DEVELOPMENT OF EXTRACTION AND SEPARATION TECHNIQUES FOR THE DETERMINATION OF FATTY ACIDS AND PHENOLIC COMPOUNDS IN STINGLESS BEE AND TUALANG HONEY

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UNIVERSITI SAINS MALAYSIA

2025

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

September 2025

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest gratitude to my supervisor, **Dr. Wong Yong Foo**, for his unwavering support, expert guidance, and invaluable insights throughout the course of my doctoral studies. His mentorship has been instrumental in shaping both my academic and personal growth.

Special thanks are extended to the **School of Chemical Sciences/Center for Research on Multidimensional Separation Science** for providing an intellectually stimulating environment and access to essential resources that were critical for the successful completion of this research. I am particularly grateful to the technical and administrative staff for their constant assistance.

To my colleagues and fellow researchers in **Dr. Wong Group**, thank you for the stimulating discussions, collaborative spirit, and the camaraderie we shared throughout this journey.

I am especially grateful to my friends **Orwa Albitar, Mohamad Agha, Zaynab Mando, Rama Ballouze,** and **Musab Alghodran** for their constant encouragement, understanding, and the much-needed laughter that helped me stay grounded during the highs and lows of this academic journey. Your friendship has been a true source of joy and support.

A special thank you goes to my friends **Samar Kharboutli and Ghaidaa Al-Khayer**, who, despite the distance, were always there for me with words of support, thoughtful messages, and a steady belief in my ability to succeed

On a more personal note, I am eternally thankful to my family, especially **my parents** and **my siblings** (**Foton, Ghesoun, and Tarek**), for their unconditional love, patience, and belief in me. Their support has been the foundation of my resilience and determination.

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C_c	Analyte concentration in the sedimented phase
C_0	The initial concentration of analyte
R_s	Resolution
t_{Rf}	Retention time of the first analyte
t_{Rs}	Retention time and the second analyte
w_f	Peak width of the first analyte
w_s	Peak width of the second analyte
π-π	Pi-Pi interaction

LIST OF ABBREVIATIONS

1D	One-dimensional
2D	Second-dimensional
$^{1}t_{R}$	Retention time of a compound in the first dimension
$^{2}t_{R}$	Retention time of a compound in the second dimension
ACN	Acetonitrile
AM	*Apis mellifera*
BA	Benzoic acid
BF_3:MeOH	Trifluoro borane with methanol
BGE	Background electrolyte
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
C18	Octadecyl
$C_{14:0}$	Myristic acid
$C_{15:0}$	Pentadecanoic acid
$C_{16:0}$	Palmitic acid
$C_{16:1}$	Palmitoleic acid
$C_{17:0}$	Heptadecanoic acid
$C_{18:0}$	Stearic acid
$C_{18:1}$	Oleic acid
$C_{18:2}$	Linoleic acid
$C_{18:3}$	α-Linolenic acid
$C_{19:0}$	Nonadecanoic acid
$C_{20:0}$	Arachidic acid
$C_{20:4}$	Arachidonic acid
$C_{20:5}$	Eicosapentaenoic acid
$C_{22:6}$	Docosahexaenoic acid
CAA	Caffeic acid
CA	Trans-cinnamic acid
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CE–ESI-MS	Capillary electrophoresis-electrospray ionization mass spectrometry

$CHCl_3$	Chloroform
CH	Catechin hydrate
CPS	Cyanopropyl polysiloxane
CZE	Capillary zone electrophoresis
DCM	Dichloromethane
d_f	Film thickness
DLLME	Dispersive liquid-liquid microextraction
ECD	Electron capture detector
EF	Enrichment factor
EOF	Electroosmotic flow
EtOAc	Ethyl acetate
EtOH	Ethanol
FAs	Fatty acids
FAMEs	Fatty acid methyl esters
GA	Gallic acid
GAC	Green analytical chemistry
GC–FID	Gas chromatography with flame ionization detection
GC×GC	Comprehensive two-dimensional gas chromatography
GC–IT/MS	Gas chromatography-ion trap mass spectrometry
HBA	4-Hydroxybenzoic acid
HCA	2-Hydroxycinnamic acid
Hesp	Hesperetin
HPLC–DAD	High-performance liquid chromatography with diode-array detection
ICH	International Council for Harmonisation
IDLLME	Inverse dispersive liquid-liquid microextraction
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LPME	Liquid phase microextraction
MAE	Microwave-assisted extraction
MDGC	Multidimensional gas chromatography

MECC	Micellar electrokinetic capillary chromatography
MEKC	Micellar electrokinetic chromatography
MeOH	Methanol
MSA	Mass-separating agent
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
MUFA	Monounsaturated fatty acids
MWNTs	Multiwalled carbon nanotubes
N	Naringin
$Na_2B_4O_7$	Sodium tetraborate dehydrate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
$(NH_4)_2SO_4$	Ammonium sulfate
NMR	Nuclear magnetic resonance
P	Principle
PA	Protocatechuic acid
PAs	Phenolic acids
p-COA	*p*-Coumaric acid
PCs	Phenolic compounds
PDA	Photodiode array detector
PEG	Polyethylene glycol
P_M	Modulation period
PS-DVB	Polystyrene-divinylbenzene
PUFA	Polyunsaturated fatty acids
Q	Quercetin
RSD	Relative standard deviation
SB	Stingless bees
SBH	Stingless bee honey
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SULLE	Sugaring-out liquid liquid extraction
SYA	Syringic acid
*T*e	Elution temperature

TH	*Apis dorsata* (Tualang honey)
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
ToF-MS	Time-of-flight mass spectrometry
UAE	Ultrasound-assisted extraction
UHPLC–LTQ–Orbitrap MS	Ultra-high performance liquid chromatography with linear ion trap-orbitrap mass spectrometry
UPLC–ESI–MS/MS	Ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry
UV	Ultraviolet
VALLLME	Vortex-assisted liquid-liquid–liquid microextraction

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PEMBANGUNAN TEKNIK PENGEKSTRAKAN DAN PEMISAHAN UNTUK PENENTUAN ASID LEMAK DAN SEBATIAN FENOLIK DALAM MADU LEBAH KELULUT DAN MADU TUALANG

