Suryati, Julaeha, Farabi, Ambarsari, and Hidayat (2023) reported comparable fatty acid profiles for BSFL oil, though with higher lauric acid concentrations, indicating that fatty acid composition is influenced by feed composition and environmental factors (Udomsil et al., 2019). This exceptionally high lauric acid concentration in BSFL directly correlates with its superior antimicrobial performance against *S. aureus* and *B. cereus* as demonstrated in Table 3.

The lauric acid content of BSFL oils (24.11 %) represented a 160-fold higher concentration compared to AHCK oils (0.15 g/100 g, $p < 0.05$) and a 345-fold higher concentration compared to SWP oils (0.07 g/100 g, $p < 0.05$). This finding aligns with recent mechanistic studies demonstrating that lauric acid exerts antimicrobial activity through membrane-disrupting mechanisms, where the hydrophobic carbon chain integrates into bacterial lipid bilayers, compromising membrane integrity and causing cytoplasmic leakage (Matsue et al., 2019; Purschke et al., 2017; Yang et al., 2021). In addition, prior reports state that medium-chain fatty acids like lauric acid and its monoglyceride (monolaurin) disrupt bacterial membranes, especially effective against gram-positive bacteria, while gram-negatives like Pseudomonas are more resistant (Casillas-Vargas et al., 2021; Sun, O'Connor, & Roberton, 2003; Yoon, Jackman, Valle-González, & Cho, 2018). BSFL oil's lauric acid makes it promising for clean-label food preservation applications, including edible coatings and emulsions (Suryati et al., 2023).

As demonstrated in Fig. 4B, the outer layer of the bacterial cell membranes is composed of certain lipid molecules. The hydrophobic part of lauric acid can attack the structure of the bacterial membranes. Consequently, the damaged cell membranes are exposed to cytoplasmic fluid that leaks out (Nitbani, Tjitda, Nitti, Jumina, & Detha, 2022). The leaking of the cytoplasmic fluid causes a decrease in the cell activity of the bacteria, leading to the death of the cells. Medium-chain fatty acids like lauric acid can disrupt bacterial cell walls by causing membrane depolarization and generating reactive oxygen species, leading to cell membrane damage (Shilling et al., 2013). The antimicrobial action involves rapid membrane depolarization, disruption of macromolecular synthesis, and release of low-molecular-weight proteins into the medium (Parsons, Yao, Frank, Jackson, & Rock, 2012). Lauric acid specifically targets gram-positive bacteria more effectively than gram-negative bacteria due to structural differences in cell wall composition, with the mechanism likely involving separation of inner and outer membranes resulting in cytoplasmic disorganization (Bergsson, Arnfinnsson, Steingrímsson, & Thormar, 2001; Skřivanová, Marounek, Benda, & Březina, 2006). Moreover, monolaurin, derived from lauric acid, possesses significantly greater antimicrobial potency than lauric acid itself due to its amphipathic structure and ability to integrate into microbial membranes (Ruzin & Novick, 2000). Therefore, the BSFL oil might be considered as a candidate for an antibacterial material.

SWP exhibited the highest omega-3 fatty acid content (27,199.59 mg/100 g), predominantly α-linolenic acid (C18:3), contributing 27.10 g/100 g of total fatty acids. Omega-3 fatty acids function as endogenous antimicrobial molecules with bactericidal and fungicidal effects (Das, 2008). The high omega-3 content in SWP explains its moderate antimicrobial activity against tested pathogens in Table 4. AHCK displayed a balanced fatty acid profile with high levels of oleic acid (31.44 g/100 g) and linoleic acid (30.83 g/100 g). Medium-chain saturated fatty acids can serve as antipersister and antibiofilm agents by increasing membrane fluidity and enhancing antimicrobial susceptibility (Desbois & Smith, 2010).

The saponification values varied significantly among species: BSFL (212.46 mgKOH/g) > SWP (190.24 mgKOH/g) > AHCK (110.25 mgKOH/g). Results were comparable to previous studies showing edible insect oil saponification values ranging from 171.00 to 191.51 mgKOH/g (Kinyuru, 2021; Susirirut, Thitipramote, & Chaiwut, 2023). Higher saponification values indicate shorter fatty acid chains and higher concentrations of medium-chain fatty acids, which are more suitable for antimicrobial applications (Kabara, Swieczkowski, Conley, & Truant, 1972). BSFL's exceptionally high saponification value reflects its abundance of medium-chain fatty acids, particularly lauric acid, directly correlating with its superior antimicrobial performance.

The unsaponifiable matter content ranged from 1.62 % (BSFL) to 3.16 % (AHCK). Unsaponifiable compounds include sterols, tocopherols, carotenoids, and triterpene alcohols that contribute anti-inflammatory, antioxidant, and antimicrobial properties (Georgel et al., 2005). AHCK's higher unsaponifiable content may contribute to its moderate antimicrobial activity through synergistic effects with fatty acids. Most

oils of normal purity contain less than 2 % unsaponifiable matter, while higher values may indicate superior bioactive compound content (Codex Alimentarius, 2019).

3.4.2. Species-specific antimicrobial peptide activity

The peptide fractions (<3 kDa) demonstrated distinct antimicrobial profiles compared to their oil counterparts, as illustrated in Fig. 5. The disk diffusion assay results show that BSFL peptides exhibited the broadest spectrum activity, with measurable inhibition zones against both gram-positive bacteria (*B. cereus*: 0.85 ± 0.04 mm) and gram-negative bacteria (*E. coli*: 0.82 ± 0.02 mm, *S. typhimurium*: 0.75 ± 0.04 mm, and *P. aeruginosa*: 1.17 ± 0.02 mm). This comprehensive activity profile distinguishes BSFL peptides from AHCK peptides, which showed selectivity for gram-positive bacteria (*B. cereus*: 0.93 ± 0.05 mm and *S. aureus*: 1.25 ± 0.04 mm), and SWP peptides, which demonstrated activity against *B. cereus* (0.88 ± 0.06 mm) and *E. coli* (0.88 ± 0.02 mm) but failed to inhibit other tested pathogens.

Most notably, BSFL peptides demonstrated activity against *P. aeruginosa* (1.17 ± 0.02 mm zone radius, corresponding to 8.34 ± 0.04 mm total diameter), a pathogen for which neither AHCK nor SWP peptides nor insect oils showed any inhibitory effects (0.00 ± 0.00 mm). This finding is particularly significant because *P. aeruginosa* is notorious for its intrinsic resistance to many antimicrobial agents due to multiple defense mechanisms including low outer membrane permeability, constitutive multidrug efflux pumps, and biofilm formation capabilities (Lister, Wolter, & Hanson, 2009; Moradali, Ghods, & Rehm, 2017). Importantly, none of the tested insect oils (from any species) exhibited measurable activity against *P. aeruginosa* (Figs. 3–4, Tables 2–4), underscoring that the anti-*Pseudomonas* activity is specifically attributable to the peptide fraction. The observed activity, while modest compared to effects on other pathogens, is noteworthy given *P. aeruginosa*'s role in food spoilage of fresh produce, dairy products, and minimally processed foods, as well as its significance as an opportunistic pathogen in healthcare settings (Silby, Winstanley, Godfrey, Levy, & Jackson, 2011). The mechanism by which BSFL peptides overcome *P. aeruginosa*'s resistance barriers likely involves the cationic, amphiphilic nature of insect antimicrobial peptides such as defensins and cecropins (Table 6), which can interact with negatively charged lipopolysaccharides in the outer membrane through self-promoted uptake pathways (Hancock & Sahl, 2006; Yeaman & Yount, 2003). However, the anti-*Pseudomonas* activity was demonstrated under in vitro disc diffusion conditions and represents preliminary evidence. Future studies should determine quantitative MIC values for *P. aeruginosa* and explore structural modifications or synergistic combinations with other antimicrobials to enhance efficacy against this resistant pathogen for practical food preservation applications.

The recovery of bioactive peptides following extensive proteolytic processing during SGD demonstrates remarkable digestive stability, with the molecular weight constraint (<3 kDa) suggesting selection for structurally optimized fragments that maintain antimicrobial efficacy despite gastrointestinal degradation. The tetrapeptide motifs identified within larger antimicrobial sequences indicate cryptic bioactive domains responsible for retained post-digestion activity, representing evolutionary optimization for oral delivery potential.

Table 6 reveals functional diversity among insect-derived AMPs recovered following SGD, representing multiple peptide families with distinct mechanistic properties and broad therapeutic potential. The identified peptides encompass α-helical cecropins (Cecropin A, B) lacking cysteine residues that exhibit broad-spectrum bacterial activity through carpet-like membrane disruption mechanisms, cysteine-rich defensins (So-D4) with characteristic β-sheet structures targeting gram-positive bacteria, and proline-rich peptides (Apidaecin, ApCe1) that employ Type II antimicrobial mechanisms targeting bacterial ribosomal machinery rather than membrane disruption (Aleksandrova et al., 2024; Stączek, Kunat-Budzyńska, Cytryńska, & Zdybicka-Barabas, 2024). These structural classifications align with comprehensive reviews indicating that insect AMPs can be categorized into three major classes: linear α-helical peptides without cysteine residues, peptides with β-sheet globular structures stabilized by intramolecular disulfide bridges, and peptides containing unusually high numbers of specific amino acid residues such as proline or glycine (Ma et al., 2023; Mylonakis, Podsiadlowski, Muhammed, & Vilcinskas, 2016). Glycine-rich regions provide flexibility for membrane insertion, while proline residues create β-turn structures essential for amphiphilic peptide folding (Pan et al.,

Table 6
Amino acid sequences and bioactivity profiles of insect-derived antimicrobial peptides (<3 kDa) recovered following SGD, demonstrating structural diversity and antimicrobial spectrum across bacterial, fungal, and viral pathogens.

Peptide name	Insect	Sequence	Active against	Reference
Caeridin 1.1	Cricket	GLLDGLLGTLGLG	Bacterial	This study
Chromacin		YPGPQAKEDSEGPSQGPASREK	Bacterial	
Apidaecin II		GNNRPIYIPQPRPPHPRL	Bacterial	
So-D4 (defensin)		MFFSSKKCKTVSKTFRGPCVRNA	Bacterial	
Antifungal peptide	Black soldier fly larvae	PSHTGMSVPPP	Fungal	
Antibacterial peptide		GNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSSTSA	Bacterial	
Caeridin 1.4		GLLDGLLGGLGLG	Bacterial	
Caprine LF	Silkworm	PEWSKCYQWQRRMRKLGAPSITCVRRTSA	Bacterial and fungal	
Melitin		GIGAVLKVLTTGLPALISWIKRKRQQ~	Bacterial	
Caeridin 7.1		GLLDMVTGLLGNLG	Bacterial	
AnFl2	Solitary potter wasp	GILRSLGWIQMPRSRRRHR	Bacterial	Richter et al. (2022)
ApCe1	Honey bee	GIYTGRLLPVYIPQPRPPHPRLRR	Bacterial	
Apidaecin	Insect	GNNRPVYIPQPRPPHPRI	Bacterial	Marcos, Muñoz, Pérez-Payá, Misra, and López-García (2008)
Cecropin A		KWKFKKIEKMGRNIRDGI VKAGPAIEVIGSAKAI	Bacterial	
Cecropin B		KWKVFKKIEKMGRNIRN GIVKAGPAIAVLGEAKAL	Precursor of antibacterial peptide	
Alloferon 1	Blow fly	HGVSGHGQHGVHG	Virus, cancer cells.	Chernysh et al. (2002)
Alloferon 2		GVSGHGQHGVHG	Virus, cancer cells.	
Mastoparan-S	Giant African Mantis	LRLKSIVSYAKKVL	Bacterial - *E. coli* - *K. pneumoniae*	Zare-Zardini et al., (2015)

Note: Amino acid sequences are presented using standard single-letter abbreviations. Blue letters indicate conserved tetrapeptide motifs identified within antimicrobial peptide sequences that may contribute to bioactivity retention following gastrointestinal digestion.

2022; Rivero-Pino, Leon, & Montserrat-de la Paz, 2024). The glycine-rich Caeridin family peptides (1.1, 1.4, 7.1) demonstrate these conserved amphipathic motifs essential for membrane permeabilization, while the unique alloferon peptides (Alloferon 1, 2) from blow fly sources represent immunomodulatory agents with antiviral and anti-cancer properties through natural killer cell activation and interferon synthesis stimulation (Appiah et al., 2024; Chernysh et al., 2002).

Insect AMPs disrupt membranes and intracellular targets through multiple mechanisms, reducing resistance development potential compared to conventional single-target antibiotics (Mylonakis et al., 2016; Wu, Patočka, & Kuča, 2018). These peptides typically exhibit cationic, amphiphilic structures that facilitate electrostatic interactions with negatively charged bacterial membranes, followed by membrane insertion and pore formation through barrel-stave, carpet, or toroidal pore mechanisms (Stączek, Cytryńska, & Zdybicka-Barabas, 2023). AMPs lead to cell lysis by interfering with various cellular processes including protein synthesis, DNA replication, and enzyme activity, without easily allowing microbes to develop drug resistance (Luo & Song, 2021; Ma et al., 2023; Wu et al., 2018; Zhang et al., 2021). The superior antimicrobial performance of BSFL peptides can be attributed to their evolutionary adaptation to highly pathogenic environments, as these larvae naturally inhabit decaying organic matter rich in diverse microbial communities (Van Moll, De Smet, Cos, & Van Campenhout, 2022; Xia, Ge, & Yao, 2021). Recent proteomic analyses have identified multiple antimicrobial peptide families in BSFL, including defensins, cecropins, and diptericins, which function through complementary membrane-disruption mechanisms (Fahmy et al., 2024; Pimchan et al., 2024). Previous research has identified several antimicrobial peptides from dipteran insects with significant bioactive properties, including StomoxynZH1 peptide from BSFL larvae demonstrating inhibitory activity against *S. aureus*, *E. coli*, *R. solani*, and *S. sclerotiorum* (Elhag et al., 2017), and three peptides isolated from BSFL digestive tract—Hidefensin-1, Hidiptericin-1, and HiCG13551—showing antimicrobial potential against *S. pneumoniae*, *E. coli*, and *S. aureus* (Xu et al., 2020).

The synergistic action of multiple peptide families likely contributes to the broad-spectrum activity observed, as recent studies demonstrate that antimicrobial peptide combinations exhibit enhanced efficacy and reduced resistance development compared to single-compound applications (Candian & Tedeschi, 2023). Therefore, combining laurate-rich BSFL oil with AMPs offers synergistic effects, supporting advanced delivery systems like nanoemulsions or active films (Casillas-Vargas et al., 2021; Weng, Marcet, Rendueles, & Díaz, 2023; Yoon et al., 2018).

4. Conclusions

This investigation demonstrates the significant potential of edible insect-derived bioactive compounds as sustainable antimicrobial alternatives in food preservation. BSFL emerged as the most promising source, with exceptional lauric acid content (24.11 g/100 g total fatty acids, representing 161-fold enrichment compared to AHCK and 345-fold compared to SWP) directly correlating with superior broad-spectrum antimicrobial performance through membrane-disrupting mechanisms. US pretreatment consistently outperformed HPP, achieving 2.4–3.8 fold improvements ($p < 0.05$) via acoustic cavitation-induced cellular disruption. The synergistic integration of US pretreatment with SC-CO_2 extraction at 35 MPa represents an optimal green technology approach, maximizing bioactive compound liberation while maintaining quality standards and eliminating organic solvent residues.

Species-specific profiles revealed mechanistic insights: BSFL's medium-chain fatty acid dominance enables potent membrane disruption against both Gram-positive and Gram-negative bacteria, SWP's omega-3 richness provides moderate antimicrobial activity with additional health benefits, and AHCK's balanced fatty acid composition enables effective inhibition of Gram-positive pathogens. The collective antimicrobial efficacy demonstrated by these insect-derived bioactives against major foodborne pathogens (*S. aureus*, *E. coli*, *B. cereus*, *S. typhimurium*) positions them as promising next-generation natural preservatives with sustainable production potential and clean-label appeal. Limitations of this study include the in vitro nature of antimicrobial testing. Future research should validate efficacy in relevant food matrices (meat, dairy, beverages) and assess stability under processing and storage conditions.

CRediT authorship contribution statement

Nantawan Boonmee: Writing – original draft, Methodology, Formal analysis, Data curation. **Andrea M. Liceaga:** Writing – review & editing, Supervision, Resources, Investigation. **Chayaphon Sriphannam:** Supervision, Resources, Conceptualization. **Khanitta Ruttarattanamongkol:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

This manuscript was edited with the assistance of Claude (Anthropic), an artificial intelligence language model, for linguistic refinement and grammatical accuracy. All content was subsequently reviewed, verified, and edited by the authors, who maintain full responsibility for the manuscript's content and accuracy.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2025.104371.

Data availability

The data that has been used is confidential.

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Effects of Ultrasound and High-Pressure Pretreatments Combined with Supercritical CO_2 Extraction on Oil Yield and Properties of House Cricket (*Acheta domesticus*)

BP-0229

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ABSTRACT

House crickets (*Acheta domesticus*) have emerged as a promising alternative source of high-quality proteins and oils with potential health benefits. This study investigated the effects of pretreatments (high-pressure processing and ultrasound) combined with supercritical CO_2 extraction (25 – 35 MPa, 30 min, 37°C) on the yield and properties of house cricket oils. The extracted oils were analyzed for yield, peroxide value, acid value, thermal properties, and fatty acid profile. The results showed that the oil yield ranged from 36.23 to 102.82% compared to the initial oil content obtained using the Soxhlet method. The peroxide value and acid value of the extracted oils ranged from 1.11 to 21.18 mEqvO_2/kg and 3.80 to 78.98 mgKOH/g, respectively. Ultrasonic pretreatment at 37 kHz for 15 min altered the thermal properties of the extracted oils compared to the control. The extracted house cricket oil presented a wide range of melting peaks and crystallization peak, from -35.26 to 22.56 °C and -53.71 to 12.90°C, respectively, which makes them liquid-like at room temperature. Ultrasound had effected on thermal properties that changed melting and crystallization of house cricket oils. The fatty acid profile of the extracted oils revealed a significant content of health-beneficial unsaturated fatty acids, such as oleic acid (ω-9) at 31.60 g/100 g and linoleic acid (ω-6) at 31.01 g/100 g.

1. INTRODUCTION

In recent years, the growing demand for alternative sources of protein and oils has been driven by concerns over the sustainability and safety of traditional meat proteins and plant oils (1,2).

Edible insects have emerged as a promising alternative, with over 2,000 species identified worldwide (3). Among these, crickets have gained popularity due to their high protein (42.0-70.0% dry basis) and fat (9.71-29.1% dry basis) content (4,5). Consequently, cricket proteins and oils have the potential to be incorporated into human diets and animal feeds. Ugar, A.E. et al. (6) reported that the major of fatty acids of cricket oil includes are omega-6 (30-40%), omega-9 (23-27%), palmitic (24-30%) and stearic (7-11%). Their content of potential oil as omega-3, omega-6, which can inhibit the production of inflammation-inducing prostaglandin hormones and shown potential of reducing the risk of coronary artery disease (7). It has been found to benefit the health of mice and pigs (8). However, to fully exploit this potential, it is crucial to understand the qualities, fatty acid compositions, and biological properties of cricket oils.

Pretreatment processes prior to oil extraction have gained increasing attention as a means to enhance extraction efficiency. Novel extraction techniques, such as high-pressure processing (HPP) and ultrasound (UL), have become popular due to their reported ability to improve oil extraction yield and reduce extraction time compared to conventional methods (9,10). The combination of these high-potential technologies with supercritical CO_2 (SC-CO_2) extraction is of particular interest. HPP can disrupt lipid bilayers (11), while ultrasound generates cavitation, resulting in the breakdown of cell walls (12). These mechanisms may enhance the efficiency of SC-CO_2 extraction.

The objective of this study was to extract oils from house crickets using SC-CO_2 extraction by comparing HPP and UL pretreatment processes. Oil yield, oil qualities, oil characteristics, and fatty acid composition of the extracted cricket oils were evaluated.

2. MATERIAL AND METHODS

2.1 Materials

House crickets (*Acheta domesticus*) were obtained from Thai Ento Food Company Limited, Thailand. The raw materials were cleaned using distilled water to remove extraneous materials and boiled at 100 °C for 5 min to reduce biocontamination. One kilogram of cleaned fresh cricket were packed in polyethylene 8 × 12 inch and stored at -18 °C until use. All reagents were of analytical grade and purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2 Pretreatment processes

UL pretreatment was performed using an ultrasonic bath (E100H, Elma, Germany). Cleaned fresh house cricket was thawed before UL pretreatment. Then, the samples were separated to three portions. Each 100 grams of house crickets were weighed, placed in a polyethylene bag (4x6 inches) and 50 ml of distilled water were added. The sample was then placed in the ultrasonic bath containing distilled water as the liquid medium. UL pretreatment was conducted at 37 kHz for 5, 10, and 15 min. The pretreatment temperature was maintained at 37 ± 1°C using an ice bath.

HPP pretreatment was carried out using a high-pressure machine (HPP600MPa/ 3-5L Pilot Scale; Baotou Kefa Inc., Baotou City, China) with a maximum operating pressure capacity of 600 MPa. Fifty grams of house crickets were placed in a polyethylene bag (4x6 inches) and then loaded into the 5 L high-pressure vessel containing distilled water as the hydrostatic fluid. HPP pretreatment was performed at pressures of 200, 400, and 600 MPa, at 37 ± 1°C for 5 min.

Non-pretreated house crickets were used as a control sample for comparing the oil extraction yield and oil quality with the pretreated samples. All samples were measured in trip After finished pretreatment processes, all samples were dried using hot air oven at 70°C for 8-10 h.

2.3 *Supercritical CO_2 extraction*

SC-CO_2 extraction was performed using a pilot-scale SC-CO_2 extraction machine (SEF-01L, CAREDDI SCF, China). The extraction process was carried out in a stainless-steel container (1 L) at various pressures (25, 30, and 35 MPa) and a constant temperature of 37 ± 1°C for 30 min. The SC-CO_2 flow rate was maintained at 24-26 L/h. For each extraction, 100 g of pretreated or non-pretreated dried house cricket, milled and sieved through 18 mesh. House cricket powders was filled in the SC-CO_2 extractor chamber and sealed. The extraction time was initiated once the SC-CO_2 system reached the desired pressure. After the extraction process was completed, the extracted house cricket oils and defatted house cricket powder were collected. The oils were then centrifuged at 6000 rpm for 20 min to remove any residual liquid contaminants.

2.4 *Oil extraction yield*

The yield of house cricket oils was calculated using the following equation (1).

$$\text{Yield (\%)} = \frac{W_1}{W_2} \text{ x } 100 \quad (1)$$

where W_1 is the total oil yield (g) of house cricket successive fraction by SC-CO_2 and W_2 is the weight of ground house cricket (g).

2.5 *Oil quality*

Peroxide value (PV) and acid value (AV) were used to indicate the quality of oils. The PV and AV of house cricket oils was determined using the acid-base titration method according to the AOAC (1990) (13). All analysis was done in triplicates.

2.6 *Oil characterization*

Differential scanning calorimeters (DSC) (TA Instruments DSC 25; New Castle, DE, USA.) was used to analyze the crystallization and melting properties of house cricket oils, according to the modified procedure by Chou, T.H. et al. (14). Ten milligrams of selected house cricket oils were weighted into an aluminium pan and sealed with aluminium lid. An empty aluminium pan was used as a reference. The DSC condition was set at a temperature range of -60 to 60 °C with a heating of 5 °C per min.

2.7 *Fatty acid profile*

Fatty acid profiles of selected house cricket oils were performed according to the procedure of AOAC (2012) 996.06 (15) using the GC-MS technique. Identification of peaks was done by comparison with relative retention times for the standard fatty acid methyl esters (FAMEs). Concentrations of each fatty acid were reported as g/100 g oil.

2.8 *Statistical analysis*

For statistical analysis, all samples were performed in triplicate. Three time of SC-CO_2 were performed for each pretreated sample. All data were analysed using the general linear model procedure in SPSS (IBM SPSS Statistics Ver. 26), are expressed as the mean ± SD. The $P<0.05$ was considered to indicate a statistically significant difference with Duncan's multiple range test.

3. RESULTS AND DISCUSSION

3.1 *Oil extraction yield*

Oil extraction yields of the control and pretreated (HPP or UL) house cricket obtained by SC-CO_2 extraction are presented in Figure 1. Among of all, UL15 combined SC-CO_2 300 MPa gave the highest of the yield that increasing oil extraction yield up to 102.82% Roselló-Soto E. et al. (16) reported the effect of UL for extracted oil that UL induces cavitation and shear forces. It can create pores on cell membrane of sample and oil can be easier released. The results of HPP combined SC-CO_2 extraction, it was expected that HPP would increase the extracted oil yield because of its ability on the cell wall disruption of samples (6). In contrast, HPP had slightly effect on oil extraction yield. Some of them were a bit decreased from control. It was hypothesized that pressure might have disrupted the structures of triglycerides (6). Kim, S.W. et al. (17) showed the oil yield of black soldier fly increased with pressure, from 16.0% at 15 MPa to 24.5% at 35 MPa, which might be due to the increasing oil solubility. Thus, more SC-CO_2 could penetrate into cells and leach more oil (6). The pores and damage allow increased diffusion and mass transfer of intracellular oil components (18).

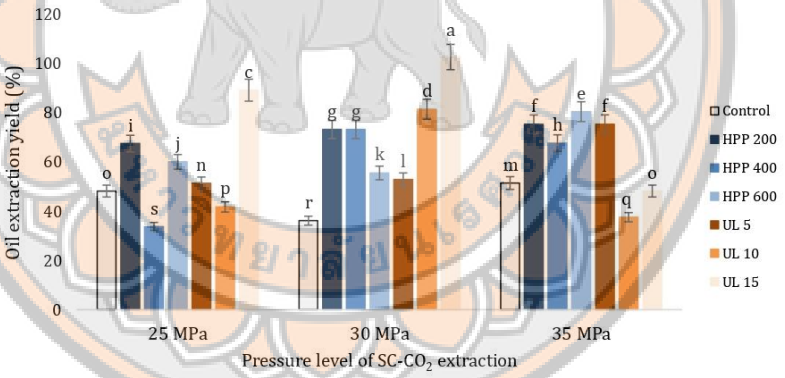

Figure 1. Oil extraction yield of pretreated house cricket using SC- CO_2 extraction. Different superscript letters of all samples were indicating statistical significance.

3.2 *Peroxide and acid value of house cricket oils*

Peroxide value of house cricket oil was evaluated and shown in Figure 2. A general rule is that the peroxide value of oils should not be above 10 mEqv.O_2/kg fat to avoid rancidity (19). Only

SC-CO_2 oils extracted from all UL pretreated samples had the peroxide value below the standard, while most of the oils obtained from HPP pretreated samples possessed peroxide values above 10 mEqv.O_2/kg fat.

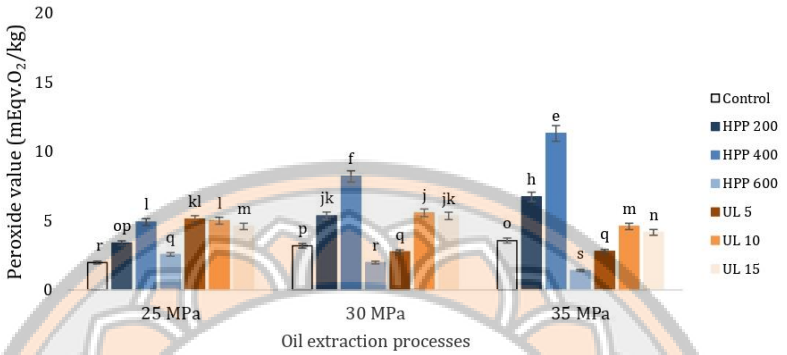

Figure 2. Peroxide value of pretreated house cricket oils obtained by SC-CO_2 extraction. Different superscript letters of all samples were indicating statistical significance.

HHP had a negative effect on the oxidation status of cricket oils. The pressure <300 MPa had a slight effect on lipid oxidation, but the oxidation increased above 300 MPa (6). A combination of CO_2 and HPP can reduce lipid oxidation (20). Increasing the pressure level can increase solvent solubility (21), which may explain why the 600 MPa HPP pretreatment resulted in lower lipid oxidation compared to the 400 MPa pretreatment. The higher pressure likely allowed for better CO_2 penetration into the samples, leading to more efficient extraction and reduced exposure of the lipids to oxidative conditions. On the other hand, Chemat et al. (22) reported that ultrasonic pretreatment might cause the release of free radicals in the sample, potentially contributing to an increase in peroxide value.

The acid value of house cricket pretreated using UL + SC-CO_2 decreased when increasing the pressure and extraction time (Figure 3). This result is similar to Kerras, H. et al. (23). HPP 200 and 400 MPa, acid value was increased when increasing pressure of SC-CO_2. Wang, H. et al. (24) found that the pressure of 200 MPa enhanced lipase activity by 18%. After the researcher received the house cricket samples. House crickets were kept at -18◦C for a long time before being boiled, which may have promoted the lipid oxidation reaction. The effects of pressure on lipid oxidation depend on the food matrix and treating conditions including pressure level, holding time, etc. In addition, the unsaturated fatty acid in oil at a pressure of 400 MPa and above became more sensitive to lipid oxidation, and lipid membranes were changed (25).

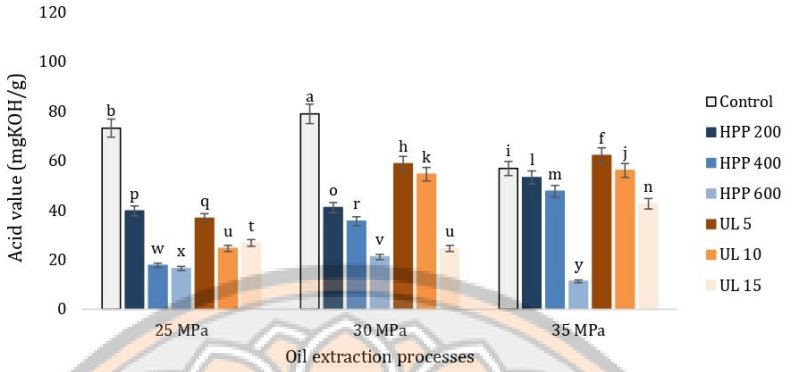

Figure 3. Acid value of pretreated house cricket oils obtained by SC-CO_2 extraction. Different superscript letters of all samples were indicating statistical significance.

3.3 Thermal properties of house cricket oils

After SC-CO_2 extraction, oil yield and oil quality were evaluated. UL15 combined SC-CO_2 at 30 MPa gave the highest oil yield and acceptable oil quality. House cricket oil pretreated by UL 37 kHz, for 15 min was determined for the melting and crystallization temperatures using DSC (Table 1 & Figure 4).

Table 1. Thermal properties of house cricket oils.

Treatments	Peak	Crystallization (°C)	Melting point (°C)
Control	1	-53.71	-21.18
	2	-44.32	-14.56
	3	-18.37	-4.70
	4	3.38	2.71
	5	-	14.67
UL15	1	-22.97	-35.26
	2	-14.53	-15.10
	3	0.34	-0.93
	4	12.90	10.74
	5	19.81	22.52

* Control = house cricket oil control extracted using SC-CO_2 30 MPa, UL15 = house cricket oil pretreated by ultrasonic 37 kHz for 5 min and extracted oil using SC-CO_2 30 MPa.

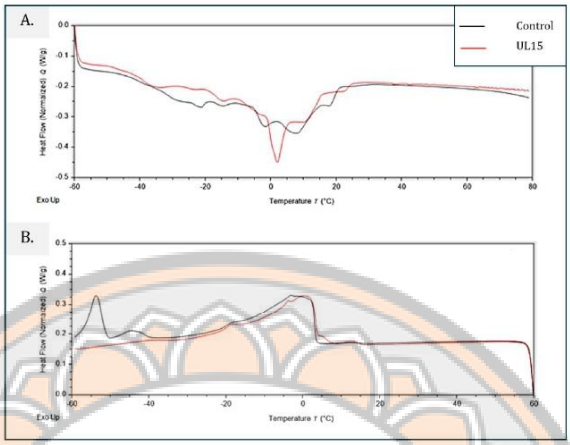

Figure 4. DSC thermogram of house cricket oil. (A.) crystal point and (B.) melting point.

House cricket oil pretreated by UL15 combined SC-CO_2 30 MPa was evaluated thermal properties and compared with control combined SC-CO_2 30 MPa. UL15 showed at least five crystal point that ranged from -22.97 °C to 19.81 °C, for control was from -53.71 °C to 3.38 °C. Changing of crystallization points indicates that the effect by different of triacylglycerol in oil composition (26). For melting point of UL15 was from -35.26 °C to 22.52 °C, control was from -21.18 °C to 14.67 °C. The fatty acid composition affected the thermal properties of oils, which has a high concentration of unsaturated fatty acids, has a low melting point (27). Ugar, A.E. et al. (6) reported that house cricket oil extracted by hexane 1: 15 w/v had crystallization and melting point at - 2. 26 and 1. 11°C, respectively. It is different from this study because used difference oil extraction method.

3.4 Fatty acid profiles

The fatty acid of house cricket oil, subjected to UL 37 kHz, for 15 min combined SC-CO_2 at 35 MPa pressure, were analyzed and presented in Table 2.

Table 2. Fatty acid composition of UL15 combined SC-CO_2.

Fatty acid compositions	Fatty acid content (g/100 g)
Oleic acid (C18:1c9)	31.44
Linoleic acid (C18:2)	30.83
Linolenic acid (C18:3)	0.84
Saturated fatty acid	35.83
Unsaturated fatty acid	64.18
Monounsaturated fatty acid	32.39
Polyunsaturated fatty acid	31.79
Tran fat	0.10
Omega 3	0.78
Omega 6	31.01
Omega 9	31.60

House cricket oil has high levels of omega-6 and omega-9 fatty acids. The fatty acid profiles of the house cricket oils also revealed significant amounts of C18:1c9, and C18:2, which are known for their health benefits. Additionally, all insect oils were found to contain high levels of omega-6 and omega-9 fatty acids, which play crucial roles in blood clotting, wound healing, immune system support, and cholesterol regulation (28,29). This highlights the potential of insect oils as nutraceuticals.

4. CONCLUSIONS

This study demonstrated that ultrasound (UL) and high-pressure processing (HPP), can enhance oil extraction from house crickets using supercritical CO_2 (SC-CO_2) extraction. The combination of UL pretreatment for 15 min with SC-CO_2 extraction at 30 MPa proved to be the most suitable condition, yielding the highest oil extraction efficiency (102.82%) while maintaining lower peroxide and acid values, indicating reduced lipid oxidation. The application of UL and HPP pretreatments in combination with SC-CO_2 extraction offers a promising approach for the efficient extraction and utilization of these valuable components from house crickets.