ABSTRAK

Madu lebah tanpa sengat (SBH) dan madu Tualang (TH) di Malaysia semakin mendapat perhatian dalam disebabkan komposisinya yang unik dan manfaat farmakologinya. Namun, penilaian kuantitatif yang tepat bagi sebatian fenolik (PCs) dan asid lemak (FAs) dalam madu ini kekal sebagai satu cabaran analitikal yang ketara disebabkan oleh kepelbagaian struktur yang tinggi serta gangguan daripada matriks madu yang kaya dengan gula. Kaedah sedia ada biasanya kurang kepekaan, kekhususan, dan kelestarian alam sekitar yang diperlukan untuk analisis moden. Tesis ini menumpukan kepada kekangan dengan membangunkan dan mengesahkan kaedah penyediaan sampel menggunakan teknik pengasingan lanjutan untuk penentuan PCs dan FAs dalam SBH, TH dan *Apis mellifera*. Satu hijau dan pendekatan yang mudah pengekstrakan mikro cecair-cecair serakan (DLLME) yang digabungkan dengan elektroforesis kapilari dan pengesanan tatasusunan diod (CE−DAD) untuk penentuan enam belas PCs dalam SBH dan TH telah diterangkan. Keadaan DLLME telah diselidiki melalui eksperimen pemboleh ubah faktor tunggal. Pengasingan dilakukan menggunakan kapilari silika bercantum menggunakan penimbal yang terdiri daripada 40 mmol L^{-1} boraks (pH 9.3) dan 10 % (v/v) etanol dalam < 32 minit. Kaedah ini menunjukkan had pengesanan yang rendah (0.0124 – 0.518 mg L^{-1}), kadar perolehan semula yang baik (85.2 – 111 %), faktor pengayaan (1.79 – 13.9), dan skor kehijauan (0.35 AGREEprep; 0.54 AGREE). Kaedah yang telah disahkan ini telah digunakan

untuk penentuan PCs dalam SBH dan TH. Selepas itu, satu kajian telah dijalankan menggunakan kaedah *pengekstrakan cecair-cecair berbantu gula* (SULLE) yang digabungkan dengan teknik pengesanan kromatografi gas dua dimensi berion nyala-api (GC×GC–FID) bagi penentuan serentak 13 FAs dalam SBH, TH, dan *A. mellifera*. Parameter penting SULLE adalah disiasat secara sistematik. Untuk pemisahan GC×GC, set turus kapilari MEGA-10 × SUPELCOWAX®10 digunakan dengan tempoh modulasi (P_M) selama 3 saat. Kapasiti puncak dan daya penyelesaian yang dipertingkatkan diperoleh berbanding analisis GC satu dimensi. Dalam keadaan yang dioptimumkan, had pengesanan adalah 0.0481 – 0.266 mg L^{-1}, dengan kadar perolehan semula 82.2 % – 111 %. Kaedah SULLE–GC×GC–FID yang disahkan telah berjaya digunakan untuk analisis kuantitatif FAs dalam SBH, TH, dan *A. mellifera*. Seterusnya, satu kaedah pengekstrakan cecair-cecair (LLE) yang dipertingkatkan dengan pendekatan GC×GC–FID telah dibangunkan untuk kuantifikasi 12 asid fenolik (PAs) dalam SBH, TH, dan *A. mellifera*. Parameter LLE telah dioptimumkan secara sistematik. Satu set turus kapilari HP-5ms × berkutub sederhana, dengan tempoh modulasi 4 saat, telah digunakan. Had pengesanan adalah 0.0234 – 0.182 mg L^{-1}, dan kadar perolehan semula 80.6 % – 102 %. Kaedah GC×GC–FID yang telah disahkan telah diaplikasikan dengan berkesan untuk analisis kuantitatif PAs dalam sampel madu SBH, *Apis dorsata*, dan *Apis mellifera*. Secara keseluruhannya, pendekatan yang dibangunkan ini adalah selaras dengan prinsip kimia analitikal hijau, terutamanya dari segi pengurangan penggunaan pelarut organik, kecekapan masa, dan kesederhanaan proses.

DEVELOPMENT OF EXTRACTION AND SEPARATION TECHNIQUES FOR THE DETERMINATION OF FATTY ACIDS AND PHENOLIC COMPOUNDS IN STINGLESS BEE AND TUALANG HONEY

ABSTRACT

Malaysian stingless bee honey (SBH) and Tualang honey (TH), have garnered increasing interest for their distinctive composition and pharmacological benefits. However, the accurate quantification of phenolic compounds (PCs) and fatty acids (FAs) in honey remains a significant analytical challenge due to their structural variability and sugar-rich honey matrix interference. Existing methods often lack the sensitivity, selectivity, and environmental sustainability required for modern analyses. This thesis addresses these limitations by developing and validating sample preparation methods with advanced separation techniques for the determination of PCs and FAs in SBH, TH and *Apis mellifera* honey. A green and simple dispersive liquid–liquid microextraction (DLLME) with capillary electrophoresis-diode array detection (CE−DAD) approach for the determination of sixteen PCs in SBH and TH was described. DLLME conditions were investigated using single-factor variable experiments. The separation was achieved using fused-silica capillary with buffer of 40 mmol L^{-1} borax (pH 9.3) and 10 % (v/v) ethanol in < 32 min. The method exhibited low detection limits (0.0124 – 0.518 mg L^{-1}), good recoveries (85.2 – 111 %), enrichment factors (1.79 – 13.9), and greenness scores (0.35 AGREEprep; 0.54 AGREE). The validated method was applied for determination of PCs in SBH and TH. Afterwards, the investigation was conducted using a sugaring-out assisted liquid-liquid extraction (SULLE) with comprehensive two-dimensional gas chromatography-

flame ionization detection (GC×GC–FID) approach for the determination of 13 FAs in SBH, TH, and *A. mellifera*. Important SULLE parameters were systematically investigated. For GC×GC separation, a MEGA-10 × SUPELCOWAX®10 column set was used, with a 3 sec modulation period (P_M). Improved peak capacity and resolving power were demonstrated compared to one-dimensional GC analyses. Under optimized conditions, detection limits were 0.0481 – 0.266 mg L^{-1}, with recoveries of 82.2 % – 111 %. The validated SULLE–GC×GC–FID method was successfully applied for quantification of FAs in SBH, TH, and *A. mellifera*. Next, an improved liquid-liquid extraction (LLE) with GC×GC–FID approach was developed for the quantification of 12 phenolic acids (PAs) in SBH, TH, and *A. mellifera*. LLE parameters were systematically optimized. A HP-5ms × mid-polar column set, with P_M of 4 sec were used. Detection limits were 0.0234 – 0.182 mg L^{-1}, with recovery of 80.6 % – 102 %. The validated GC×GC–FID method was effectively applied for quantification of PAs in SBH, *Apis dorsata*, and *A. mellifera* honey. Overall, the developed approaches are in agreement with green analytical chemistry principles, particularly reduction of solvents, time and simplicity.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

In recent years, honey derived from stingless bees (SB) and *Apis* bees has attracted substantial scientific and commercial interest owing to its unique physicochemical characteristics and potential health benefits (Gadge et al., 2024). Stingless bee honey (SBH), in particular, is produced by a diverse group of Meliponini bees native to tropical regions such as Malaysia, and is recognized for its rich bioactive composition and pronounced therapeutic properties (Rozman et al., 2022). Unlike honey from *Apis dorsata* and *mellifera*, SBH contains higher concentrations of phenolic compounds (PCs) and fatty acids (FAs), both of which contribute significantly to its antioxidant, antimicrobial, anti-inflammatory, and anticancer activities (Zulkifli et al., 2023). These distinctive features not only elevate the nutritional and medicinal value of SBH but also position it as a premium natural product in the global market (Ranneh et al., 2018).