5. ACKNOWLEDGEMENTS

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แบบ สป/สผ/อสป/001-ก
หน้า 1 ของจำนวน 2 หน้า

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☑ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ. 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ. 2535 และ พระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ. 2542

สำหรับเจ้าหน้าที่	
วันรับคำขอ 17/01/2566	เลขที่คำขอ 2303000116
วันยื่นคำขอ	
สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ใช้กับแบบผลิตภัณฑ์ ประเภทผลิตภัณฑ์	
วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
ลายมือชื่อเจ้าหน้าที่	

1. ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์ กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน

2. คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☐ บุคคลธรรมดา ☐ นิติบุคคล ☑ หน่วยงานรัฐ ☐ มูลนิธิ ☐ อื่นๆ
ชื่อ สำนักงานการวิจัยแห่งชาติ
ที่อยู่ 196 ถ.พหลโยธิน
ตำบล/แขวง ลาดยาว อำเภอ/เขต จตุจักร จังหวัด กรุงเทพมหานคร รหัสไปรษณีย์ 10900 ประเทศ ไทย
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☐ เลขประจำตัวประชาชน ☐ เลขทะเบียนนิติบุคคล ☑ เลขประจำตัวผู้เสียภาษีอากร 0 0 9 4 0 0 0 1 5 9 9 5 1 ☑ เพิ่มเติม (ดังแนบ)
ในกรณีที่กรมฯ สื่อสารกับท่าน ท่านสะดวกใช้ทาง ☐ อีเมลผู้ขอ ☑ อีเมลตัวแทน

3.1 สัญชาติ ไทย	
3.2 โทรศัพท์	
3.3 โทรสาร	

4. สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5. ตัวแทน (ถ้ามี)
ชื่อ นางสาวกัญญารัตน์ ประทุมศิริ
ที่อยู่ กองส่งเสริมการบริการวิชาการ มหาวิทยาลัยนเรศวร 99
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เลขประจำตัวประชาชน 3 6 5 9 9 0 0 6 4 3 7 9 7 ☐ เพิ่มเติม (ดังแนบ)

5.1 ตัวแทนเลขที่ 2350	
5.2 โทรศัพท์ 055-968617	
5.3 โทรสาร 055-968603	

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ ☐ ชื่อและที่อยู่เดียวกันกับผู้ขอ
ชื่อ นางสาวชนิษฐา รุดรัตนมงคล
ที่อยู่ คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติ และสิ่งแวดล้อม มหาวิทยาลัยนเรศวร หมู่ที่ 99 หมู่ 9
ตำบล/แขวง ท่าโพธิ์ อำเภอ/เขต เมืองพิษณุโลก จังหวัด พิษณุโลก รหัสไปรษณีย์ 65000 ประเทศ ไทย
อีเมล
เลขประจำตัวประชาชน 3 6 0 0 1 0 1 1 2 6 6 0 2 ☑ เพิ่มเติม (ดังแนบ)

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร
เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

สำหรับเจ้าหน้าที่

จำแนกประเภทสิทธิบัตร/อนุสิทธิบัตร

☐ กลุ่มวิศวกรรม	☐ กลุ่มเคมี	สิทธิบัตรการออกแบบ	อนุสิทธิบัตร
สิทธิบัตรการประดิษฐ์ (วิศวกรรม)	สิทธิบัตรการประดิษฐ์ (เคมีเทคนิค)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 1)	☐ อนุสิทธิบัตร (วิศวกรรม)
สิทธิบัตรการประดิษฐ์ (ไฟฟ้า)	สิทธิบัตรการประดิษฐ์ (ปิโตรเคมี)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 2)	☐ อนุสิทธิบัตร (เคมี)
สิทธิบัตรการประดิษฐ์ (ฟิสิกส์)	สิทธิบัตรการประดิษฐ์ (เทคโนโลยีชีวภาพ)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 3)	
	สิทธิบัตรการประดิษฐ์ (เภสัชภัณฑ์)		

Signed by DIP-CA

8. การยื่นคำขอนอกราชอาณาจักร ☐ PCT ☐ เพิ่มเติม (ดังแนบ)

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย
☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9. การแสดงการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด
วันแสดง วันเปิดงานแสดง ผู้จัด

10. การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียนฝากเก็บ	10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ

11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้
โดยขอยื่นเป็นภาษา
☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ

12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา

13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย

ก. แบบพิมพ์คำขอ	3	หน้า
ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์	4	หน้า
ค. ข้อถือสิทธิ	1	หน้า
ง. รูปเขียน	รูป	หน้า
จ. ภาพแสดงแบบผลิตภัณฑ์		
☐ รูปเขียน	รูป	หน้า
☐ ภาพถ่าย	รูป	หน้า
ฉ. บทสรุปการประดิษฐ์	1	หน้า

14. เอกสารประกอบคำขอ
☑ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์
☐ หนังสือมอบอำนาจ
☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ
☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย
☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ
☐ เอกสารอื่นๆ

15. ข้าพเจ้าขอรับรองว่า
☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน
☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก

16. ลายมือชื่อ
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☑ ตัวแทน
(นางสาวกัญญารัตน์ ประทุมศิริ)

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

แบบ สป/สผ/อสป/012-ก
หน้า 1 ของจำนวน 1 หน้า

ใบแนบต่อท้าย สป/สผ/001-ก

2. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร

2. ชื่อ มหาวิทยาลัยนเรศวร

ที่อยู่ 99 หมู่ที่ 9 ต.ท่าโพธิ์ อ.เมืองพิษณุโลก จ.พิษณุโลก 65000 ประเทศ ไทย

อีเมล suleeratc@nu.ac.th สัญชาติ ไทย โทรศัพท์ 055968617

เลขประจำตัวผู้เสียภาษีอากร

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์

2. ชื่อ นางสาวนันทวรรณ บุญมี

ที่อยู่ คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติ และสิ่งแวดล้อม มหาวิทยาลัยนเรศวร หมู่ที่ 99 หมู่ 9 ต.ท่าโพธิ์ อ.เมืองพิษณุโลก จ.พิษณุโลก 65000 ประเทศไทย

สัญชาติ ไทย

เลขประจำตัวประชาชน 1601200098717

รายละเอียดการประดิษฐ์

ชื่อที่แสดงถึงการประดิษฐ์

กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน

สาขาวิทยาการที่เกี่ยวข้องกับการประดิษฐ์

วิทยาศาสตร์และเทคโนโลยีการอาหารในส่วนที่เกี่ยวข้องกับกรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน

ภูมิหลังของศิลปะหรือวิทยาการที่เกี่ยวข้อง

แมลงมีความสัมพันธ์กับมนุษย์ทั้งในด้านที่ก่อให้เกิดประโยชน์และโทษ โดยจากการสำรวจพบว่าในจำนวนแมลงทั้งหมดที่พบอยู่ในโลกมีเพียงร้อยละ 0.01 ที่เป็นแมลงที่ก่อให้เกิดโทษต่อมนุษย์และอีกร้อยละ 99.99 เป็นแมลงที่ก่อให้เกิดประโยชน์กับมนุษย์ (สุปาณี, 2550) ในเบลเยี่ยม หน่วยงานรัฐบาลกลางด้านความปลอดภัยของห่วงโซ่อาหาร (FASFC) ได้ตีพิมพ์รายชื่อแมลงที่อนุญาตให้บริโภคได้จำนวน 12 ชนิด คือ *Acheta domesticus, Achroia grisella, Alphitobius diaperinus, Alphitobius laevigatua, Bombyx mori, Galleria mellonella, Gryllodes sigillatus, Gryllus assimilis, Locusta migratoria, Schistocerca americana, Tenebrio molitor* และ *Zophobas atratus* (Claeys และคณะ, 2015) มีรายงานเกี่ยวกับแมลงเพื่อการบริโภคในการเป็นแหล่งโปรตีนที่มีคุณภาพ โดยพบว่า จิ้งหรีดบ้านหรือสะดิ้ง มีปริมาณโปรตีนเฉลี่ยอยู่ในช่วงร้อยละ 55.00 – 70.70 มีปริมาณไขมันร้อยละ 9.80 - 22.80 ปริมาณแร่ธาตุร้อยละ 3.60 - 9.10 อีกทั้งยังมีกรดไขมันจำเป็น เช่น Linoleic acid และ Linolenic acid เป็นต้น (Laroche และคณะ, 2019)

จิ้งหรีด (Cricket) อยู่ในวงศ์ Gryllidae เป็นแมลงขนาดลำตัวขนาดกลางถึงขนาดใหญ่เมื่อเทียบกับแมลงทั่วไป สามารถพบได้ทั่วโลก โดยเฉพาะในเขตร้อนชื้น พบแล้วประมาณ 900 ชนิด (Wikipedia.org, 2565) จิ้งหรีดเป็นแหล่งของโปรตีนที่มีคุณภาพสูง โดยมีปริมาณโปรตีนอยู่ในช่วงร้อยละ 57.50 – 65.00 (Churchward-Venne , 2017) จากการคาดการณ์การเพิ่มขึ้นของประชากรโลกที่คาดว่าจะเพิ่มขึ้นเป็น 9.6 พันล้านคน ภายในปี 2050 (Huis และคณะ, 2013) แหล่งโปรตีนทางเลือกอื่นที่นอกเหนือไปจากแหล่งโปรตีนดั้งเดิม เช่น เนื้อหมู เนื้อวัว ไข่ เนื้อไก่ ถั่วเหลือง ข้าวสาลีและข้าวโพดจึงถูกพิจารณาเพื่อใช้เป็นแหล่งโปรตีนทดแทน ในขณะเดียวกันดักแด้ไหม (Silkworm) ที่เป็นแมลงเศรษฐกิจที่น่าสนใจอีกชนิดหนึ่งโดยรู้จักกันดีในอุตสาหกรรมผ้าไหม (พัชรินทร์, 2559) นอกจากนี้ ดักแด้ไหมยังสามารถนำมารับประทานได้ โดยพบว่าดักแด้มีโปรตีนสูงถึงร้อยละ 54.19 - 68.97 มีไขมันร้อยละ 11.67 - 12.59 ซึ่งเป็นไขมันชนิดที่ดี (อุไรวรรณ และชนะ, 2550) และมีคุณค่าทางโภชนาการสูง (ทิพย์วดี, 2556) และแมลงอีกหนึ่งชนิดที่กำลังได้รับความนิยมในปัจจุบันในการนำมันสกัดไขมันและโปรตีน คือ หนอนทหารเสือ หรือ หนอนแมลงวันลาย (Black soldier fly) ซึ่งเป็นหนอนแมลงวันชนิดหนึ่งสามารถช่วยย่อยสลายอินทรียวัตถุ สามารถพบได้ทั่วไปในสภาพภูมิอากาศเขตร้อนและเขตอบอุ่น ไม่ใช่แมลงที่เป็นพาหะของโรคหรือศัตรูพืช (กุลชาติ และทัศนีย์, 2554) ลักษณะของตัวหนอนหลังฟักออกจากไข่มีลักษณะแบนยาว ไม่มีขา เคลื่อนที่โดยใช้ตะขอที่บริเวณปากในการคืบคลาน หลังจากออกจากไข่จะสีขาวขุ่น และจะค่อยเปลี่ยนสีผนังลำตัวไปเป็นสีน้ำตาล

เมื่อมีอายุเพิ่มขึ้น ขนาดของหนอนนทหารเสือพบตั้งแต่ 5 – 19 มิลลิเมตร มีการเข้าดักแด้ก่อนเข้าสู่ระยะตัวเต็มวัย (รุ่งทิพย์, 2561) มีปริมาณโปรตีนในช่วงร้อยละ 43.16 – 44.36 ปริมาณไขมันร้อยละ 27.75 – 32.12 เยื่อใยร้อยละ 7.47 – 7.62 และเถ้าร้อยละ 9.41 – 7.63 นอกจากนี้ ยังประกอบด้วยแร่ธาตุที่สำคัญ เช่น แคลเซียม ฟอสฟอรัส เป็นต้น (Park, K., 2013) ด้วยปริมาณโปรตีนและไขมันที่สูง เมื่อเปรียบเทียบกับแหล่งโปรตีนจากอาหารเดิม พบว่า หนอนนทหารเสือมีปริมาณโปรตีนใกล้เคียงกับโปรตีนจากพืชและสัตว์ อีกทั้ง ยังมีรายงานองค์ประกอบกรดอะมิโนใกล้เคียงกับกรดอะมิโนจากเนื้อสัตว์ และองค์ประกอบกรดไขมันที่มีคุณสมบัติเชิงหน้าที่ที่น่าสนใจ เช่น น้ำมันหนอนนทหารเสือประกอบด้วยกรดลอริกร้อยละ 38.43 (Ushakova และคณะ, 2016) ซึ่งเป็นที่ทราบกันดีว่ากรดลอริกมีคุณสมบัติที่โดดเด่นในด้านของการเป็นสารต้านจุลินทรีย์ โดยทั่วไปสามารถพบกรดลอริกได้ในน้ำมันมะพร้าวและนมแม่ ด้วยเหตุนี้ แมลงทั้ง 3 ชนิดนี้จึงเป็นแมลงที่น่าสนใจในการนำมาสกัดโปรตีนและไขมันเพื่อใช้ประโยชน์ในอุตสาหกรรมอาหาร อาหารสัตว์ ตลอดจนอุตสาหกรรมเครื่องสำอาง

กระบวนการสกัดน้ำมันที่นิยมใช้กันอย่างแพร่หลาย เช่น กระบวนการสกัดด้วยตัวทำละลายอินทรีย์ เครื่องบีบอัด กระบวนการสกัดด้วยของไหลวิกฤติยิ่งยวด แต่กระบวนการสกัดน้ำมันดังกล่าวไม่เพียงพอในการสกัดน้ำมันออกจากตัวอย่างให้มีปริมาณต่ำกว่าร้อยละ 10 เพื่อใช้ในกระบวนการผลิตโปรตีนผงสำหรับการบริโภคได้ ดังนั้น กระบวนการปรับสภาพผนังเซลล์ของตัวอย่างก่อนการสกัดน้ำมันจึงมีความจำเป็น จากการสืบค้นข้อมูลจากงานวิจัย พบว่า กระบวนการปรับสภาพตัวอย่างที่น่าสนใจ ได้แก่ กระบวนการแปรรูปโดยใช้แรงดันสูง กระบวนการสนามไฟฟ้าแบบพัลซ์ กระบวนการให้ความร้อนแบบโอห์มมิค การประยุกต์ใช้คลื่นอัลตราโซนิก ซึ่งกระบวนการที่เหมาะสำหรับการศึกษานี้ คือ กระบวนการแปรรูปโดยใช้แรงดันสูง เนื่องจากเป็นกระบวนการที่ไม่ใช้ความร้อน จึงไม่มีผลต่อลักษณะทางประสาทสัมผัสของตัวอย่าง (อิรินันท์ และณัฐพร, 2562) แต่สามารถส่งผลต่อผนังเซลล์ของตัวอย่างโดยความดันสูงมีผลทำให้เกิดรูพรุนบนผนังเซลล์ตัวอย่าง ในขั้นตอนการคลายแรงดันเมื่อเสร็จสิ้นกระบวนการแปรรูป (Uhm and Yoon, 2011) การเกิดรูพรุนนี้เป็นผลดีในขั้นตอนการสกัดน้ำมัน คือ สารที่ใช้สกัดหรือตัวทำละลายสามารถแทรกผ่านเข้าไปภายในเซลล์ของตัวอย่างแล้วชะสารตัวอย่างออกมากับสารสกัดได้ดีมากขึ้น

กระบวนการแปรรูปโดยใช้แรงดันสูงเป็นกระบวนการแปรรูปอาหารที่ไม่ใช้ความร้อน (Non-thermal process) แต่ใช้ความดันในระดับที่สูงกว่าความดันบรรยากาศในช่วง 100 – 900 MPa ซึ่งสามารถทำลายเชื้อจุลินทรีย์ที่เป็นสาเหตุให้อาหารเสื่อมเสีย จุลินทรีย์ก่อโรค (Pathogen) และเอนไซม์ที่ทำให้อาหารเสื่อมคุณภาพ โดยกระบวนการแปรรูปโดยใช้แรงดันสูงเป็นกระบวนการที่ยังคงรสชาติและความสดของอาหารไว้ จึงถือได้ว่าเป็นการฆ่าเชื้อด้วยวิธีการพาสเจอร์ไรเซชันแบบเย็น (Cold pasteurization) (สุทธินี, 2564) หลักการทำงานของกระบวนการ คือ การให้แรงดันกับอาหารผ่านตัวกลาง เช่น น้ำ หรือของเหลวอื่นๆ เมื่อจุลินทรีย์ได้รับความดันที่สูงขึ้นจะส่งผลให้ผนังเซลล์ถูกทำลาย เกิดการรั่วไหลของสารจากภายในและภายนอกเซลล์ สารพันธุกรรมต่างๆ เช่น ไรโบโซม กรดนิวคลีอิก สังเคราะห์ได้ไม่สมบูรณ์และถูกทำลาย เซลล์จุลินทรีย์จึงไม่สามารถทำงานได้และตายในที่สุด (สุพรรณี, 2546; วิริยา, 2553)

จากข้อดีในด้านความสามารถในการทำให้เกิดรูพรุนหรือการแตกของผนังเซลล์ของเชื้อจุลินทรีย์ จึงเป็นไปได้ว่ากระบวนแปรรูปโดยใช้แรงดันสูงจะช่วยทำให้เกิดรูพรุนหรือการแตกของผนังเซลล์ของตัวอย่างและก่อให้เกิดการเพิ่มประสิทธิของกระบวนการสกัดน้ำมันได้ และจากการศึกษาการเปิดเผยต่างๆ ของกระบวนการแปรรูปโดยใช้แรงดันสูง พบว่า มีความเป็นไปได้ในการนำมาประยุกต์ใช้ในกระบวนการปรับสภาพแมลงสำหรับการบริโภคก่อนกระบวนการสกัดน้ำมันและผลิตโปรตีนผง ดังนั้น จึงเป็นที่มาของการประดิษฐ์นี้ ที่เป็นการศึกษากรรมวิธีการปรับสภาพแมลงสำหรับการบริโภคด้วยเทคนิคการแปรรูปด้วยการใช้แรงดันสูงก่อนกระบวนการสกัดน้ำมันเพื่อผลิตโปรตีนผงต่อคุณสมบัติทางเคมี – กายภาพ คุณสมบัติทางชีวภาพ และผลิตภัณฑ์ที่ได้จากกรรมวิธีดังกล่าว

ลักษณะและความมุ่งหมายของการประดิษฐ์

กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน เป็นการปรับสภาพแมลงสดด้วยการใช้แรงดันสูง ก่อนกระบวนการสกัดน้ำมันด้วยตัวทำละลายอินทรีย์หรือเทคนิคของไหลวิกฤติยิ่งยวดเพื่อผลิตโปรตีน โดยนำแมลงมาทำความสะอาด และบรรจุลงในถังของเครื่องแปรรูปโดยใช้แรงดันสูงที่ระดับความดันในช่วง 200 – 600 เมกะปาสคาล (MPa) เป็นเวลา 5 – 15 นาที แล้วนำไปอบแห้งและบดเป็นผง

ความมุ่งหมายของการประดิษฐ์นี้ คือ การเพิ่มประสิทธิภาพของกระบวนการสกัดน้ำมันออกจากแมลง ด้วยกรรมวิธีการปรับสภาพแมลงสดด้วยเทคนิคการแปรรูปด้วยการใช้แรงดันสูงทำให้สามารถสกัดน้ำมันออกจากผงแมลงได้มากขึ้น อีกทั้งยังช่วยลดการปนเปื้อนของเชื้อจุลินทรีย์ในวัตถุดิบเริ่มต้น รวมถึงสามารถต่อยอดและขยายกำลังการผลิตไปสู่ระดับอุตสาหกรรมได้

การเปิดเผยการประดิษฐ์โดยสมบูรณ์

กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน มีขั้นตอนดังนี้

ก. นำแมลงที่เลือกได้จาก จิ้งหรีด หนอนนกหารเสือ ดักแด้ไหม อย่างใดอย่างหนึ่งหรือรวมกัน มาทำความสะอาดทั้งตัวด้วยการล้างด้วยน้ำกลั่นและบรรจุตัวอย่างในถุงพลาสติกชนิดความหนาแน่นต่ำ (LDPE)

ข. นำแมลงที่ได้จากขั้นตอน ก. บรรจุลงในถังของเครื่องแปรรูปโดยใช้แรงดันสูง ที่ระดับความดันในช่วง 200 – 600 เมกะปาสคาล (MPa) เป็นเวลา 5 – 15 นาที ที่อุณหภูมิโดยรอบ (ambient temperature)

ค. นำแมลงที่ได้จากขั้นตอน ข. อบให้แห้งด้วยตู้อบลมร้อนที่อุณหภูมิ 65 - 75 องศาเซลเซียส เป็นเวลา 10 – 12 ชั่วโมง หรือจนกระทั่งแมลงมีความชื้นในช่วง 5 – 8 %

ง. นำแมลงอบแห้งที่ได้จากขั้นตอน ค.ไปบดเป็นผงและร่อนผ่านตะแกรงขนาด 15-20 เมช (Mesh) จะได้ผงแมลงสำหรับนำไปผลิตเป็นโปรตีนต่อไป

กระบวนการสกัดน้ำมันด้วยตัวทำละลาย (Solvent extraction)

ก. นำผงแมลงที่ผ่านการปรับสภาพด้วยการใช้แรงดันสูงใส่ในถังสกัดในอัตราส่วนผงแมลง:ตัวทำละลาย เท่ากับ 1:10 w/v

ข. สกัดตัวอย่างโดยใช้ magnetic bar สำหรับกวนตัวอย่างและตัวทำละลาย เป็นระยะเวลา 2 ชั่วโมง

ค. เมื่อครบระยะเวลาที่กำหนด ทำการแยกส่วนของเหลวที่มีแมลงอบแห้งละลายอยู่ กับส่วนตะกอนออกจากกันด้วยเครื่องปั่นเหวี่ยง (Centrifuge) ที่ความเร็วรอบ 6,000 รอบต่อนาที(rpm) เป็นระยะเวลา 15 นาที

ง. นำส่วนของเหลวใส่ในบีกเกอร์และทำการระเหยตัวทำละลายภายในตู้ดูดควัน เป็นระยะเวลา 24 ชั่วโมง และส่วนตะกอนนำใส่ภาชนะ และนำไประเหยตัวทำละลายออกเช่นเดียวกันกับส่วนใส

จ. เมื่อระเหยตัวทำละลายออกจนหมด ในส่วนของเหลวจะเหลือเพียงน้ำมันที่สกัดออกมาจากแมลงอบแห้ง และส่วนตะกอนจะได้เป็นผงแมลงแห้ง จากนั้นส่วนน้ำมันและผงแมลงหลังกระบวนการสกัดจะถูกนำไปวิเคราะห์คุณสมบัติทางเคมี - กายภาพ คุณสมบัติทางด้านชีวภาพ และส่วนตะกอนจะได้เป็นผงแมลงสำหรับใช้ในกระบวนการผลิตโปรตีนผงสำหรับการบริโภคในขั้นตอนการผลิตโปรตีนไอโซเลตหรือไฮโดรไลเซทจากแมลงสำหรับการบริโภคต่อไป

กระบวนการสกัดน้ำมันด้วยเทคนิคของไหลวิกฤติยิ่งยวด (Supercritical fluid extraction)

ก. นำผงแมลงที่ผ่านการปรับสภาพด้วยการใช้แรงดันสูงใส่ในถังสกัด

ข. ทำการสกัดน้ำมันด้วยคาร์บอนไดออกไซด์วิกฤติยิ่งยวด ที่ระดับความดันในช่วง 250 – 350 บาร์ (bar) ระยะเวลาในการสกัด 30 – 90 นาที ที่อุณหภูมิ 35 – 40 องศาเซลเซียส อัตราการไหลในช่วง 24 – 28 ลิตรต่อชั่วโมง (L/h.)

ค. เมื่อครบระยะเวลาสกัด ทำการเก็บน้ำมันจากถังบรรจุสารสกัด และเก็บกากแมลงหลังสกัดในถัง

ง. ส่วนน้ำมันและกากแมลงหลังสกัดจะถูกนำไปวิเคราะห์คุณสมบัติทางเคมี - กายภาพ คุณสมบัติทางด้านชีวภาพ และส่วนของกากแมลงจะนำไปผลิตโปรตีนผงต่อไป

วิธีการในการประดิษฐ์ที่ดีที่สุด

ดังได้บรรยายไว้ในหัวข้อการเปิดเผยการประดิษฐ์โดยสมบูรณ์

ข้อถือสิทธิ

1. กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน มีขั้นตอนดังนี้

ก) นำแมลงมาทำความสะอาดทั้งตัวด้วยการล้างด้วยน้ำกลั่นและบรรจุตัวอย่างในถุงพลาสติกชนิดความหนาแน่นต่ำ (LDPE)

ข) นำแมลงที่ได้จากขั้นตอน ก. บรรจุลงในถังของเครื่องแปรรูปโดยใช้แรงดันสูง ที่ระดับความดันในช่วง 200 – 600 เมกะปาสคาล (MPa) เป็นเวลา 5 – 15 นาที ที่อุณหภูมิโดยรอบ (ambient temperature)

ค) นำแมลงที่ได้จากขั้นตอน ข. อบให้แห้งด้วยตู้อบลมร้อนที่อุณหภูมิ 65 - 75 องศาเซลเซียส เป็นเวลา 10 – 12 ชั่วโมง หรือจนกระทั่งแมลงมีความชื้นในช่วง 5 – 8 %

ง) นำแมลงอบแห้งที่ได้จากขั้นตอน ค. ไปบดเป็นผงและร่อนผ่านตะแกรงขนาด 15-20 เมช (Mesh) จะได้ผงแมลงสำหรับนำไปผลิตเป็นโปรตีนต่อไป

2. กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน ตามข้อถือสิทธิ 1 ที่ซึ่ง แมลง เลือกได้จาก จิ้งหรีด หนอนทหารเสือ ดักแด้ไหม อย่างใดอย่างหนึ่งหรือรวมกัน

บทสรุปการประดิษฐ์

กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน เป็นการปรับสภาพแมลงสดด้วยการใช้แรงดันสูง ก่อนกระบวนการสกัดน้ำมันเพื่อผลิตโปรตีน โดยนำแมลงมาทำความสะอาด และบรรจุลงในถังของเครื่องแปรรูปโดยใช้แรงดันสูง ที่ระดับความดันในช่วง 200 – 600 เมกะปาสคาล (MPa) เป็นเวลา 5 – 15 นาที ที่อุณหภูมิโดยรอบ (ambient temperature) แล้วนำไปอบแห้งและบดเป็นผง ทำให้สามารถสกัดน้ำมันออกจากผงแมลงได้มากขึ้น อีกทั้งยังช่วยลดการปนเปื้อนของเชื้อจุลินทรีย์ในวัตถุดิบเริ่มต้น รวมถึงสามารถต่อยอดและขยายกำลังการผลิตไปสู่ระดับอุตสาหกรรมได้

หนังสือมอบอำนาจ

ทำที่ มหาวิทยาลัยนเรศวร
เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์
อำเภอเมืองพิษณุโลก
จังหวัดพิษณุโลก 65000

วันที่ 29 เดือน ธันวาคม พ.ศ. 2565

โดยหนังสือฉบับนี้ **สำนักงานการวิจัยแห่งชาติ** โดย ดร.วิภารัตน์ ดีอ่อง ตำแหน่ง ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ ที่อยู่เลขที่ 196 ถนนพหลโยธิน แขวงลาดยาว เขตจตุจักร กรุงเทพฯ 10900 และ

มหาวิทยาลัยนเรศวร โดย รองศาสตราจารย์ ดร.ศรินทร์ทิพย์ แทนธานี ตำแหน่ง รักษาราชการแทนอธิการบดีมหาวิทยาลัยนเรศวร ที่อยู่ เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000

ขอมอบอำนาจและแต่งตั้งให้ **นางสาวกัญญารัตน์ ประทุมศิริ** ตัวแทนสิทธิบัตรเลขที่ 2350 เป็นผู้ดำเนินการเป็นตัวแทนในนามของข้าพเจ้าอันแท้จริงที่จะยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตร **ชื่อผลงาน "กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน"** จดทะเบียน เปลี่ยนแปลงชื่อ ในส่วนที่เกี่ยวข้อง ถอนคำขอรับสิทธิบัตร/อนุสิทธิบัตร ยื่นคำคัดค้าน จดทะเบียนสิทธิบัตร/อนุสิทธิบัตร หรือยื่นคำขอให้มีการเพิกถอนสิทธิบัตร/อนุสิทธิบัตร ใดที่คล้ายหรือขัดกับสิทธิบัตร/อนุสิทธิบัตรดังกล่าว ให้มีอำนาจลงนาม และยื่นเอกสาร หรือข้อความใด ๆ ในนามของ ผู้มอบอำนาจซึ่งตัวแทนในฐานะดังกล่าวข้างต้นเห็นว่าจำเป็นหรือเหมาะสมเปลี่ยนแปลงแก้ไขเอกสารดังกล่าว และให้มีอำนาจที่จะดำเนินการใด ๆ ดังกล่าวข้างต้นซึ่งตัวแทนพิจารณาเห็นสมควรเพื่อดำเนินการตามหนังสือนี้

เพื่อเป็นหลักฐานแห่งการนี้ ข้าพเจ้าได้ลงลายมือชื่อ และประทับตรา (ถ้ามี) ไว้เป็นสำคัญ

ลงชื่อ.....ผู้มอบอำนาจ
(ดร.วิภารัตน์ ดีอ่อง)
ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ

ลงชื่อ.....ผู้มอบอำนาจ
(รองศาสตราจารย์ ดร.ศรินทร์ทิพย์ แทนธานี)
รักษาราชการแทนอธิการบดีมหาวิทยาลัยนเรศวร

ลงชื่อ.....ผู้รับมอบอำนาจ
(นางสาวกัญญารัตน์ ประทุมศิริ)

ลงชื่อ.....พยาน
(นางสาวธรรมภรณ์ ประภาสะวัต)
ผู้อำนวยการกองบริหารทุนวิจัยและนวัตกรรม 3

ลงชื่อ.....พยาน
(ดร.พิสุทธิ์ อภิชยกุล)
รองอธิการบดีฝ่ายวิเทศสัมพันธ์และการถ่ายทอดเทคโนโลยี

หนังสือสัญญาโอนสิทธิรับสิทธิบัตร/อนุสิทธิบัตร

ทำที่ มหาวิทยาลัยนเรศวร
เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์
อำเภอเมืองพิษณุโลก
จังหวัดพิษณุโลก 65000

วันที่ 29 เดือน ธันวาคม พ.ศ. 2565

หนังสือสัญญาฉบับนี้ทำขึ้นระหว่าง **"ผู้โอนสิทธิ"** คือ

1.รองศาสตราจารย์ ดร.ขนิษฐา รุตรัตนมงคล คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติ และสิ่งแวดล้อม มหาวิทยาลัยนเรศวร เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000

2.นางสาวนันทวรรณ บุญมี คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติ และสิ่งแวดล้อม มหาวิทยาลัยนเรศวร เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000

ผู้รับโอนสิทธิ คือ

สำนักงานการวิจัยแห่งชาติ โดย ดร.วิภารัตน์ ดีอ่อง ตำแหน่ง ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ ที่อยู่เลขที่ 196 ถนนพหลโยธิน แขวงลาดยาว เขตจตุจักร กรุงเทพฯ 10900 และ

มหาวิทยาลัยนเรศวร โดย รองศาสตราจารย์ ดร.ศรินทร์ทิพย์ แทนธานี ตำแหน่ง รักษาราชการแทนอธิการบดีมหาวิทยาลัยนเรศวร ที่อยู่ เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000

โดยหนังสือสัญญานี้ ผู้โอนซึ่งเป็นผู้ประดิษฐ์ **"กรรมวิธีการปรับสภาพแมลงด้วยการใช้แรงดันสูงเพื่อการผลิตโปรตีน"** ขอโอนสิทธิในการประดิษฐ์ดังกล่าว ซึ่งรวมถึงสิทธิขอรับสิทธิบัตร/อนุสิทธิบัตร และ/หรือสิทธิอื่นๆ ที่เกี่ยวข้องให้แก่ผู้รับโอน

เพื่อเป็นหลักฐานแห่งการนี้ผู้โอนและผู้รับโอนจึงลงลายมือชื่อไว้เป็นหลักฐานต่อหน้าพยาน

ลงชื่อ.....ผู้โอน
(รองศาสตราจารย์ ดร.ขนิษฐา รุตรัตนมงคล)

ลงชื่อ.....ผู้รับโอน
(ดร.วิภารัตน์ ดีอ่อง)
ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ

ลงชื่อ.....ผู้โอน
(นางสาวนันทวรรณ บุญมี)

ลงชื่อ.....ผู้รับโอน
(รองศาสตราจารย์ ดร.ศรินทร์ทิพย์ แทนธานี)
รักษาราชการแทนอธิการบดีมหาวิทยาลัยนเรศวร

ลงชื่อ.....พยาน
(นางสาวธรรมภรณ์ ประภาสะวัต)
ผู้อำนวยการกองบริหารทุนวิจัยและนวัตกรรม 3

ลงชื่อ.....พยาน
(ดร.พิสุทธิ์ อภิชยกุล)
รองอธิการบดีฝ่ายวิเทศสัมพันธ์และการถ่ายทอดเทคโนโลยี

แบบ สป/สผ/อสป/001-ก
หน้า 1 ของจำนวน 2 หน้า

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☑ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้
ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ. 2522
แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ. 2535
และ พระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ. 2542

สำหรับเจ้าหน้าที่

วันรับคำขอ 17/01/2566	เลขที่คำขอ 2303000118
วันยื่นคำขอ	
สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ใช้กับแบบผลิตภัณฑ์ ประเภทผลิตภัณฑ์	
วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
ลายมือชื่อเจ้าหน้าที่	

1. ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์ กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน

2. คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่
ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☐ บุคคลธรรมดา ☐ นิติบุคคล ☑ หน่วยงานรัฐ ☐ มูลนิธิ ☐ อื่นๆ
ชื่อ สำนักงานการวิจัยแห่งชาติ
ที่อยู่ 196 ถ.พหลโยธิน
ตำบล/แขวง ลาดยาว อำเภอ/เขต จตุจักร จังหวัด กรุงเทพมหานคร รหัสไปรษณีย์ 10900 ประเทศ ไทย
อีเมล saraban@nrct.go.th
☐ เลขประจำตัวประชาชน ☐ เลขทะเบียนนิติบุคคล ☑ เลขประจำตัวผู้เสียภาษีอากร 0 0 9 4 0 0 0 1 5 9 9 5 1 ☑ เพิ่มเติม (ดังแนบ)
ในกรณีที่กรมฯ สื่อสารกับท่าน ท่านสะดวกใช้ทาง ☐ อีเมลผู้ขอ ☑ อีเมลตัวแทน

3.1 สัญชาติ ไทย
3.2 โทรศัพท์
3.3 โทรสาร

4. สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5. ตัวแทน (ถ้ามี)
ชื่อ นางสาวกัญญารัตน์ ประทุมศิริ
ที่อยู่ กองส่งเสริมการบริการวิชาการ มหาวิทยาลัยนเรศวร 99
ตำบล/แขวง ท่าโพธิ์ อำเภอ/เขต เมืองพิษณุโลก จังหวัด พิษณุโลก รหัสไปรษณีย์ 65000 ประเทศ
อีเมล kanyaratp@nu.ac.th
เลขประจำตัวประชาชน 3 6 5 9 9 0 0 6 4 3 7 9 7 ☐ เพิ่มเติม (ดังแนบ)