The increasing global demand for natural health products and functional foods has catalyzed the need for reliable methods to characterize and authenticate honey based on its bioactive content. PCs and FAs in honey serve as key markers for botanical origin, geographical provenance, and therapeutic efficacy (Cheung et al., 2019; Massaro et al., 2024). However, their accurate quantification poses analytical challenges due to their substantial structural variability, low concentrations, the complexity of the honey matrix, and the presence of interfering substances such as sugars, proteins, and waxes (Hassan et al., 2022; Jarukas et al., 2020). These factors necessitate the development of highly selective and sensitive analytical methodologies

capable of extracting, separating, and detecting these target analytes with precision and reproducibility.

Typically, the extraction of PCs and FAs from complex honey matrices often begins with sample pretreatment methods aimed at isolating target analytes from interfering substances such as sugars, proteins, and waxes (Gómez-Caravaca et al., 2006; Kostić et al., 2017; Pyrzynska & Biesaga, 2009). Conventional extraction techniques, including liquid-liquid extraction (LLE) (Boutoub et al., 2021), solid phase extraction (SPE) (Dżugan et al., 2020) for PCs, Soxhlet extraction (Ghosh & Jung, 2017), and the Bligh and Dier method (Ferreira et al., 2023) for FAs, have been widely utilized due to their simplicity and ability to recover a broad range of analytes. However, these methods often require large volumes of hazardous organic solvents, extended extraction times, and intensive sample handling steps, which not only increase environmental burden but also introduce variability and risk of analyte degradation (Ghosh & Jung, 2017; Hassan et al., 2022; Lucci et al., 2017). Moreover, the lack of selectivity in some conventional methods can lead to co-extraction of matrix components, resulting in low analytical sensitivity and potential interference during chromatographic analysis (Badawy et al., 2022; Campo et al., 2006). These challenges underscore the need for more selective, efficient, and environmentally sustainable extraction approaches when analyzing PCs and FAs in honey samples (Hashemirad et al., 2024; Hassan et al., 2022; Zhang et al., 2023).

Conventional techniques such as high-performance liquid chromatography (HPLC) (Pyrzynska & Sentkowska, 2015) and one-dimensional gas chromatography (1D-GC) have been widely used for honey analysis (Chiu & Kuo, 2020). Nevertheless, these methods often suffer from limitations including lengthy preparation steps, significant solvent consumption, limited separation power, and inadequate sensitivity

for trace-level detection (Jalali-Heravi & Parastar, 2011; Shi et al., 2022; Stalikas, 2007; Waktola et al., 2020). In response to these challenges, green extraction and separation approaches have been proposed, focusing on minimizing environmental impact while enhancing analytical performance.

This thesis presents a systematic study on the development and validation of extraction and chromatographic techniques tailored for the quantitative and qualitative assessment of PCs and FAs in Malaysian stingless bee and Tualang honey, and *Apis mellifera* honey. The research encompasses three major methodological advancements. First, a dispersive liquid-liquid microextraction (DLLME) method was developed and optimized for the extraction of sixteen PCs, followed by detection using capillary electrophoresis with diode array detection (CE–DAD). This method provides a rapid, environmentally friendly, and cost-effective alternative to conventional solvent-intensive techniques. Secondly, the study introduces a sugaring-out assisted liquid-liquid extraction (SULLE) approach, designed to isolate FAs from honey matrices. Coupled with comprehensive two-dimensional gas chromatography-flame ionization detection (GC×GC–FID), this method achieves superior separation efficiency and peak resolution compared to traditional 1D-GC methods. The modulation of extraction parameters such as pH, solvent volume, salt concentration, and extraction cycles was systematically conducted to ensure optimal recovery and reproducibility. Thirdly, a refined liquid-liquid extraction (LLE) protocol is proposed for the determination of twelve phenolic acids (PAs) in honey, also utilizing GC×GC–FID for detection. This technique further strengthens the analytical capability for complex honey matrices, offering improved selectivity and analytical throughput. All developed methods were rigorously validated based on international guidelines,

evaluating critical parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, reproducibility, and green assessment metrics.

The integrated analytical strategies presented in this work not only advance the state-of-the-art in honey analysis but also contribute to the broader field of green analytical chemistry. By reducing solvent usage, enhancing extraction selectivity, and increasing separation power, these methods align with current sustainability goals in analytical sciences. Furthermore, the findings from this study offer valuable insights into the chemical fingerprinting of Malaysian SBH, Tualang honey, and *Apis mellifera* honey, supporting quality control, authentication, and value-added applications in food and pharmaceutical.

1.2 Stingless bees

Stingless bees (SB), belonging to the *Meliponini* tribe, have garnered substantial attention within the realm of entomology and apiculture due to their unique ecological and economic significance (Engel et al., 2023). These diminutive insects, characterized by their lack of a defensive sting, play a pivotal role in pollination dynamics and biodiversity maintenance across diverse ecosystems, and comprise about 550 species (56 genera) that are found across various tropical and subtropical zones around the globe (Grüter, 2020; Heard, 1999; Pimentel et al., 2021).

In Malaysia, the indigenous species of SB, such as *Heterotrigona itama* and *Geniotrigona thoracica*, play an essential role in the pollination of various crops, contributing to the biodiversity and sustainability of local ecosystems (Azmi et al., 2022; Zawawi et al., 2022). These bees exhibit unique foraging behaviors and are instrumental in sustaining the reproductive success of various crops (Slaa et al., 2006). Moreover, the Malaysian government has recognized the potential of stingless

beekeeping as a sustainable source of income for rural communities due to their role in producing honey with unique therapeutic properties, promoting initiatives to enhance beekeeping practices and conserve these vital pollinators (Isah et al., 2019).

In Brazil, a diverse array of SB species, including *Melipona quadrifasciata*, *Melipona scutellaris*, *Tetragonisca angustula*, *Nannotrigona testaceicornis*, and *Trigona spinipes*, are found across different regions, which contribute to the pollination of native flora and agricultural crops (Cham et al., 2019). Brazilian researchers and beekeepers have been actively engaged in studying the behaviour, ecology, and potential therapeutic properties of these bees, opening new avenues for scientific research and economic development (Jaffé et al., 2015). Academic studies have underscored the intricate social structures and communication mechanisms exhibited by SB colonies, revealing their sophisticated organization and cooperative foraging strategies (Devkota et al., 2024).

In the professional context of apiculture, the cultivation of SB colonies has emerged as an alternative and sustainable practice for pollination services and honey production (Roubik, 2018). With an increasing global awareness of the ecological importance of pollinators, the SB has garnered attention for its potential role in enhancing agricultural productivity (Bueno et al., 2023). However, challenges such as habitat loss, climate change, and the need for specialized knowledge in managing SB colonies pose significant hurdles for their widespread adoption (Toledo-Hernández et al., 2022). As research endeavors continue to unfold the intricacies of their behavior, physiology, and ecological impact, the SB remains a focal point for both academic exploration and practical applications in sustainable agriculture (Aldasoro Maya et al., 2023). Beyond their pollination services, SB have become subjects of investigation for

their unique products, including medicinal honey with potential therapeutic properties, further stimulating research initiatives at the intersection of apiculture and biomedicine.

1.3 Stingless bee honey

Stingless bee honey (SBH), a multifaceted and unique apicultural product, has increasingly captivated the attention of researchers and professionals alike due to its distinctive composition and potential applications in various domains (Rozman et al., 2022). Derived from the nectar of diverse floral sources by SB, this honey exhibits a remarkable profile characterized by its rich blend of sugars, enzymes, organic acids, and bioactive compounds (Biluca et al., 2016; Mahmad et al., 2023). One of the advantages of SBH lies in its diverse phenolic composition, encompassing flavonoids, PAs, and tannins, which contribute not only to its sensory attributes but also to its therapeutic potential (Zulkifli et al., 2023). The antioxidant properties inherent in these PCs make SBH an intriguing subject of investigation for its role in mitigating oxidative stress and its associated health benefits (Cheng et al., 2023; Gadge et al., 2024). Beyond its chemical composition, the production of SBH is a fascinating process intricately tied to the ecological dynamics of the surrounding environment (Rozman et al., 2022). SB, as meticulous pollinators, navigate the floral landscape, collecting nectar and transforming it into honey through a complex series of enzymatic activities within their hives (Salomón et al., 2024).

The symbiotic relationship between SB and their floral sources not only underscores the ecological importance of these pollinators but also shapes the unique flavor profile and botanical origin of the honey they produce (Ávila et al., 2019; Ng et al., 2021). From a nutritional standpoint, SBH stands out as more than a simple

sweetener. It possesses inherent antibacterial and antifungal properties, attributed to the presence of hydrogen peroxide and other antimicrobial compounds, making it a potential natural remedy for various health ailments (Mwangi et al., 2024; Zulkhairi Amin et al., 2018). Furthermore, the potential economic impact of SBH production can be overstated (Ab Hasan et al., 2023; Soh et al., 2021). As interest in natural, locally sourced products continues to rise, the cultivation of SB colonies for honey production presents an enticing opportunity for beekeepers and entrepreneurs (Mustafa et al., 2018; Soh et al., 2021). However, challenges such as hive management, species-specific behaviors, and environmental factors necessitate a nuanced approach to optimize production and ensure the sustainability of SBH harvesting (Rozman et al., 2022). SBH emerges as a complex and dynamic entity, intertwining ecological, nutritional, medicinal, and economic dimensions (Al-Hatamleh et al., 2020).

1.4 Tualang honey

Tualang honey is a highly prized natural honey produced by the giant honeybee (*Apis dorsata*), which builds its hives high on the branches of the Tualang tree (Koompassia excelsa), native to the rainforests of Southeast Asia, particularly Malaysia (Ahmed & Othman, 2013). This honey is renowned not only for its unique floral composition but also for its rich antioxidant, antibacterial, and anti-inflammatory properties (Devasvaran & Yong, 2016). Its bioactivity is largely attributed to the PCs, flavonoids, and organic acids it contains, which vary depending on the floral sources and environmental conditions of the region (Chew et al., 2018; Kishore et al., 2011). Tualang honey is typically dark amber in color, a characteristic that correlates with antioxidant activity compared to lighter honeys (Kamal et al., 2021). However, stingless bee honey has demonstrated significantly higher antioxidant capacities,

showing stronger radical scavenging activity against a range of reactive oxygen species than Tualang honey (Ranneh et al., 2018). It has traditionally been used in Malay folk medicine for treating wounds, infections, and gastrointestinal ailments (Ahmed & Othman, 2013; Kamal et al., 2021). Furthermore, Tualang honey has been investigated as a natural alternative to synthetic antioxidants in functional foods and nutraceuticals (Al-Kafaween et al., 2023). Its complex matrix and varying composition, however, pose analytical challenges, necessitating advanced methods for characterization and quality control (Chew et al., 2018; Pascual-Maté et al., 2018). Overall, Tualang honey stands out as a unique natural product with promising therapeutic applications and significant value in traditional and modern medicine (Azman et al., 2024).

1.5 *Apis mellifera*

Apis mellifera honey is a natural sweet substance produced by the Western honeybee (*Apis mellifera*) from the nectar of flowering plants, which includes more than 20 recognised subspecies (Bastos & Sampaio, 2012; Nawrocka et al., 2018). This type of honey is the most widely produced and consumed globally, largely due to the widespread domestication and management of *A. mellifera* colonies (Visick & Ratnieks, 2023). The composition of *A. mellifera* honey is complex, comprising mainly carbohydrates such as fructose and glucose, along with small amounts of enzymes, amino acids, vitamins, minerals, and PCs (Machado De-Melo et al., 2018). It possesses well-documented antibacterial, antioxidant, anti-inflammatory, and wound-healing properties, making it valuable in both nutrition and traditional medicine (Bouacha et al., 2024). The physicochemical and bioactive properties of this honey vary depending on factors such as floral source, geographical origin, climate,

and processing methods (Ayton et al., 2025). *A. mellifera* honey typically exhibits antioxidant activity due to its rich phenolic and flavonoid content, which contribute to its therapeutic effects (Cianciosi et al., 2018; Machado De-Melo et al., 2018). Given its versatility and health-promoting benefits, *A. mellifera* honey continues to be a focus of extensive scientific research. Overall, it remains a cornerstone of apicultural products with both economic and medicinal importance (da Silva et al., 2025).

1.6 Phenolic compounds

PCs constitute a varied class of secondary metabolites that are widely distributed in plant species (Ramawat & Mérillon, 2013). These compounds are distinguished by the presence of one or more hydroxyl functional groups linked to an aromatic ring structure (Vuolo et al., 2019). These compounds are broadly classified into flavonoids, PAs, tannins, and lignins (Rispail et al., 2005). PCs are essential for plant defense mechanisms against stress, ultraviolet radiation, and pathogens, and play a significant role in the flavor, color, and nutritional quality of fruits and vegetables (Saini et al., 2024). The biosynthesis of PCs in plants occurs via the shikimate pathway, resulting in the formation of phenylalanine, a precursor to many PCs (Yahia & Ornelas-Paz, 2009). Due to their diverse biological activities, PCs are of high interest in the fields of nutrition and medicine (Rahman et al., 2022). The antioxidant activity of PCs is well documented, as they effectively neutralize free radicals and chelate metal ions, thereby preventing diseases related to oxidative stress (Rudrapal et al., 2022). In addition, PCs have anti-inflammatory, antimicrobial, and anticancer properties, which contribute to their therapeutic potential (Rahman et al., 2022; Sun & Shahrajabian, 2023). They are added to food products as natural preservatives and functional ingredients (Bouarab Chibane et al., 2019). Despite the well-known benefits

of PCs, there are challenges in their extraction, stability, and bioavailability (Albuquerque et al., 2021). Current research is focused on improving extraction techniques and enhancing the stability of PCs within food matrices.