5.1 ตัวแทนเลขที่ 2350
5.2 โทรศัพท์ 055-968617
5.3 โทรสาร 055-968603

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ ☐ ชื่อและที่อยู่เดียวกันกับผู้ขอ
ชื่อ นางสาวชนิษฐา รุดรัตนมงคล
ที่อยู่ คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติ และสิ่งแวดล้อม มหาวิทยาลัยนเรศวร หมู่ที่ 99 หมู่ 9
ตำบล/แขวง ท่าโพธิ์ อำเภอ/เขต เมืองพิษณุโลก จังหวัด พิษณุโลก รหัสไปรษณีย์ 65000 ประเทศ ไทย
อีเมล
เลขประจำตัวประชาชน 3 6 0 0 1 0 1 1 2 6 6 0 2 ☑ เพิ่มเติม (ดังแนบ)

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร
เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

สำหรับเจ้าหน้าที่

จำแนกประเภทสิทธิบัตร/อนุสิทธิบัตร

☐ กลุ่มวิศวกรรม
สิทธิบัตรการประดิษฐ์ (วิศวกรรม)
สิทธิบัตรการประดิษฐ์ (ไฟฟ้า)
สิทธิบัตรการประดิษฐ์ (ฟิสิกส์)

☐ กลุ่มเคมี
สิทธิบัตรการประดิษฐ์ (เคมีเทคนิค)
สิทธิบัตรการประดิษฐ์ (ปิโตรเคมี)
สิทธิบัตรการประดิษฐ์ (เทคโนโลยีชีวภาพ)
สิทธิบัตรการประดิษฐ์ (เภสัชภัณฑ์)

สิทธิบัตรการออกแบบ
☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 1)
☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 2)
☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 3)

อนุสิทธิบัตร
☐ อนุสิทธิบัตร (วิศวกรรม)
☐ อนุสิทธิบัตร (เคมี)

Signed by DIP-CA

แบบ สป/สผ/อสป/001-ก (ใบต่อ)
หน้า 2 ของจำนวน 2 หน้า

8. การยื่นคำขอนอกราชอาณาจักร ☐ PCT ☐ เพิ่มเติม (ดังแนบ)

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย
☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9. การแสดงการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด
วันแสดง วันเปิดงานแสดง ผู้จัด

10. การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียนฝากเก็บ	10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ

11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้
โดยขอยื่นเป็นภาษา
☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ

12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือวันที่ประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ในการประกาศโฆษณา

13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย

ก. แบบพิมพ์คำขอ	3	หน้า
ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์	4	หน้า
ค. ข้อถือสิทธิ	1	หน้า
ง. รูปเขียน	รูป	หน้า
จ. ภาพแสดงแบบผลิตภัณฑ์		
☐ รูปเขียน	รูป	หน้า
☐ ภาพถ่าย	รูป	หน้า
ฉ. บทสรุปการประดิษฐ์	1	หน้า

14. เอกสารประกอบคำขอ
☑ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์
☑ หนังสือมอบอำนาจ
☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ
☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย
☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ
☐ เอกสารอื่นๆ

15. ข้าพเจ้าขอรับรองว่า
☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน
☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก

16. ลายมือชื่อ
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☑ ตัวแทน
(นางสาวกัญญารัตน์ ประทุมศิริ)

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

Signed by DIP-CA

แบบ สป/สผ/อสป/012-ก
หน้า 1 ของจำนวน 1 หน้า

ใบแนบต่อท้าย สป/สผ/001-ก

2. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร

2. ชื่อ มหาวิทยาลัยนเรศวร

ที่อยู่ 99 หมู่ที่ 9 ต.ท่าโพธิ์ อ.เมืองพิษณุโลก จ.พิษณุโลก 65000 ประเทศ ไทย

อีเมล suleeratc@nu.ac.th สัญชาติ ไทย โทรศัพท์ 055968617

เลขประจำตัวผู้เสียภาษีอากร

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์

2. ชื่อ นางสาวนันทวรรณ บุญมี

ที่อยู่ คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติ และสิ่งแวดล้อม มหาวิทยาลัยนเรศวร หมู่ที่ 99 หมู่ 9 ต.ท่าโพธิ์ อ.เมืองพิษณุโลก จ.พิษณุโลก 65000 ประเทศไทย

สัญชาติ ไทย

เลขประจำตัวประชาชน 1601200098717

รายละเอียดการประดิษฐ์

ชื่อที่แสดงถึงการประดิษฐ์

กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน

สาขาวิทยาการที่เกี่ยวข้องกับการประดิษฐ์

วิทยาศาสตร์และเทคโนโลยีการอาหารในส่วนที่เกี่ยวข้องกับกรรมวิธีการปรับสภาพแมลงด้วยเทคนิคการให้คลื่นอัลตราโซนิกเพื่อการผลิตโปรตีน

ภูมิหลังของศิลปะหรือวิทยาการที่เกี่ยวข้อง

แมลงสำหรับการบริโภคจัดได้ว่าเป็นแหล่งโปรตีนที่มีคุณภาพและมีแนวโน้มที่น่าสนใจในการเป็นแหล่งโปรตีนทางเลือกในอนาคต เช่น จิ้งหรีดบ้าน มีปริมาณโปรตีนเฉลี่ยอยู่ในช่วง 55.0 – 70.7% w/w มีปริมาณไขมันเท่ากับ 9.8 - 22.8% ปริมาณแร่ธาตุ 3.6 - 9.1% อีกทั้ง ยังมีกรดไขมันจำเป็น เช่น Linoleic และ Linolenic Acids เป็นต้น (Laroche และคณะ, 2019) จากการคาดการณ์การเพิ่มขึ้นของประชากรโลกที่คาดว่าจะเพิ่มขึ้นเป็น 9.6 พันล้านคน ภายในปี 2050 (Huis และคณะ, 2013) แหล่งโปรตีนทางเลือกอื่นที่นอกเหนือไปจากแหล่งโปรตีนดั้งเดิม เช่น เนื้อหมู เนื้อวัว ไข่ เนื้อไก่ ถั่วเหลือง ข้าวสาลีและข้าวโพดจึงถูกพิจารณาเพื่อใช้เป็นแหล่งโปรตีนทดแทน แมลงสำหรับการบริโภคอีกหนึ่งชนิดที่น่าสนใจ คือ ดักแด้ไหม (Silkworm) ปกติเรารู้จักดักแด้ไหมในด้านของการเป็นแมลงเศรษฐกิจที่สำหรับอุตสาหกรรมผ้าทอ (พัชรินทร์, 2559) แต่ดักแด้ไหมยังสามารถนำมารับประทานเป็นอาหารได้ โดยพบว่าดักแด้มีโปรตีนสูงถึง 54.19 - 68.97 % มีไขมัน 11.67 - 12.59 % เป็นไขมันที่ดี (อุไรวรรณ และชนะ, 2550) และมีคุณค่าทางโภชนาการสูง (ทิพย์วดี, 2556) นอกจากนี้ แมลงสำหรับการบริโภคที่กำลังได้รับความสนใจในปัจจุบัน คือ หนอนทหารเสือ หรือ หนอนแมลงวันลาย (Black soldier fly) เนื่องจากตัวอ่อนของแมลงชนิดนี้สามารถช่วยย่อยสลายอินทรียวัตถุ หรือของเสียในอุตสาหกรรม ซึ่งนับได้ว่าเป็นช่วยลดปริมาณขยะในโลกได้ (กุลชาติ และทัศนีย์, 2554) โดยหนอนทหารเสือมีปริมาณโปรตีนในช่วง 43.16 – 44.36 % ปริมาณไขมัน 27.75 – 32.12 % เยื่อใย 7.47 – 7.62 % และเถ้า 9.41 – 7.63 % อีกทั้ง ยังประกอบด้วยแร่ธาตุที่สำคัญ เช่น แคลเซียม ฟอสฟอรัส เป็นต้น (Park, K., 2013) ด้วยปริมาณโปรตีนและไขมันที่สูงของแมลงสำหรับการบริโภคเหล่านี้ เมื่อเปรียบเทียบกับแหล่งโปรตีนจากอาหารดั้งเดิม พบว่า แมลงมีปริมาณโปรตีนใกล้เคียงกับโปรตีนจากพืชและสัตว์ อีกทั้ง ยังมีรายงานองค์ประกอบกรดอะมิโนใกล้เคียงกับกรดอะมิโนจากเนื้อสัตว์ และองค์ประกอบกรดไขมันที่มีคุณสมบัติเชิงหน้าที่ที่น่าสนใจ เช่น น้ำมันจิ้งหรีดมีองค์ประกอบของกรดไขมันชนิดที่มีประโยชน์ต่อร่างกาย เช่น โอเมก้า 3 และ 6 ในระดับที่สูง น้ำมันหนอนทหารเสือประกอบด้วยกรดลอริก 38.43% (Ushakova และคณะ, 2016) ซึ่งเป็นที่ทราบกันดีว่ากรดลอริกมีคุณสมบัติที่โดดเด่นในด้านของการเป็นสารต้านจุลินทรีย์ โดยทั่วไปสามารถพบกรดลอริกได้ในน้ำมันมะพร้าวและนมแม่ ด้วยเหตุนี้ แมลงทั้ง 3 ชนิดนี้จึงเป็นแมลงที่น่าสนใจในการนำมาสกัดโปรตีนและไขมันเพื่อใช้ประโยชน์ในอุตสาหกรรมอาหาร อาหารสัตว์ ตลอดจนอุตสาหกรรมเครื่องสำอาง

กระบวนการสกัดน้ำมันที่นิยมใช้กันอย่างแพร่หลาย เช่น กระบวนการสกัดด้วยตัวทำละลายอินทรีย์ เครื่องบีบอัด กระบวนการสกัดด้วยของไหลวิกฤติยิ่งยวด แต่กระบวนการสกัดน้ำมันดังกล่าวไม่เพียงพอในการสกัดน้ำมันออกจากตัวอย่างให้มีปริมาณต่ำกว่า 10 % เพื่อใช้ในกระบวนการผลิตโปรตีนผงสำหรับการบริโภคได้ ดังนั้น กระบวนการปรับสภาพผนังเซลล์ของตัวอย่างก่อนการสกัดน้ำมันจึงมีความจำเป็น จากการสืบค้นข้อมูลจากงานวิจัย พบว่า กระบวนการปรับสภาพตัวอย่างที่มีการใช้งานในปัจจุบัน ได้แก่ กระบวนการแปรรูปโดยใช้แรงดันสูง กระบวนการสนามไฟฟ้าแบบพัลซ์ กระบวนการให้ความร้อนแบบโอห์มมิค และการประยุกต์ใช้คลื่นอัลตราโซนิก เป็นต้น ซึ่งกระบวนการที่น่าสนใจในการศึกษานี้ คือ กระบวนการปรับสภาพแมลงสำหรับการบริโภคก่อนการสกัดน้ำมันด้วยการใช้คลื่นอัลตราโซนิก เนื่องจากมีรายงานการวิจัยว่าการสั่นสะเทือนหรือเสียดสีกันเป็นความร้อนที่เกิดจากการให้คลื่นอัลตราโซนิกต่อตัวอย่างขณะแปรรูป ส่งผลให้เกิดการสกัดและเกิดการปลดปล่อยสารที่สนใจออกมาจากตัวอย่างได้ง่ายขึ้น (อารีรัตน์, 2560) โดยกระบวนการที่เกิดขึ้นภายในโครงสร้างของตัวอย่างระหว่างการให้คลื่นอัลตราโซนิก คือ คลื่นส่งผลให้เกิดรูพรุนบนผนังเซลล์ตัวอย่างในขั้นตอนการสั่นที่เกิดจากการเคลื่อนที่ของคลื่น และการเกิดรูพรุนนี้เป็นผลดีในขั้นตอนการสกัดน้ำมัน เพราะตัวทำละลายสามารถแทรกผ่านเข้าไปภายในเซลล์ของตัวอย่างแล้วชะน้ำมันออกมากับสารสกัดได้ดีมากขึ้น

กระบวนการสกัดโดยการใช้คลื่นอัลตร้าโซนิค (Ultrasound Extraction หรือ Sonication) เป็นการใช้คลื่นเสียงความถี่สูงในช่วง 20 - 2,000 กิโลเฮิรตซ์ เพื่อทำให้เกิดการสั่นสะเทือนหรือเสียดสีกันเป็นความร้อน ทำให้เกิดการสกัดและเกิดการปลดปล่อยสารที่สนใจออกมาจากตัวอย่าง (อารีรัตน์, 2560) Ultrasound extraction มีผลทางกลกับตัวอย่าง โดยการทำให้การแพร่ของตัวทำละลายเข้าไปในตัวอย่างได้สูงขึ้น จึงช่วยเพิ่มพื้นที่ในการสัมผัสระหว่างเฟสของแข็งและเฟสของเหลวเป็นผลให้ตัวถูกละลายถ่ายโอนจากเฟสของแข็งไปยังตัวทำละลายได้เร็วขึ้น (สุภาษิต และคณะ, 2556)

ดังนั้น จึงมีความเป็นไปได้สูงที่การปรับสภาพแมลงสำหรับการบริโภคก่อนการสกัดน้ำมันจะช่วยเพิ่มประสิทธิภาพของกระบวนการสกัดน้ำมันและจากการศึกษาการเปิดเผยต่างๆ ของการประยุกต์ใช้งานคลื่นอัลตราโซนิก พบว่า มีความเป็นไปได้ในการนำมาประยุกต์ใช้ในกระบวนการปรับสภาพแมลงสำหรับการบริโภคก่อนกระบวนการสกัดน้ำมันและผลิตโปรตีนผง ดังนั้น จึงเป็นที่มาของการประดิษฐ์นี้ ที่เป็นการศึกษากรรมวิธีการปรับสภาพแมลงสำหรับการบริโภคด้วยการให้คลื่นอัลตราโซนิกก่อนกระบวนการสกัดน้ำมันเพื่อผลิตโปรตีนต่อคุณสมบัติทางเคมี – กายภาพ คุณสมบัติทางชีวภาพ และผลิตภัณฑ์ที่ได้จากกรรมวิธีดังกล่าว

ลักษณะและความมุ่งหมายของการประดิษฐ์

กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน เป็นการปรับสภาพแมลงสดด้วยการให้คลื่นอัลตราโซนิก ก่อนกระบวนการสกัดน้ำมันด้วยตัวทำละลายอินทรีย์หรือเทคนิคของไหลวิกฤติยิ่งยวดเพื่อผลิตโปรตีน โดยนำแมลงมาทำความสะอาด และบรรจุลงในอ่างของเครื่องกำเนิด

คลื่นอัลตราโซนิก ทำการให้คลื่นอัลตราโซนิกที่ระดับความถี่ในช่วง 50 – 100 กิโลเฮิรตซ์ (kHz) เป็นเวลา 5 – 15 นาที โดยใช้น้ำเป็นตัวกลางในการส่งผ่านคลื่นไปยังแมลง แล้วนำไปอบแห้งและบดเป็นผง

ความมุ่งหมายของการประดิษฐ์นี้ คือ การเพิ่มประสิทธิภาพของกระบวนการสกัดน้ำมันออกจากแมลง ด้วยกรรมวิธีการปรับสภาพแมลงด้วยการให้คลื่นอัลตราโซนิก ทำให้สามารถสกัดน้ำมันออกจากแมลงได้มากขึ้น อีกทั้ง กระบวนการนี้ยังเป็นเทคโนโลยีที่สามารถต่อยอดและขยายกำลังการผลิตไปสู่ระดับอุตสาหกรรมเพื่อการผลิตโปรตีนสำหรับใช้ในอุตสาหกรรมอาหารสัตว์ อาหารคน และอุตสาหกรรมอื่นๆ ต่อไปในอนาคตได้

การเปิดเผยการประดิษฐ์โดยสมบูรณ์

กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน มีขั้นตอนดังนี้

ก. นำแมลงที่เลือกได้จาก จิ้งหรีด หนอนทหารเสือ ดักแด้ไหม อย่างใดอย่างหนึ่งหรือรวมกัน มาทำความสะอาดทั้งตัวด้วยการล้างด้วยน้ำกลั่นและบรรจุตัวอย่างในถุงพลาสติกชนิดความหนาแน่นต่ำ (LDPE)

ข. นำแมลงที่ได้จากขั้นตอน ก. บรรจุลงในอ่างของเครื่องกำเนิดคลื่นอัลตราโซนิก ทำการให้คลื่นอัลตราโซนิกที่ระดับความถี่ในช่วง 50 – 100 กิโลเฮิรตซ์ (kHz) เป็นเวลา 5 – 15 นาที โดยใช้น้ำเป็นตัวกลางในการส่งผ่านคลื่นไปยังแมลง อุณหภูมิของน้ำตัวกลางเท่ากับอุณหภูมิโดยรอบ (Ambient temperature)

ค. นำแมลงที่ได้จากขั้นตอน ข. อบให้แห้งด้วยตู้อบลมร้อนที่อุณหภูมิ 65 - 75 องศาเซลเซียส เป็นเวลา 10 – 12 ชั่วโมง หรือจนกระทั่งแมลงมีความชื้นในช่วง 5 – 8 %

ง. นำแมลงอบแห้งที่ได้จากขั้นตอน ค.ไปบดเป็นผงและร่อนผ่านตะแกรงขนาด 15-20 เมช(Mesh) จะได้ผงแมลงสำหรับนำไปผลิตเป็นโปรตีนต่อไป

กระบวนการสกัดน้ำมันด้วยตัวทำละลาย (Solvent extraction)

ก. นำผงแมลงที่ผ่านการปรับสภาพด้วยการใช้แรงดันสูงใส่ในถังสกัดในอัตราส่วนผงแมลง:ตัวทำละลาย เท่ากับ 1:10 w/v

ข. สกัดตัวอย่างโดยใช้ magnetic bar สำหรับกวนตัวอย่างและตัวทำละลาย เป็นระยะเวลา 2 ชั่วโมง

ค. เมื่อครบระยะเวลาที่กำหนด ทำการแยกส่วนของเหลวที่มีแมลงอบแห้งละลายอยู่ กับส่วนตะกอนออกจากกันด้วยเครื่องปั่นเหวี่ยง (Centrifuge) ที่ความเร็วรอบ 6,000 รอบต่อนาที(rpm) เป็นระยะเวลา 15 นาที

ง. นำส่วนของเหลวใส่ในบีกเกอร์และทำการระเหยตัวทำละลายภายในตู้ดูดควัน เป็นระยะเวลา 24 ชั่วโมง และส่วนตะกอนนำใส่ภาชนะ และนำไประเหยตัวทำละลายออกเช่นเดียวกันกับส่วนใส

จ. เมื่อระเหยตัวทำละลายออกจนหมด ในส่วนของเหลวจะเหลือเพียงน้ำมันที่สกัดออกมาจากแมลงอบแห้ง และส่วนตะกอนจะได้เป็นผงแมลงแห้ง จากนั้นส่วนน้ำมันและผงแมลงหลังกระบวนการสกัดจะถูกนำไปวิเคราะห์คุณสมบัติทางเคมี - กายภาพ คุณสมบัติทางด้านชีวภาพ และส่วนตะกอนจะได้เป็นผงแมลงสำหรับใช้ในกระบวนการผลิตโปรตีนผงสำหรับการบริโภคในขั้นตอนการผลิตโปรตีนไอโซเลตหรือไฮโดรไลเซทจากแมลงสำหรับการบริโภคต่อไป

กระบวนการสกัดน้ำมันด้วยเทคนิคของไหลวิกฤติยิ่งยวด (Supercritical fluid extraction)

ก. นำผงแมลงที่ผ่านการปรับสภาพด้วยการใช้แรงดันสูงใส่ในถังสกัด

ข. ทำการสกัดน้ำมันด้วยคาร์บอนไดออกไซด์วิกฤติยิ่งยวด ที่ระดับความดันในช่วง 250 – 350 บาร์ (bar) ระยะเวลาในการสกัด 30 – 90 นาที ที่อุณหภูมิ 35 – 40 องศาเซลเซียส อัตราการไหลในช่วง 24 – 28 ลิตรต่อชั่วโมง (L/h.)