1.7 Phenolic compounds in stingless bee honey

SBH has gained increasing attention for its unique composition, particularly in terms of PCs (Biluca et al., 2016). In SBH, these compounds play an essential role in conferring medicinal attributes to the honey, making it a subject of intense scientific investigation (Esa et al., 2022). Phenolic profile of SBH encompasses a diverse array of compounds, including PAs and flavonoids (Santos et al., 2021). Common PAs identified in SBH, TH and *A. mellifera* include benzoic acid, vanillic acid, caffeic acid, ferulic acid, gallic acid and *p*-coumaric acid, while flavonoids include quercetin, kaempferol, chrysin, and apigenin, each contributing distinct bioactive properties (dos Santos et al., 2025; Silva et al., 2019). These compounds contribute not only to the honey's distinctive flavor and aroma but also to its therapeutic potential (Becerril-Sánchez et al., 2021). Studies have shown that PCs in SBH exhibit potent antioxidant activity, scavenging free radicals and reducing oxidative stress, which is implicated in various chronic diseases (Majid et al., 2020; Ranneh et al., 2018; Ya'akob et al., 2019). Furthermore, these compounds have demonstrated anti-inflammatory, antimicrobial, and anticancer properties, elevating the therapeutic value of SBH beyond its role as a natural sweetener (Cianciosi et al., 2018; Zulkifli et al., 2023). Additionally, PCs serve as critical markers for determining the botanical and geographical origins of SBH, providing invaluable insights into its composition and quality (Shamsudin et al., 2022; Da Silva et al., 2013). Figure 1.1 illustrates the list of studied PAs. These compounds, derived from plant sources, are transferred into honey through the bees' interaction

with floral nectar and pollen (Bankova et al., 2018; Becerril-Sánchez et al., 2021). The composition of PCs in honey is influenced by the diversity of plant species in the bees' foraging area, which reflects the botanical source (Ng et al., 2021; Zaldivar-Ortega et al., 2024). Additionally, regional environmental factors such as climate, soil, and vegetation patterns affect the phenolic profile, making it a valuable geographical indicator (Ayoub et al., 2023; Zaldivar-Ortega et al., 2024). As a result, the unique phenolic fingerprint of a honey sample can serve as a chemical indicator that not only identifies the types of plants the bees visited but also correlates to the specific geographical area in which the honey was produced (Kaškoniene & Venskutonis, 2010; Wang et al., 2022) Understanding the intricate interplay between the PCs in SBH and their health-promoting effects holds great potential for applications in both nutrition and medicine, highlighting the necessity of continued research in this expanding field.

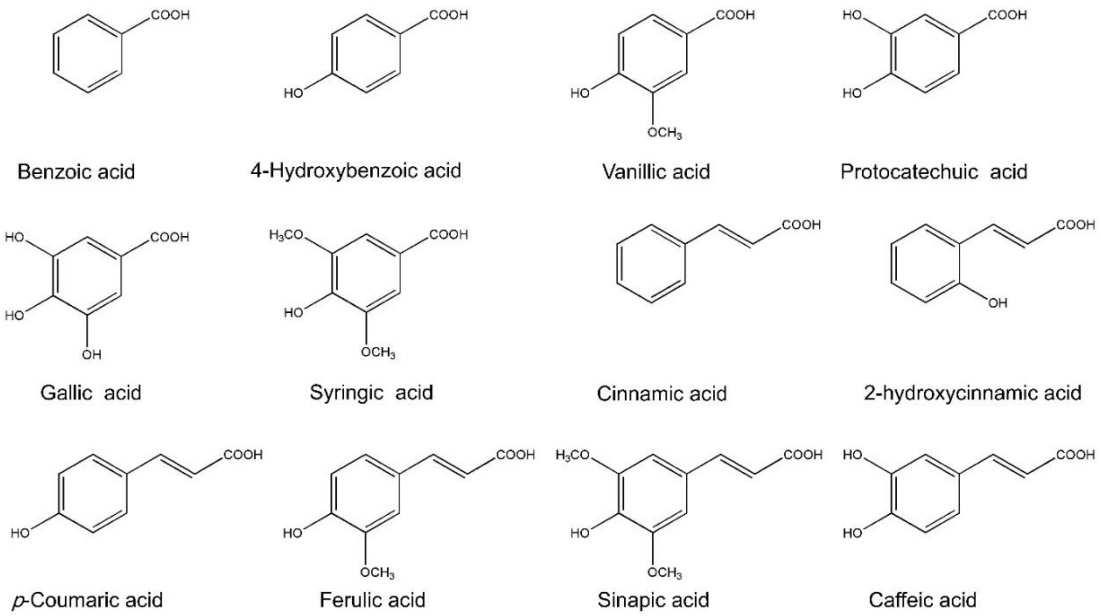

Figure 1.1 Structures of PAs employed in this study.

1.8 Fatty acids

FAs serve as fundamental components in the biological and biochemical processes of various organisms, including humans and bees, and play a crucial role in the production and quality of honey (Furse et al., 2023). Chemically, FAs are categorized as either saturated or unsaturated, depending on whether their hydrocarbon chains contain double bonds between carbon atoms (Siram et al., 2019). Saturated FAs, characterized by the absence of double bonds, generally remain solid at room temperature. In contrast, unsaturated FAs, comprising monounsaturated and polyunsaturated variants, contain one or more double bonds, which contribute to their liquid state under the same conditions (Clarke, 2008). A comprehensive knowledge of FAs in lipids is essential for evaluating alterations in metabolomic pathways within biomedical and nutritional samples (Waktola et al., 2020). In bees, FAs (from lipids) sourced from pollen are essential for their development, health, reproduction, and productivity, as they provide vital nutrients that support energy requirements and physiological functions, as well as enhance immune responses against pathogens (Bryś et al., 2021; Stephen et al., 2024). The presence of FAs in pollen influences its nutritional and health value (Alcalá-Orozco et al., 2024; Mărgăoan et al., 2014). For humans, FAs are indispensable in maintaining cellular structure and function, particularly as components of cell membranes that ensure fluidity and flexibility (Nicolson & Ash, 2014). Essential FAs (omega-3 and omega-6) are crucial for numerous physiological processes, including inflammation regulation, neuronal function, and cardiovascular health, and must be obtained through dietary sources due to the body's inability to synthesize them (Mititelu et al., 2024). These FAs are involved in synthesizing lipid mediators and regulating gene expression, underscoring their importance in preventing health disorders related to deficiency (De Carvalho &

Caramujo, 2018). Overall, the study of FAs reveals their integral role in energy storage, cellular integrity, and systemic health across diverse biological systems, highlighting their universal significance in both ecological and human contexts (De Carvalho & Caramujo, 2018; FAO, 2010).

1.9 Fatty acids in stingless bee honey

FAs in honey, although present in minimal concentrations, are contributors to its bioactivity and nutritional profile (Cucu et al., 2021). These compounds have been identified as minor but influential components in overall bee product chemical composition (Čeksterytė et al., 2008; Mărgăoan et al., 2021). The most commonly identified FAs in honey comprise saturated FAs like palmitic acid and stearic acid, as well as unsaturated FAs such as oleic acid, linoleic acid, and linolenic acid (Juárez-Trujillo et al., 2024). These FAs are primarily sourced from the pollen and nectar that bees collect (Thakur & Nanda, 2020), and their presence varies depending on the botanical origin of the bee product (Mărgăoan et al., 2014). These compounds, despite being present in trace amounts, may contribute to value-added and therapeutic properties. For instance, unsaturated FAs, particularly linoleic acid, have been associated with modulating inflammatory responses and supporting immune function (Jarukas et al., 2020). The study of FAs in honey, though relatively less explored compared to other components like PCs or sugars, offers insights into the intricate chemical composition of honey and its potential therapeutic applications. Understanding the diversity of FAs in honey across different varieties can further enhance our appreciation of its nutritional properties and quality.

1.10 Sample preparation techniques for analysis of phenolic compounds

1.10.1 Liquid-liquid extraction (LLE)

LLE is a widely used separation technique that relies on the differential solubility of analytes between an aqueous phase and a water-immiscible organic solvent (Marín-San Román et al., 2020), widely employed in chemical and biological fields (in routine analysis) due to its advantages such as operation at room temperature and simplicity (Tshepelevitsh et al., 2017). This process involves using immiscible liquid extractants to separate components in a solution (Bokhary et al., 2021). Commonly employs water-immiscible organic solvents like chloroform, ether, dichloromethane or ethyl acetate to extract target compounds, separated, evaporated, and reconstituted in minimal volumes of a suitable solvent (Bokhary et al., 2021; Pawliszyn, 2012). In one of the previous studies, ethyl acetate was employed to isolate PCs from Muğla thyme and pine honey. The LLE method proved effective in isolating significant amounts of PAs like chlorogenic acid, syringic acid, protocatechuic acid, and others with satisfied recovery (99.0 – 109.0 %) (Sıcak et al., 2021). In a previous study, the eleven PCs were extracted from Turkish honey utilizing a mixture of n-butanol, water, and chloroform as the extracting solvent, and then determined by liquid chromatography tandem-mass spectrometry (LC-MS/MS). Acceptable recoveries were obtained (91.8 – 100.2) (Kocyigit et al., 2019). Table 1.1 presents a few of the reported studies for the analysis of PCs in honey using the LLE method.

Despite its extensive applications, LLE has some shortcomings, such as difficulty in extracting certain polar compounds, the requirement for considerable volumes of hazardous organic solvents and large sample volumes, which contribute to the generation of large amounts of pollutants (Alahmad et al., 2023; Pena-Pereira et al., 2009). These limitations make the process costly, time-intensive, environmentally

unsustainable, cumbersome, labor-intensive, and susceptible to sample contamination, particularly when ultra-trace analyses are required (Badawy et al., 2022; Płotka-Wasylka et al., 2015). Furthermore, the extraction efficiency of PCs is significantly influenced by factors including the choice of solvents, extraction temperature, solvent-to-sample ratio, and the frequency of extractions (Alara et al., 2021; Pinelo et al., 2005). The drawbacks associated with this method have highlighted the need to develop and improve this approach to obtain a simple, green, effective, sensitive, affordable, and highly efficient recovery method (Hassan et al., 2022; Zhang et al., 2023).

Table 1.1 Some reported studies for the quantification analysis of PCs in honey using the LLE method. Linearity, R^2, limit of detection (LOD), limit of quantification (LOQ), and recovery of the methods are listed.

Sample matrix	Analyte	Extractant solvent	Instrument	Linearity	R^2	LOD	LOQ	Recovery	Ref.
Acacia, sunflower, linden, basil, buckwheat, oilseed rape, and goldenrod honey	Quercetin, apigenin, kaempferol, luteolin, chrysin, pinocembrin, galangin and rutin	Ethyl acetate	UHPLC–LTQ–Orbitrap MS	0.10 – 1.00 mg L^{-1}	> 0.99	0.01 – 0.05 µg kg^{-1}	0.04 – 0.18 µg kg^{-1}	83 – 113 %	(Kečkeš et al., 2013)
Cedar honey, eucalyptus, multifloral, rhododendron, vitex, carob, clover, pine, sunflower, citrus, heather, thyme, chestnut, sideritis, acacia, lavender, cotton, euphorbia, and squash blossoms honey	Pyrogallol, homogentisic acid, 3,4-dihydroxybenzoic acid, gentisic acid, pyrocatechol, galantamine, 4-hydroxy benzoic acid, 3,4-dihydroxybenzaldehyde, catechin hydrate, vanillic acid, caffeic acid, syringic acid, vanillin, epicatechin, catechin gallate, *p*-coumaric acid, ferulic acid, rutin, trans-2-hydroxy cinnamic acid, myricetin, resveratrol, trans-cinnamic acid, luteolin, quercetin, naringenin, genistein, apigenin, kaempferol, hesperetin, chlorogenic acid, and chrysin	Ethyl acetate, chloroform and dichloromethane	UPLC–ESI–MS/MS	0.05 – 10.00 mg L^{-1}	> 0.911208	0.009 – 0.020 mg kg^{-1}	-	80 – 108.9 %	(Kıvrak & Kıvrak, 2017)
Brazilian *Apis mellifera*	Gallic acid, protocatechuic acid, para-hydroxybenzoic acid, (+)-catechin, genic acid, vanillic acid, caffeic acid, chlorogenic acid, (-)-epicatechin, *p*-coumaric acid, ferulic acid, benzoic acid, synaptic, o-coumaric acid, rutin, rosmarinic acid, myricetin, cinnamic acid, naringin, quercetin and kaempferol	Butanol-ethyl acetate	HPLC-UV	-	-	-	-	-	(Nascimento et al., 2018)

Table 1.1 (Continued)

Sample matrix	Analyte	Extractant solvent	Instrument	Linearity	R^2	LOD	LOQ	Recovery	Ref.
Pine, chestnut, and sunflower honey	Gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, quercetin, kaempferol, galangin, and caffeic acid phenethyl ester	Ethanol-water	HPLC–DAD	0.5 –50 μg mL^{-1}	> 0.9979	0.06 – 0.79 μg mL^{-1}	0.21 – 2.62 μg mL^{-1}	74.92 – 115.9 %	(Guzelmeric et al., 2020)
Brazilian stingless bee honey	Benzoic acid, *p*-aminobenzoic acid, ferulic acid, syringic acid, salicylic acid, *p*-coumaric acid, 2,4-dyhydroxybenzoic acid, 2,5-dyhydroxybenzoic acid, 3,4-dyhydroxybenzoic acid, gallic acid, caffeic acid, sinapic acid, hesperidin, pinocembrin, galangin, apigenin, kaempferol, catechin, epicatechin, naringenin, pinobanksin, quercetin, naringin, rutin, taxifolin, chrysin, quercetin-3-glucoside, quercetin-3-rhamnoside, kaempferol-3-rutinoside, sinapaldehyde, coumarin, syringaldehyde, chlorogenic acid, 4-methylumbelliferone, coniferaldehyde epigallocatechin gallate	Water and mobile phase (98:2; A:B); solvent A (water with 0.1 % formic acid) and solvent B (acetonitrile with 0.1 % formic acid)	LC-ESI-MS/MS	0.2 – 250 μg L^{-1}	> 0.98	0.03 – 3.20 μg L^{-1}	0.20 – 12.8 μg L^{-1}	-	(dos Santos et al., 2025)