ค. เมื่อครบระยะเวลาสกัด ทำการเก็บน้ำมันจากถังบรรจุสารสกัด และเก็บกากแมลงหลังสกัดในถัง

ง. ส่วนน้ำมันและกากแมลงหลังสกัดจะถูกนำไปวิเคราะห์คุณสมบัติทางเคมี - กายภาพ คุณสมบัติทางด้านชีวภาพ และส่วนของกากแมลงจะนำไปผลิตโปรตีนผงต่อไป

วิธีการในการประดิษฐ์ที่ดีที่สุด

ดังได้บรรยายไว้ในหัวข้อการเปิดเผยการประดิษฐ์โดยสมบูรณ์

หน้า 1 ของจำนวน 1 หน้า

ข้อถือสิทธิ

1. กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน มีขั้นตอนดังนี้

ก. นำแมลงที่เลือกได้จาก จิ้งหรีด หนอนทหารเสือ ดักแด้ไหม อย่างใดอย่างหนึ่งหรือรวมกัน มาทำความสะอาดทั้งตัวด้วยการล้างด้วยน้ำกลั่นและบรรจุตัวอย่างในถุงพลาสติกชนิดความหนาแน่นต่ำ (LDPE)

ข. นำแมลงที่ได้จากขั้นตอน ก. บรรจุลงในอ่างของเครื่องกำเนิดคลื่นอัลตราโซนิก ทำการให้คลื่นอัลตราโซนิกที่ระดับความถี่ในช่วง 50 – 100 กิโลเฮิรตซ์ (kHz) เป็นเวลา 5 – 15 นาที โดยใช้น้ำเป็นตัวกลางในการส่งผ่านคลื่นไปยังแมลง อุณหภูมิของน้ำตัวกลางเท่ากับอุณหภูมิโดยรอบ (Ambient temperature)

ค. นำแมลงที่ได้จากขั้นตอน ข. อบให้แห้งด้วยตู้อบลมร้อนที่อุณหภูมิ 65 - 75 องศาเซลเซียส เป็นเวลา 10 – 12 ชั่วโมง หรือจนกระทั่งแมลงมีความชื้นในช่วง 5 – 8 %

ง. นำแมลงอบแห้งที่ได้จากขั้นตอน ค.ไปบดเป็นผงและร่อนผ่านตะแกรงขนาด 15-20 เมช(Mesh) จะได้ผงแมลงสำหรับนำไปผลิตเป็นโปรตีนต่อไป

2. กรรมวิธีการปรับสภาพแมลงด้วยเทคนิคการให้คลื่นอัลตราโซนิกเพื่อการผลิตโปรตีน ตามข้อถือสิทธิ 1 ที่ซึ่ง แมลง เลือกได้จาก จิ้งหรีด หนอนทหารเสือ ดักแด้ไหม อย่างใดอย่างหนึ่งหรือรวมกัน

บทสรุปการประดิษฐ์

กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน เป็นการปรับสภาพแมลงสดด้วยการให้คลื่นอัลตราโซนิก ก่อนกระบวนการสกัดน้ำมันด้วยตัวทำละลายอินทรีย์หรือเทคนิคของไหลวิกฤติยิ่งยวดเพื่อผลิตโปรตีน โดยนำแมลงมาทำความสะอาด และบรรจุลงในอ่างของเครื่องกำเนิดคลื่นอัลตราโซนิก ทำการให้คลื่นอัลตราโซนิกที่ระดับความถี่ในช่วง 50 – 100 กิโลเฮิรตซ์ (kHz) เป็นเวลา 5 – 15 นาที โดยใช้น้ำเป็นตัวกลางในการส่งผ่านคลื่นไปยังแมลง แล้วนำไปอบแห้งและบดเป็นผง ทำให้สามารถสกัดน้ำมันออกจากแมลงได้มากขึ้น อีกทั้ง กระบวนการนี้ยังเป็นเทคโนโลยีที่สามารถต่อยอดและขยายกำลังการผลิตไปสู่ระดับอุตสาหกรรมเพื่อการผลิตโปรตีนสำหรับใช้ในอุตสาหกรรมอาหารสัตว์ อาหารคน และอุตสาหกรรมอื่นๆ ต่อไปในอนาคตได้

หนังสือมอบอำนาจ

ทำที่ มหาวิทยาลัยนเรศวร
เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์
อำเภอเมืองพิษณุโลก
จังหวัดพิษณุโลก 65000

วันที่ 29 เดือน ธันวาคม พ.ศ. 2565

โดยหนังสือฉบับนี้ **สำนักงานการวิจัยแห่งชาติ** โดย ดร.วิภารัตน์ ดีอ่อง ตำแหน่ง ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ ที่อยู่เลขที่ 196 ถนนพหลโยธิน แขวงลาดยาว เขตจตุจักร กรุงเทพฯ 10900 และ

มหาวิทยาลัยนเรศวร โดย รองศาสตราจารย์ ดร.ศรินทร์ทิพย์ แทนธานี ตำแหน่ง รักษาราชการแทนอธิการบดีมหาวิทยาลัยนเรศวร ที่อยู่ เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000

ขอมอบอำนาจและแต่งตั้งให้ **นางสาวกัญญารัตน์ ประทุมศิริ** ตัวแทนสิทธิบัตรเลขที่ 2350 เป็นผู้ดำเนินการเป็นตัวแทนในนามของข้าพเจ้าอันแท้จริงที่จะยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตร **ชื่อผลงาน "กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน"** จดทะเบียน เปลี่ยนแปลงชื่อ ในส่วนที่เกี่ยวข้อง ถอนคำขอรับสิทธิบัตร/อนุสิทธิบัตร ยื่นคำคัดค้าน จดทะเบียนสิทธิบัตร/อนุสิทธิบัตร หรือยื่นคำขอให้มีการเพิกถอนสิทธิบัตร/อนุสิทธิบัตร ใดที่คล้ายหรือขัดกับสิทธิบัตร/อนุสิทธิบัตรดังกล่าว ให้มีอำนาจลงนาม และยื่นเอกสาร หรือข้อความใด ๆ ในนามของ ผู้มอบอำนาจซึ่งตัวแทนในฐานะดังกล่าวข้างต้นเห็นว่าจำเป็นหรือเหมาะสมเปลี่ยนแปลงแก้ไขเอกสารดังกล่าว และให้มีอำนาจที่จะดำเนินการใด ๆ ดังกล่าวข้างต้นซึ่งตัวแทนพิจารณาเห็นสมควรเพื่อดำเนินการตามหนังสือนี้

เพื่อเป็นหลักฐานแห่งการนี้ ข้าพเจ้าได้ลงลายมือชื่อ และประทับตรา (ถ้ามี) ไว้เป็นสำคัญ

ลงชื่อ.....ผู้มอบอำนาจ
(ดร.วิภารัตน์ ดีอ่อง)
ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ

ลงชื่อ.....ผู้มอบอำนาจ
(รองศาสตราจารย์ ดร.ศรินทร์ทิพย์ แทนธานี)
รักษาราชการแทนอธิการบดีมหาวิทยาลัยนเรศวร

ลงชื่อ.....ผู้รับมอบอำนาจ
(นางสาวกัญญารัตน์ ประทุมศิริ)

ลงชื่อ.....พยาน
(นางสาวธรรมภรณ์ ประภาสะวัต)
ผู้อำนวยการกองบริหารทุนวิจัยและนวัตกรรม 3

ลงชื่อ.....พยาน
(ดร.พิสุทธิ์ อภิชยกุล)
รองอธิการบดีฝ่ายวิเทศสัมพันธ์และการถ่ายทอดเทคโนโลยี

หนังสือสัญญาโอนสิทธิรับสิทธิบัตร/อนุสิทธิบัตร

ทำที่ มหาวิทยาลัยนเรศวร
เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์
อำเภอเมืองพิษณุโลก
จังหวัดพิษณุโลก 65000

วันที่ 29 เดือน ธันวาคม พ.ศ. 2565

หนังสือสัญญาฉบับนี้ทำขึ้นระหว่าง **"ผู้โอนสิทธิ"** คือ

1.รองศาสตราจารย์ ดร.ขนิษฐา รุตรัตนมงคล คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติ และสิ่งแวดล้อม มหาวิทยาลัยนเรศวร เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000

2.นางสาวนันทวรรณ บุญมี คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติ และสิ่งแวดล้อม มหาวิทยาลัยนเรศวร เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000

ผู้รับโอนสิทธิ คือ

สำนักงานการวิจัยแห่งชาติ โดย ดร.วิภารัตน์ ดีอ่อง ตำแหน่ง ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ ที่อยู่เลขที่ 196 ถนนพหลโยธิน แขวงลาดยาว เขตจตุจักร กรุงเทพฯ 10900 และ

มหาวิทยาลัยนเรศวร โดย รองศาสตราจารย์ ดร.ศรินทร์ทิพย์ แทนธานี ตำแหน่ง รักษาราชการแทนอธิการบดีมหาวิทยาลัยนเรศวร ที่อยู่ เลขที่ 99 หมู่ 9 ตำบลท่าโพธิ์ อำเภอเมือง จังหวัดพิษณุโลก 65000

โดยหนังสือสัญญานี้ ผู้โอนซึ่งเป็นผู้ประดิษฐ์ **"กรรมวิธีการปรับสภาพแมลงด้วยการใช้คลื่นอัลตร้าโซนิกเพื่อการผลิตโปรตีน"** ขอโอนสิทธิในการประดิษฐ์ดังกล่าว ซึ่งรวมถึงสิทธิขอรับสิทธิบัตร/อนุสิทธิบัตร และ/หรือสิทธิอื่นๆ ที่เกี่ยวข้องให้แก่ผู้รับโอน

เพื่อเป็นหลักฐานแห่งการนี้ผู้โอนและผู้รับโอนจึงลงลายมือชื่อไว้เป็นหลักฐานต่อหน้าพยาน

ลงชื่อ.....ผู้โอน
(รองศาสตราจารย์ ดร.ขนิษฐา รุตรัตนมงคล)

ลงชื่อ.....ผู้รับโอน
(ดร.วิภารัตน์ ดีอ่อง)
ผู้อำนวยการสำนักงานการวิจัยแห่งชาติ

ลงชื่อ.....ผู้โอน
(นางสาวนันทวรรณ บุญมี)

ลงชื่อ.....ผู้รับโอน
(รองศาสตราจารย์ ดร.ศรินทร์ทิพย์ แทนธานี)
รักษาราชการแทนอธิการบดีมหาวิทยาลัยนเรศวร

ลงชื่อ.....พยาน
(นางสาวธรรมภรณ์ ประภาสะวัต)
ผู้อำนวยการกองบริหารทุนวิจัยและนวัตกรรม 3

ลงชื่อ.....พยาน
(ดร.พิสุทธิ์ อภิชยกุล)
รองอธิการบดีฝ่ายวิเทศสัมพันธ์และการถ่ายทอดเทคโนโลยี

มาตรฐานสินค้าเกษตร

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วันที่

คณะกรรมการวิชาการพิจารณามาตรฐานสินค้าเกษตร
เรื่อง ผลิตภัณฑ์อาหารแปรรูปจากจิ้งหรีด

1. อธิบดีกรมปศุสัตว์ หรือผู้ที่อธิบดีมอบหมาย ประธานกรรมการ
   นายรักษ์ไทย งามภักดิ์ ผู้อำนวยการสำนักพัฒนาระบบและรับรองมาตรฐานสินค้าปศุสัตว์
   นายอภินันท์ คงนุรัตน์ ผู้อำนวยการกลุ่มรับรองด้านการปศุสัตว์
2. ผู้แทนกรมส่งเสริมการเกษตร กรรมการ
   นายวีรศักดิ์ บุญเชิญ
   นางสาวเมธินี กีรเกียรติ
3. ผู้แทนสำนักงานคณะกรรมการอาหารและยา กรรมการ
   นางสาวอรเกตน์เก้า พัฒนากุล
   นายวรพจน์ ฤทธิ์ดี
4. ผู้แทนสำนักงานมาตรฐานสินค้าเกษตรและอาหารแห่งชาติ กรรมการ
   นางสาวยุพา เหล่าจินดาพันธ์
   นางสาววิรัชนี โลหะชุมพล
5. ผู้แทนสำนักพัฒนาระบบและรับรองมาตรฐานสินค้าปศุสัตว์ กรมปศุสัตว์ กรรมการ
   นายอนุชา มุมอ่อน
6. ผู้แทนสถาบันสุขภาพสัตว์แห่งชาติ กรมปศุสัตว์ กรรมการ
   นายนพพร โต๊ะมี
7. ผู้แทนคณะอุตสาหกรรมเกษตร มหาวิทยาลัยเกษตรศาสตร์ กรรมการ
   ผู้ช่วยศาสตราจารย์วศะพร เพรททิเชย์ จันทร์พุฒ
8. ผู้แทนคณะอุตสาหกรรมเกษตร มหาวิทยาลัยเชียงใหม่ กรรมการ
   รองศาสตราจารย์ยุทธนา พิมลศิริผล
   ผู้ช่วยศาสตราจารย์ภัทวรา ปฐมรังษิยังกุล
9. ผู้แทนสภาเกษตรกรแห่งชาติ กรรมการ
   นายณรงค์รัตน์ ม่วงประเสริฐ
10. ผู้แทนสภาหอการค้าแห่งประเทศไทย กรรมการ
    นายธนาภูมิ ม่วงเอี่ยม
11. ผู้แทนกลุ่มอุตสาหกรรมอาหารและเครื่องดื่ม สภาอุตสาหกรรมแห่งประเทศไทย กรรมการ
    นายปราโมทย์ ตาหวัฒน์
12. ผู้แทนอุตสาหกรรมมูลนิธิเพื่อสถาบันอาหาร กรรมการ
    นางสาวมยุรา ปรารถนาเปลี่ยน

13. ผู้แทนสมาคมการค้าอาหารอนาคตไทย กรรมการ
นายนนทวัฒน์ บางเอี่ยม

14. ผู้แทนสมาคมอุตสาหกรรมแมลงไทย กรรมการ
นายณธัชพงศ์ รักศรี

15. ผู้แทนสำนักกำหนดมาตรฐาน กรรมการและเลขานุการ
สำนักงานมาตรฐานสินค้าเกษตรและอาหารแห่งชาติ
นางสาวจีรจิต ดิศสนะ

(4)

จิ้งหรีดเป็นแมลงที่สามารถใช้เป็นแหล่งโปรตีนทดแทนเนื้อสัตว์ จึงมีความสำคัญทางเศรษฐกิจด้านอาหาร สามารถนำไปแปรรูปเป็นจิ้งหรีดแห้งที่ใช้เป็นส่วนผสมของอาหารและเครื่องดื่มหลายชนิด มีศักยภาพสูงด้านการส่งออกเป็นอาหารอนาคต คณะกรรมการมาตรฐานสินค้าเกษตรจึงเห็นสมควรจัดทำมาตรฐานสินค้าเกษตร เรื่อง จิ้งหรีดแห้ง เพื่อส่งเสริมและยกระดับการผลิตจิ้งหรีดแห้งให้มีคุณภาพและความปลอดภัยด้านอาหาร นอกจากนี้ยังสามารถใช้เป็นเกณฑ์อ้างอิงทางการค้าและสร้างความเชื่อมั่นแก่ผู้บริโภค

มาตรฐานสินค้าเกษตรนี้ กำหนดขึ้นโดยใช้เอกสารต่อไปนี้เป็นแนวทาง

มกษ. 9023-2564. หลักการทั่วไปด้านสุขลักษณะอาหาร: การปฏิบัติทางสุขลักษณะที่ดี.