Table 1.1 (Continued)

Sample matrix	Analyte	Extractant solvent	Instrument	Linearity	R^2	LOD	LOQ	Recovery	Ref.
Stingless honey (*Meliponinae*) honey	Salicylic acid, protocatechuic acid, *p*-coumaric acid, caffeic acid, ferulic acid, 4-amino benzoic acid, chlorogenic acid, rosmarinic acid, vanillic acid, mandelic acid, naringenin, aromadendrin, taxifolin, isoquercetin, scopoletin, quercetin, and eriodictiol	Ethyl acetate	HPLC–ESI-MS/MS	-	-	-	-	-	(Biluca et al., 2020)
Mugga ironbark, grey ironbark, brush box, yapunyah, coolibah, spotted gum, and macadamia	Kojic acid, gallic acid, 3,4-dihydroxybenzoic acid, DL-*p*-hydroxyphenyllactic acid, epigallocatechin, *p*-hydroxybenzoic acid, catechin hydrate, vanillic acid, syringic acid, 4-methylpyrocatechol, *p*-coumaric acid, ellagic acid, taxifolin, benzoic acid, phenylacetic acid, methyl syringate, tricetin, abscisic acid, luteolin, 3-*o*-methyl quercetin, methyl ferulate, pinobanksin, kaempferol, isorhamnetin, and pinocembrin.	Acidified aqueous solution and acetonitrile	HPLC–DAD	-	≥ 0.9855	0.012 – 0.25 mg kg^{-1}	0.040 – 2.99 mg kg^{-1}	-	(Moore et al., 2025)

UHPLC–LTQ–Orbitrap MS, ultra-high performance liquid chromatography with linear ion trap-orbitrap mass spectrometry; UPLC–ESI–MS/MS, ultra-performance liquid chromatography-electrospray ionization tandem mass spectrometry; HPLC–DAD, high-performance liquid chromatography with photodiode-array detection; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry.

1.10.2 Solid-phase extraction (SPE)

SPE was introduced in the mid-1970s as an alternative to LLE for isolating and concentrating analytes from complex matrices (Liška, 2000; Poole, 2002). The principle of SPE is based on selective adsorption and desorption, where target compounds interact with a sorbent material packed in a cartridge or disk (Badawy et al., 2022; Płotka-Wasylka et al., 2023; Zhang et al., 2023). The analytes are retained on the sorbent (stationary phase) through mechanisms such as hydrophobic interactions, ion exchange, hydrogen bonding, or π–π interactions, and are subsequently eluted using an appropriate solvent that disrupts these interactions (Badawy et al., 2022; Erger & Schmidt, 2014). Figure 1.2 shows a schematic diagram of the SPE system. SPE offers several advantages, including improved sample cleanliness, reduced solvent usage, and enhanced sensitivity and reproducibility (Li et al., 2006; S. Mandal et al., 2023; Samanidou, 2019). However, its disadvantages include the potential for sorbent saturation, loss of analytes during the elution step, and higher initial costs for sorbent materials and equipment (Poole, 2003; Rodríguez et al., 2000; Zhang et al., 2023). The selectivity of SPE to separate the desired analyte from other sample components is based on the nature of the sample, the choice of solvents, and the adsorbent type employed (Li et al., 2017; Marín-San Román et al., 2020). Common sorbents used in SPE include silica-based materials (e.g., C18; C8), polymeric resins (e.g., polystyrene-divinylbenzene (PS-DVB); Strata-X; Amberlite XAD-2; Oasis HLB), or mixed-mode ion-exchange polymer (Fontanals et al., 2007, 2019), also multiwalled carbon nanotubes (MWNTs) (Ahmed Elbashir et al., 2017), each chosen based on the chemical properties of the target analytes (Fontanals et al., 2019; Zhou et al., 2024), and were employed to analyze PCs in honey (SBH or other types of honey) (Ávila et al., 2019; Ciucure & Geană, 2019; Di Marco et al., 2018;

Dimitrova et al., 2007; Michalkiewicz et al., 2008; Nešović et al., 2020; Sergiel et al., 2014; Wabaidur et al., 2015; Wang et al., 2014; Yung An et al., 2016). Table 1.2 presents some of the published studies for the determination of PCs in honey using the SPE method. Across the spectrum of honey varieties (from chaste, rape, and Yemeni to European and Chinese monofloral types), researchers have favored methanol under acidic conditions (typically pH 2) for optimal analyte solubilization. Zhou et al. (2014) and Badjah Hadj Ahmed et al. (2014) demonstrated the effectiveness of conventional sorbents such as Oasis HLB and MWCNTs, respectively, yielding high recoveries and exceptional linearity ($R^2 > 0.99$), even when targeting diverse polyphenolic profiles including flavonoids and PAs. Similarly, Stanek & Jasicka-Misiak (2018) employed Amberlite XAD-2 to robustly extract PCs from various floral honey, achieving low detection (0.010 – 0.218 μg mL^{-1}), further substantiating the material's affinity for broad-spectrum analyte retention. Notably, each study underscores the critical interplay between extraction solvent, pH adjustment, and sorbent selectivity in achieving methodological robustness, analytical sensitivity, and reproducibility across vastly differing honey matrices.

Building upon this methodological foundation, subsequent investigations have adapted SPE workflows to accommodate more complex phenolic landscapes and emerging honey sources. Anand et al. (2019) expanded the analytical scope through the use of Strata-X cartridges with high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS), capturing phenolic variability in manuka and related honey with recoveries up to 100 %. In the domain of stingless bee honey, Ávila et al. (2019) and Majid et al. (2020) employed Amberlite XAD-2 and ISOLUTEC18, respectively, marking a pivotal shift toward evaluating tropical honey varieties and confirming the technique's adaptability to

regional biodiversity. In 2023, Wang et al. introduced the Oasis HLB column with HPLC–MS/MS for comprehensive phenolic profiling in Chinese honey, achieving low detection (0.2 – 1.0 μg kg^{-1}) and wide quantification ranges. This versatility has made SPE widely applicable in environmental, pharmaceutical, and food analysis, such as determination of PCs in SBH (Majid et al., 2020), wine (Pérez-Magariño et al., 2008), olive oil (Deflaoui et al., 2021), water (Paun et al., 2024), fruit (Palma et al., 2002), milk (Palacios Colón et al., 2023), plant (Mencin et al., 2021), and plasma (Vasilakopoulou et al., 2022).