สำนักงานการวิจัยแห่งชาติ. 2566, รายงานฉบับสมบูรณ์โครงการยกระดับมาตรฐานกระบวนการแปรรูปจิ้งหรีดผงสู่เชิงพาณิชย์. 159 หน้า.

ประกาศกระทรวงเกษตรและสหกรณ์
เรื่อง กำหนดมาตรฐานสินค้าเกษตร : จิ้งหรีดแห้ง
ตามพระราชบัญญัติมาตรฐานสินค้าเกษตร พ.ศ. ๒๕๕๑

ด้วยคณะกรรมการมาตรฐานสินค้าเกษตร เห็นสมควรกำหนดมาตรฐานสินค้าเกษตร เรื่อง จิ้งหรีดแห้ง เป็นมาตรฐานทั่วไป ตามพระราชบัญญัติมาตรฐานสินค้าเกษตร พ.ศ. ๒๕๕๑ เพื่อส่งเสริมสินค้าเกษตรให้ได้คุณภาพ มาตรฐาน และปลอดภัย

อาศัยอำนาจตามความในมาตรา ๕ มาตรา ๑๕ วรรคสอง และมาตรา ๑๖ แห่งพระราชบัญญัติมาตรฐานสินค้าเกษตร พ.ศ. ๒๕๕๑ ประกอบมติคณะกรรมการมาตรฐานสินค้าเกษตร ในการประชุมครั้งที่ ๑/๒๕๖๘ เมื่อวันที่ ๒๗ กุมภาพันธ์ ๒๕๖๘ รัฐมนตรีว่าการกระทรวงเกษตรและสหกรณ์จึงออกประกาศกำหนดมาตรฐานสินค้าเกษตร : จิ้งหรีดแห้ง มาตรฐานเลขที่ มกษ. 8006-2568 ไว้เป็นมาตรฐานทั่วไป ดังมีรายละเอียดแนบท้ายประกาศนี้

ทั้งนี้ ให้ใช้บังคับตั้งแต่วันถัดจากวันประกาศในราชกิจจานุเบกษาเป็นต้นไป

ประกาศ ณ วันที่ ๑๐ เมษายน พ.ศ. ๒๕๖๘

(นางนฤมล ภิญโญสินวัฒน์)
รัฐมนตรีว่าการกระทรวงเกษตรและสหกรณ์

มกษ. 8006-2568

มาตรฐานสินค้าเกษตร
จิ้งหรีดแห้ง

1. ขอบข่าย

มาตรฐานสินค้าเกษตรนี้ใช้กับจิ้งหรีดแห้งตามนิยามผลิตภัณฑ์ข้อ 2.1 ที่บรรจุในภาชนะบรรจุที่ป้องกันการปนเปื้อนและความชื้นเพื่อจำหน่าย สำหรับนำไปแปรรูปหรือปรุงอาหาร

2. คำอธิบายสินค้า

2.1 นิยามผลิตภัณฑ์

2.1.1 จิ้งหรีดแห้ง (dried cricket) ในมาตรฐานนี้ได้มาจากจิ้งหรีดที่อยู่ในวงศ์ Gryllidae ทั้งนี้ มาตรฐานนี้ครอบคลุมชนิดจิ้งหรีด ดังนี้

1) จิ้งหรีดบ้าน หรือสะดิ้ง มีชื่อวิทยาศาสตร์ว่า *Acheta domesticus* L.

2) จิ้งหรีดทองดำ มีชื่อวิทยาศาสตร์ว่า *Gryllus bimaculatus* De Geer

3) จิ้งหรีดทองแดง มีชื่อวิทยาศาสตร์ว่า *Teleogryllus mitratus* Burmeister

4) จิ้งหรีดทองลาย มีชื่อวิทยาศาสตร์ว่า *Gryllus locorojo* Weissman and Gray

2.1.2 จิ้งหรีดแห้งตามมาตรฐานนี้มี 3 ประเภท ได้แก่ จิ้งหรีดแห้งทั้งตัว ผงจิ้งหรีด และผงจิ้งหรีดที่สกัดไขมันออกบางส่วน

2.2 นิยามกระบวนการผลิต

2.2.1 จิ้งหรีดที่ใช้ผลิตจิ้งหรีดแห้งตามข้อ 2.1 เตรียมจากวัตถุดิบจิ้งหรีดสด ดังนี้

2.2.1.1 เตรียมจากจิ้งหรีดสด สะอาด ไม่เน่าเสีย; หรือ

2.2.1.2 เตรียมจากจิ้งหรีดตามข้อ 2.2.1.1 ที่ผ่านกระบวนการอื่นมาก่อน เช่น การให้ความร้อน แช่เย็น หรือแช่แข็ง

2.2.2 จิ้งหรีดแห้งทั้งตัว (whole dried cricket) เตรียมจากจิ้งหรีดตามข้อ 2.2.1 ล้างด้วยน้ำสะอาด ให้ความร้อน เช่น ต้ม นึ่ง เพื่อกำจัดจุลินทรีย์ นำไปทำแห้ง เช่น อบ คั่ว แล้วนำไปบรรจุในภาชนะบรรจุที่ป้องกันการปนเปื้อนและความชื้น

2.2.3 ผงจิ้งหรีด (cricket powder) เตรียมจากจิ้งหรีดแห้งทั้งตัวตามข้อ 2.2.2 มาทำให้เป็นผง หรืออาจนำจิ้งหรีดตามข้อ 2.2.1 ล้างด้วยน้ำสะอาด หลังจากนั้นนำมาผ่านกระบวนการต่าง ๆ เช่น บด ทำแห้ง จนผลิตภัณฑ์สุดท้ายมีลักษณะเป็นผง แล้วบรรจุในภาชนะบรรจุที่ป้องกันการปนเปื้อนและความชื้น

2.2.4 ผงจิ้งหรีดที่สกัดไขมันออกบางส่วน (partially defatted cricket powder) เตรียมจาก:

1) จิ้งหรีดแห้งทั้งตัวตามข้อ 2.2.2 บดหยาบ ผ่านกระบวนการบีบหรือสกัดไขมันออกบางส่วน และทำให้เป็นผง แล้วบรรจุในภาชนะบรรจุที่ป้องกันการปนเปื้อนและความชื้น; หรือ

2) จิ้งหรีดตามข้อ 2.2.1 ล้างด้วยน้ำสะอาด ทำแห้ง บดหยาบ ผ่านกระบวนการบีบหรือสกัดไขมันออกบางส่วน และทำให้เป็นผง แล้วบรรจุในภาชนะบรรจุที่ป้องกันการปนเปื้อนและความชื้น

3. ส่วนประกอบสำคัญและเกณฑ์คุณภาพ

3.1 ส่วนประกอบสำคัญ

จิ้งหรีดแห้งตามข้อ 2

3.2 เกณฑ์คุณภาพ

3.2.1 ข้อกำหนดทั่วไป

3.2.1.1 จิ้งหรีดแห้งทุกชนิดต้องมีสีและกลิ่นปกติตามลักษณะเฉพาะของจิ้งหรีดแห้ง ซึ่งอาจแตกต่างกันตามชนิดของจิ้งหรีด อาหารที่ใช้เลี้ยงจิ้งหรีด และกระบวนการผลิต

3.2.1.2 กรณีเป็นผงจิ้งหรีดหรือผงจิ้งหรีดที่สกัดไขมันออกบางส่วนต้องร่วน ไม่เกาะกันเป็นก้อน

3.2.2 เกณฑ์คุณภาพทางเคมี

หากมิได้มีการตกลงกันไว้เป็นอย่างอื่นในทางการค้า ให้เกณฑ์คุณภาพทางเคมีของผงจิ้งหรีดและผงจิ้งหรีดที่สกัดไขมันออกบางส่วนเป็นไปตามตารางที่ 1

ตารางที่ 1 เกณฑ์คุณภาพทางเคมีของจิ้งหรีดแห้งแบ่งตามชนิดผลิตภัณฑ์

ชนิดผลิตภัณฑ์	ปริมาณโปรตีน (ร้อยละโดยมวล)	ปริมาณความชื้น (ร้อยละโดยมวล)
จิ้งหรีดแห้งทั้งตัว	-	≤ 10
ผงจิ้งหรีด	≥ 55	≤ 10
ผงจิ้งหรีดที่สกัดไขมันออกบางส่วน	≥ 60	≤ 10

3.2.3 ข้อบกพร่องและเกณฑ์การยอมรับ

ต้องไม่พบสิ่งแปลกปลอมจากภายนอก[1/] เช่น แมลงชนิดอื่น เศษวัสดุในการเลี้ยง

[1/] สิ่งแปลกปลอมจากภายนอก (extraneous material/matter) หมายถึง สิ่งแปลกปลอมใด ๆ ในผลิตภัณฑ์ที่เกี่ยวข้องกับสภาวะหรือการปฏิบัติที่ไม่พึงประสงค์ในการผลิต เก็บรักษา หรือการกระจายสินค้า รวมถึงฟิลท์ วัสดุที่สลายตัว (เนื้อเยื่อที่เสื่อมลงที่มีสาเหตุจากปรสิตหรือไม่ใช่ปรสิต) และสิ่งอื่น ๆ เช่น ทราย ดิน เศษแก้ว สนิม หรือสารแปลกปลอมอื่น ทั้งนี้ ไม่รวมถึงการนับจำนวนแบคทีเรีย

4. วัตถุเจือปนอาหาร

4.1 กรณีเป็นจิ้งหรีดแห้งทั้งตัว ไม่อนุญาตให้ใช้วัตถุเจือปนอาหาร

4.2 สารป้องกันการจับเป็นก้อน (anticaking agent) สารป้องกันการเกิดออกซิเดชัน (antioxidant) และสารช่วยทำละลายหรือช่วยพา (carrier) ที่ใช้ ให้เป็นไปตามบัญชีหมายเลข 1 และบัญชีหมายเลข 2 ของประกาศกระทรวงสาธารณสุข (ฉบับที่ 444) พ.ศ. 2566 ออกตามความในพระราชบัญญัติอาหาร พ.ศ. 2522 เรื่อง กำหนดหลักเกณฑ์ เงื่อนไข วิธีการใช้ และอัตราส่วนของวัตถุเจือปนอาหาร (ฉบับที่ 3) ในรหัสหมวดอาหาร 08.3.2 เนื้อสัตว์บด ทำให้สุกโดยใช้ความร้อน

5. สารปนเปื้อน

ชนิดและปริมาณโลหะหนักของจิ้งหรีดแห้งตรวจพบได้ไม่เกินปริมาณสูงสุดตามที่กำหนดในตารางที่ 2

ตารางที่ 2 ข้อกำหนดปริมาณสูงสุดของโลหะหนัก

ชนิดของโลหะหนัก	ปริมาณสูงสุด (mg/kg)
1. สารหนูทั้งหมด	2
2. ปรอททั้งหมด	0.02
3. ตะกั่ว	1

ที่มา: ประกาศกระทรวงสาธารณสุข (ฉบับที่ 414) พ.ศ.2563 ออกตามความในพระราชบัญญัติอาหาร พ.ศ. 2522 เรื่อง มาตรฐานอาหารที่มีสารปนเปื้อน

6. สุขลักษณะ

6.1 การผลิตและการปฏิบัติทุกขั้นตอนในการเลี้ยง รวมถึงการปฏิบัติหลังการเก็บเกี่ยว เพื่อให้ได้จิ้งหรีดที่มีคุณภาพ เหมาะสมสำหรับเป็นวัตถุดิบในการแปรรูปเป็นผลิตภัณฑ์ที่ปลอดภัยต่อการบริโภค ต้องปฏิบัติอย่างถูกสุขลักษณะ โดยให้เป็นไปตามหรือได้รับการรับรองตาม มกษ. 8202 มาตรฐานสินค้าเกษตร เรื่อง การปฏิบัติทางการเกษตรที่ดีสำหรับฟาร์มจิ้งหรีด

6.2 การผลิตจิ้งหรีดแห้งต้องปฏิบัติอย่างถูกสุขลักษณะที่เป็นไปตามประกาศกระทรวงสาธารณสุข (ฉบับที่ 420) พ.ศ. 2563 ออกตามความในพระราชบัญญัติอาหาร พ.ศ. 2522 เรื่อง วิธีการผลิต เครื่องมือเครื่องใช้ในการผลิต และการเก็บรักษาอาหาร หรือตาม มกษ. 9023 มาตรฐานสินค้าเกษตร เรื่อง หลักการทั่วไปด้านสุขลักษณะอาหาร: การปฏิบัติทางสุขลักษณะที่ดี หรือได้รับการรับรองตาม มกษ. 9023 หรือมาตรฐานที่เทียบเท่า

6.3 จิ้งหรีดแห้งต้องบรรจุในภาชนะบรรจุที่สะอาด ถูกสุขลักษณะ มีคุณสมบัติป้องกันไม่ให้จิ้งหรีดแห้งมีความชื้นเพิ่มขึ้น และไม่ส่งผลกระทบต่อคุณภาพของจิ้งหรีดแห้งในระหว่างการเก็บรักษาและการขนส่ง

6.4 ปริมาณฮิสทามีน (histamine) ต้องพบไม่เกิน 100 mg/kg

6.5 ข้อกำหนดด้านจุลินทรีย์ ให้เป็นไปตามข้อกำหนดในตารางที่ 3 ดังนี้

ตารางที่ 3 เกณฑ์ทางจุลชีววิทยาสำหรับจิ้งหรีดแห้ง

ชนิดจุลินทรีย์	แผนการชักตัวอย่าง		เกณฑ์กำหนด	
	n	c	m	M
1. จุลินทรีย์ที่เติบโตได้ (Aerobic plate count)	5	2	10^4 cfu/g	10^5 cfu/g
2. *แซลโมเนลลา* (*Salmonella* spp.)	5	0	ไม่พบในตัวอย่าง 25 g	-
3. *สแตฟิโลค็อกคัส ออเรียส* (*Staphylococcus aureus*)	5	1	10^2 cfu/g	10^4 cfu/g
4. *แบซิลลัส ซีเรียส* (*Bacillus cereus*)	5	1	10^2 cfu/g	10^4 cfu/g
5. *คลอสทริเดียม เพอร์ฟริงเจนส์* (*Clostridium perfringens*)	5	1	10^2 cfu/g	10^3 cfu/g
6. ยีสต์และรา	5	2	10^2 cfu/g	10^3 cfu/g
7. แบคทีเรียชนิดโคลิฟอร์ม	5	2	10 cfu/g	10^2 cfu/g

หมายเหตุ: n หมายถึง จำนวนตัวอย่างขั้นต่ำที่ต้องนำมาตรวจสอบจากสินค้าแต่ละรุ่น (lot)
c หมายถึง จำนวนตัวอย่างสูงสุดที่ยอมให้พบจุลินทรีย์ในระดับสูงกว่าค่าที่กำหนด
m หมายถึง จำนวนจุลินทรีย์ที่ยอมรับได้ในตัวอย่างที่ตรวจสอบ
M หมายถึง จำนวนจุลินทรีย์ที่พบในตัวอย่างที่ตรวจสอบและมีผลให้ไม่ยอมรับสินค้ารุ่นที่ตรวจสอบ
cfu/g ย่อมาจาก colony forming unit per gram

7. น้ำหนักและการวัด

จิ้งหรีดแห้งที่บรรจุในแต่ละหีบห่อ ต้องมีน้ำหนักสุทธิไม่น้อยกว่าที่ระบุบนฉลาก

8. การแสดงฉลาก

การแสดงฉลากให้เป็นไปตามข้อ 3 ของ มกษ. 9060 มาตรฐานสินค้าเกษตร เรื่อง การแสดงฉลากสินค้าเกษตร และมีรายละเอียดข้อกำหนดการแสดงฉลากสำหรับหีบห่อสำหรับผู้บริโภคและภาชนะบรรจุที่ไม่ได้จำหน่ายโดยตรงต่อผู้บริโภค ดังต่อไปนี้

8.1 หีบห่อสำหรับผู้บริโภค

อย่างน้อยต้องมีรายการดังต่อไปนี้

1) ชื่อสินค้า อาจแสดงชนิด (species) ของจิ้งหรีดเพิ่มเติมได้

2) น้ำหนักสุทธิ ให้ใช้ระบบเมตริก

3) ชื่อและที่อยู่ ดังนี้

ก) กรณีจิ้งหรีดแห้งที่ผลิตในประเทศ ให้แสดงชื่อและที่ตั้งของผู้ผลิตหรือผู้แบ่งบรรจุ หรือแสดงชื่อและที่ตั้งของสำนักงานใหญ่ของผู้ผลิตหรือผู้แบ่งบรรจุก็ได้

ข) กรณีจิ้งหรีดแห้งที่นำเข้าจากต่างประเทศ ให้แสดงชื่อและที่ตั้งของผู้นำเข้า และแสดงชื่อและประเทศของผู้ผลิตด้วย

4) ประเทศถิ่นกำเนิด ยกเว้นกรณีผลิตเพื่อจำหน่ายในประเทศ

5) การระบุรุ่น

6) การแสดงวันที่

ก) ให้แสดงวันที่ผลิต

ข) ให้แสดงวันที่ควรบริโภคก่อน[2/]

7) คำแนะนำในการเก็บรักษา (ถ้ามี)

8) คำเตือนสำหรับผู้บริโภคที่มีอาการแพ้อาหารจากสัตว์พวกกุ้งกั้งปู อาจมีความเสี่ยงต่อการบริโภคผลิตภัณฑ์นี้

8.2 ภาชนะบรรจุที่ไม่ได้จำหน่ายโดยตรงต่อผู้บริโภค

ต้องแสดงรายการดังนี้บนฉลาก ยกเว้นรายการที่มีเครื่องหมาย * กำกับ สามารถแสดงในเอกสารกำกับหรือวิธีอื่นได้

1) ชื่อสินค้า อาจแสดงชนิด (species) ของจิ้งหรีดเพิ่มเติมได้

2) น้ำหนักสุทธิ ให้ใช้ระบบเมตริก

3) ชื่อและที่อยู่ ดังนี้

ก) กรณีจิ้งหรีดแห้งที่ผลิตในประเทศ ให้แสดงชื่อและที่ตั้งของผู้ผลิตหรือผู้แบ่งบรรจุ หรือแสดงชื่อและที่ตั้งของสำนักงานใหญ่ของผู้ผลิตหรือผู้แบ่งบรรจุก็ได้

ข) กรณีจิ้งหรีดแห้งที่นำเข้าจากต่างประเทศ ให้แสดงชื่อและที่ตั้งของผู้นำเข้า และแสดงชื่อและประเทศของผู้ผลิตด้วย

4) ประเทศถิ่นกำเนิด ยกเว้นกรณีผลิตเพื่อจำหน่ายในประเทศ*

5) การระบุรุ่น*

6) การแสดงวันที่

ก) ให้แสดงวันที่ผลิต

ข) ให้แสดงวันที่ควรบริโภคก่อน

[2/] วันที่ควรบริโภคก่อน (best before date) หมายถึง วันที่สิ้นสุดระยะเวลาที่สินค้าเกษตรยังคงคุณภาพภายใต้เงื่อนไขการเก็บรักษาที่แสดงไว้และยังไม่เปิดใช้ หลังจากวันที่ระบุ สินค้าเกษตรอาจยังคงบริโภคได้ แต่คุณภาพหรือคุณลักษณะเฉพาะบางประการของสินค้าเกษตรอาจไม่เป็นไปตามที่ได้ระบุไว้

7) คำแนะนำในการเก็บรักษา (ถ้ามี)*
8) การแสดงข้อความว่า “เป็นวัตถุดิบสำหรับแปรรูปอาหารหรือปรุงอาหาร”
9) คำเตือนสำหรับผู้บริโภคที่มีอาการแพ้อาหารจากสัตว์พวกกุ้งกั้งปู อาจมีความเสี่ยงต่อการบริโภค

9. วิธีวิเคราะห์และชักตัวอย่าง

9.1 วิธีวิเคราะห์

วิธีวิเคราะห์ให้เป็นไปตามที่กำหนดในตารางที่ 4

ตารางที่ 4 วิธีวิเคราะห์ผลิตภัณฑ์จิ้งหรีดแห้ง

รายการ	วิธีวิเคราะห์ [1/ 2/]	หลักการ
1. สี กลิ่น และการเกาะกันเป็นก้อน (ข้อ 3.2.1.1 และข้อ 3.2.1.2)	ตรวจโดยวิธีประสาทสัมผัส ขนาดตัวอย่างไม่น้อยกว่า 50 g	Sensory analysis
2. ปริมาณความชื้น (ข้อ 3.2.2)	AOAC 950.46B	Air drying
3. ปริมาณโปรตีน (ข้อ 3.2.2)	AOAC 992.15 AOAC 981.10	Titrimetry (Kjeldahl) โดยใช้ conversion factor เท่ากับ 6.25
4. สิ่งแปลกปลอม (ข้อ 3.2.3)	ตรวจพินิจขนาดตัวอย่างไม่น้อยกว่า 200 g	Visual inspection
5. สารหนูทั้งหมด (ข้อ 5 รายการที่ 1)	AOAC 986.15	Atomic absorption spectrophotometry
6. ปรอททั้งหมด (ข้อ 5 รายการที่ 2)	AOAC 986.15	Atomic absorption spectrophotometry
	AOAC 977.15	Flameless atomic absorption spectrophotometry
7. ตะกั่ว (ข้อ 5 รายการที่ 3)	AOAC 999.10 | NMKL 139 AOAC 972.25	Atomic absorption spectrophotometry
	AOAC 934.07	Colorimetry (dithizone)
8. หีบห่อหรือภาชนะบรรจุ (ข้อ 6.3)	ตรวจพินิจ	Visual inspection
9. ปริมาณฮิสทามีน (ข้อ 6.4)	AOAC 977.13	Fluorimetry
10. จุลินทรีย์ที่เติบโตได้ (ข้อ 6.5 รายการที่ 1)	BAM (Bacteriological Analytical Manual) Chapter 3	Conventional Plate Count Method
11. *แซลโมเนลลา* (ข้อ 6.5 รายการที่ 2)	ISO 6579-1 BAM (Bacteriological Analytical Manual) Chapter 5	Detection method

ตารางที่ 4 วิธีวิเคราะห์จิ้งหรีดแห้ง (ต่อ)

รายการ	วิธีวิเคราะห์ [1/ 2/]	หลักการ
12. *สแตฟิโลค็อกคัส ออเรียส* (ข้อ 6.5 รายการที่ 3)	BAM (Bacteriological Analytical Manual) Chapter 12	Conventional plate count method
13. *แบซิลลัส ซีเรียส* (ข้อ 6.5 รายการที่ 4)	BAM (Bacteriological Analytical Manual) Chapter 14	Conventional plate count method
14. *คลอสทริเดียม เพอร์ฟริงเจนส์* (ข้อ 6.5 รายการที่ 5)	BAM (Bacteriological Analytical Manual) Chapter 16	Conventional plate count method
15. ยีสต์และรา (ข้อ 6.5 รายการที่ 6)	BAM (Bacteriological Analytical Manual) Chapter 18	Conventional plate count method
16. แบคทีเรียชนิดโคลิฟอร์ม (ข้อ 6.5 รายการที่ 7)	BAM (Bacteriological Analytical Manual) Chapter 4	Conventional plate count method
17. น้ำหนักสุทธิ (ข้อ 7)	ชั่งน้ำหนักของแต่ละหีบห่อหรือภาชนะบรรจุเปรียบเทียบกับข้อมูลที่ระบุในฉลากหรือใบกำกับสินค้า	Gravimetry

หมายเหตุ

1/ วิธีวิเคราะห์ให้อ้างอิงเอกสารฉบับล่าสุด

2/ กรณีไม่สามารถใช้วิธีวิเคราะห์ตามตารางที่ 4 ให้เลือกวิธีอื่นที่พิจารณาแล้วว่าเป็นวิธีวิเคราะห์ที่มีคุณสมบัติการใช้งาน (performance characteristics) เหมาะสม และเป็นไปตามหลักเกณฑ์ข้อใดข้อหนึ่ง ดังต่อไปนี้

1) เป็นวิธีวิเคราะห์ที่ประกาศโดยองค์การแห่งชาติ หรือองค์การระหว่างประเทศด้านมาตรฐาน หรือตีพิมพ์ในเอกสารคู่มือ หรือสิ่งตีพิมพ์ที่เป็นที่ยอมรับระดับสากล
2) เป็นวิธีวิเคราะห์ที่มีผลการประเมินความใช้ได้ (validation) ของผลการทดสอบว่ามีความถูกต้องและเหมาะสม โดยห้องปฏิบัติการที่มีการร่วมศึกษากับเครือข่าย (collaborative study) ตามหลักเกณฑ์ที่สอดคล้องกับองค์การระหว่างประเทศซึ่งเป็นที่ยอมรับทั่วไป
3) กรณีไม่มีวิธีวิเคราะห์ตามข้อ 1) หรือ 2) ให้ใช้วิธีวิเคราะห์ที่ได้ประเมินความใช้ได้ของผลการทดสอบว่ามีความถูกต้องและเหมาะสมโดยห้องปฏิบัติการแห่งเดียวที่มีระบบคุณภาพ (single laboratory validation) ตามหลักเกณฑ์ที่เป็นที่ยอมรับในระดับสากล

9.2 การชักตัวอย่าง

การชักตัวอย่างให้เป็นไปตามภาคผนวก ก

9.3 เกณฑ์การตัดสิน

รุ่น (lot) ของผลิตภัณฑ์จิ้งหรีดแห้งจะยอมรับได้เมื่อผ่านเกณฑ์ที่กำหนดตามข้อ 3 ถึง ข้อ 8

ภาคผนวก ก
(เป็นส่วนหนึ่งของข้อกำหนด)

การชักตัวอย่าง

ก.1 นิยาม

ความหมายของคำที่ใช้มีดังต่อไปนี้

ก.1.1 รุ่น (lot) หมายถึง ปริมาณที่แน่นอนของสินค้าที่ผลิตแบบอุตสาหกรรม (manufactured) หรือผลิตภายใต้เงื่อนไขต่างๆ ที่สันนิษฐานว่ากระบวนการผลิตมีความสม่ำเสมอ (uniform of process)

ก.1.2 การชักตัวอย่าง (sampling) หมายถึง ขั้นตอนการดำเนินงานที่ใช้ดึงหรือจัดชุดตัวอย่าง

ก.2 การชักตัวอย่าง

ก.2.1 การชักตัวอย่างผลิตภัณฑ์จิ้งหรีดแห้ง ควรเป็นไปตามข้อกำหนด มาตรฐาน หรือกฎระเบียบของหน่วยงานภายในประเทศ เช่น

1) แนวทางปฏิบัติการเก็บตัวอย่างสำหรับโรงฆ่าสัตว์และโรงงานผลิตภัณฑ์เนื้อสัตว์เพื่อการส่งออก กรมปศุสัตว์
2) ขั้นตอนการปฏิบัติในข้อ ก.2.2

ก.2.2 การชักตัวอย่างควรดำเนินการเพื่อให้ได้ตัวอย่างที่เป็นตัวแทนของรุ่นมากที่สุดเท่าที่จะปฏิบัติได้ โดยชักตัวอย่างให้ตำแหน่งกระจายทั่วถึงทั้งรุ่น มีรายละเอียดดังนี้

1) ให้ชักตัวอย่างโดยวิธีสุ่มจากรุ่นเดียวกัน จำนวน 3 หน่วยภาชนะบรรจุ เพื่อตรวจสอบน้ำหนักสุทธิและการแสดงฉลาก
2) ชักตัวอย่างจากแต่ละภาชนะบรรจุจากข้อ 1) ในปริมาณเท่า ๆ กัน นำตัวอย่างมาผสมกันอย่างระมัดระวังและรวมเป็นตัวอย่างแบบผสมรวม (composite sample) ให้ได้อย่างน้อย 1,000 g
3) ลดขนาดตัวอย่างผสมรวมดังกล่าว เพื่อให้ได้ตัวอย่างผลิตภัณฑ์จิ้งหรีดแห้งอย่างน้อย 500 g เป็นตัวอย่างสำหรับห้องปฏิบัติการ (laboratory sample) เพื่อส่งตรวจวิเคราะห์ปริมาณโปรตีน ปริมาณความชื้น สิ่งแปลกปลอม ปริมาณยีสทามีน ปริมาณโลหะหนัก และเก็บตัวอย่างที่เหลือจากตัวอย่างสำหรับห้องปฏิบัติการไว้เพื่อใช้ในการทวนสอบ
4) ให้ชักตัวอย่างเพิ่มเติมโดยวิธีสุ่มจากรุ่นเดียวกันสำหรับการทดสอบด้านจุลินทรีย์ จำนวน 5 หน่วยภาชนะบรรจุ หน่วยละ 200 g โดยใช้วิธีการเก็บตัวอย่างและภาชนะบรรจุที่ไม่ก่อให้เกิดการปนเปื้อน ต้องเก็บรักษาตัวอย่างอย่างเหมาะสมเพื่อป้องกันการเสื่อมสภาพของตัวอย่างที่อาจส่งผลกระทบต่อผลการวิเคราะห์

ภาคผนวก ข
(ให้ไว้เป็นข้อมูล)

หน่วย

หน่วยและสัญลักษณ์ที่ใช้ในมาตรฐานนี้ และหน่วย SI (International System of Units หรือ *Le Systeme International d' Unites*) ที่ยอมรับให้ใช้ได้ มีดังนี้

รายการ	ชื่อหน่วย	สัญลักษณ์หน่วย
มวล	มิลลิกรัม (milligram)	mg
	กรัม (gram)	g
	กิโลกรัม (kilogram)	kg

บัณฑิตวิทยาลัย มหาวิทยาลัยนเรศวร

มอบเกียรติบัตรฉบับนี้ให้ไว้เพื่อแสดงว่า

นันทวรรณ บุญมี

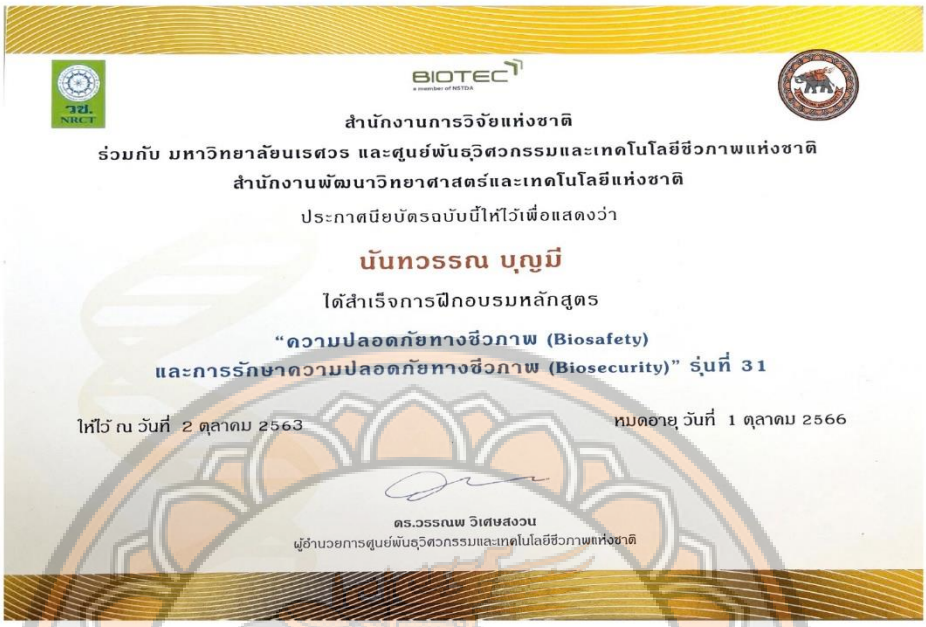
ได้เข้าร่วมโครงการอบรมจริยธรรมการวิจัยระดับบัณฑิตศึกษา

วันพฤหัสบดีที่ 22 สิงหาคม 2562

ณ ห้องสัมมนา 301 อาคารเอกภาทศรถ มหาวิทยาลัยนเรศวร

(ศาสตราจารย์ ดร.ไพศาล มุณีสร่าง)

คณบดีบัณฑิตวิทยาลัย มหาวิทยาลัยนเรศวร



สำนักงานการวิจัยแห่งชาติ
ร่วมกับ มหาวิทยาลัยนเรศวร และศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

ประกาศนียบัตรฉบับนี้ให้ไว้เพื่อแสดงว่า

นันทวรรณ บุญมี

ได้สำเร็จการฝึกอบรมหลักสูตร

"ความปลอดภัยทางชีวภาพ (Biosafety)
และการรักษาความปลอดภัยทางชีวภาพ (Biosecurity)" รุ่นที่ 31

ให้ไว้ ณ วันที่ 2 ตุลาคม 2563 หมดอายุ วันที่ 1 ตุลาคม 2566

ดร.วรรณพ วิเศษสงวน
ผู้อำนวยการศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

ใบรับรองด้านความปลอดภัยทางชีวภาพ

ชื่อโครงการ (ภาษาไทย)	การศึกษาสมบัติเชิงเทคนิค-หน้าที่ชีวเคมีและการออกฤทธิ์ทางชีวภาพของโปรตีนและน้ำมันสกัดโดยของไหลวิกฤตยิ่งยวดจากแมลงทางการค้าเพื่อการใช้ประโยชน์ในเชิงพาณิชย์
ชื่อโครงการ (ภาษาอังกฤษ)	Study of tech-functional properties, bioavailability and biological activities of supercritical extracted protein and oil from commercial insects for commercialization
ชื่อผู้วิจัย	นางสาวนันทวรรณ บุญมี
ชื่ออาจารย์ที่ปรึกษา	ผู้ช่วยศาสตราจารย์ ดร.ชนิษฐา รุดรัตนมงคล
สังกัดหน่วยงาน/คณะ	คณะเกษตรศาสตร์ ทรัพยากรธรรมชาติและสิ่งแวดล้อม
เลขสำคัญโครงการ	NUIBC MI 64-06-20
เลขที่รับรองโครงการ	64-75
ประเภทการรับรอง	งานประเภทที่ 2
การรับรองครั้งที่ 1	ข้อเสนอการวิจัยนี้ เฉพาะกิจกรรมการทดสอบฤทธิ์การต้านจุลินทรีย์ของโปรตีนและน้ำมันสกัดจากแมลงสำหรับการบริโภค ได้ผ่านการพิจารณาและรับรองจากคณะกรรมการเพื่อความปลอดภัยทางชีวภาพ มหาวิทยาลัยนเรศวร ครั้งที่ 1/2565 เมื่อวันที่ 6 ตุลาคม 2564 เห็นว่ามีความสอดคล้องกับแนวทางปฏิบัติเพื่อความปลอดภัยทางชีวภาพ จึงเห็นควรให้ดำเนินการวิจัยด้านความปลอดภัยทางชีวภาพตามข้อเสนอการวิจัยนี้ได้
วันหมดอายุครั้งที่ 1	6 ตุลาคม 2565

ลงนาม

(ดร.วิสาขี สุเรารัมย์)
ประธานคณะกรรมการพิจารณาเพื่อความปลอดภัยทางชีวภาพ
มหาวิทยาลัยนเรศวร

หมายเหตุ ใบรับรองฉบับนี้ใช้ควบคู่กับหนังสือราชการ เลขที่ อว 0603.01.13(1)/ว 2122 ลงวันที่ 8 ตุลาคม 2564