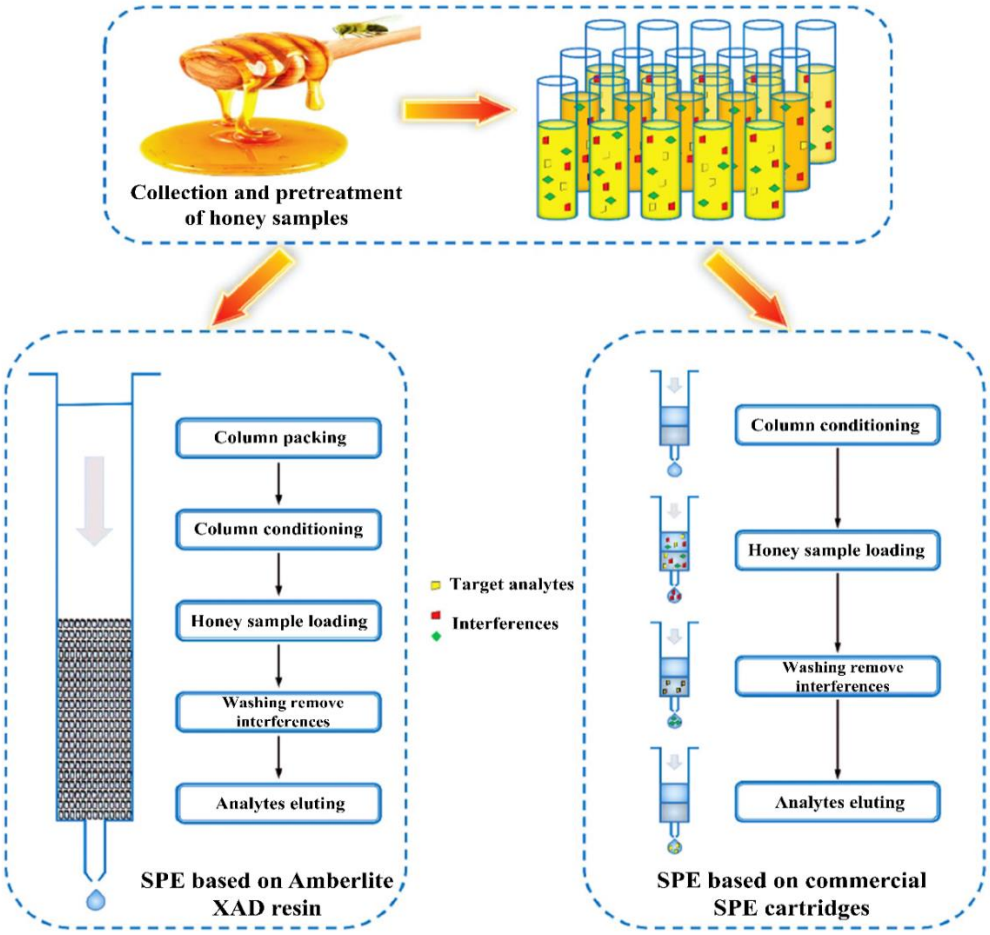

Figure 1.2 Schematic diagram of SPE system using Amberlite resin or cartridge (Zhang et al., 2023).

Table 1.2 Some reported studies for the quantification analysis of PCs in honey using the SPE method. Linearity, R^2, LOD, LOQ, and recovery of the methods are listed.

Sample matrix	Analyte	Extractant solvent	pH	Sorbent	Instrument	Linearity	R^2	LOD	LOQ	Recovery	Ref.
Chaste and rape honey	Rutin, myricetin, quercetin, kaempferol, apigenin, pinocembrine, chrysin, galangin, morin, ferulic acid, naringenin, and caffeic acid phenethyl ester	Methanol	2	Oasis HLB cartridge column	HPLC–DAD–MS/MS	0.008 – 88 μg mL^{-1}	0.9981	0.1 – 1.0 μg 100 g^{-1}	0.3 – 3.0 μg 100 g^{-1}	74.9 – 98.3 %	(Zhou et al., 2014)
Yemeni honey	Maleic acid, gallic acid, chlorogenic acid, 4-hydroxybenzoic acid, 4-hydroxyphenyl acetic acid, caffeic acid, vanillic acid, syringic acid, *p*-coumaric acid, phenol, ferulic acid, sinapic acid; naringin; luteolin, myricetin, benzoic acid, quercetin, naringenin, cinnamic acid, kaempferol, apigenin, thymol, chrysin, and galangin	Methanol	2	MWCNTs	HPLC–PDA	0.05 – 200 μg mL^{-1}	0.997	0.015 – 0.041 μg mL^{-1}	0.051 – 1.35 μg mL^{-1}	90.0 – 101.0 %	(Badjah Hadj Ahmed et al., 2014)
Willow, heather, buckwheat, pine honeydew, and manuka honey	Chlorogenic acid, gallic acid, caffeic acid, ferulic acid, *p*-coumaric acid, rosmarinic acid, ellagic acid, 3,4-dihydroxybenzoic acid, abscisic acid, myricetin, and chrysin	Methanol	2	Amberlite XAD-2 column	HPLC–PDA	0.05 – 200 μg mL^{-1}	0.9972	0.010 – 0.218 μg mL^{-1}	0.029 – 0.662 μg mL^{-1}	-	(Stanek & Jasicka-Misiak, 2018)

Table 1.2 (Continued)

Sample matrix	Analyte	Extractant solvent	pH	Sorbent	Instrument	Linearity	R^2	LOD	LOQ	Recovery	Ref.
Agastache, manuka, super manuka, tea tree, jarrah and jelly bush	Gallic acid, protocatechuic acid, 4, hydroxybenzoic acid, catechin, 2,4, dihydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, syringic acid, phenyllactic acid, *p*-coumaric acid, ferulic acid, sinapic acid, rutin, methyl syringate, rosmarinic acid, cinnamic acid, quercetin, hesperetin, and kaempferol	Methanol	2	Strata-X cartridges	HPLC–ESI–MS/MS	0 – 25 µg mL^{-1}	0.90	-	-	52.56 – 100 %	(Anand et al., 2019)
Brazilian stingless bee honey	*p*-coumaric acid, ellagic acid, quercetin and hesperitin	Methanol	2	Amberlite XAD-2 resin	HPLC–PDA	2.5 – 100 µg mL^{-1}	0.9873	-	-	-	(Ávila et al., 2019)
Heterotrigona itama Honey	Catechin, chlorogenic acid, epicatechin, o-coumaric acid, *p* coumaric acid, protocatechuic acid, quercetin, and rutin	Methanol-water		ISOLUTE C18	HPLC–DAD	-	-	-	-	-	(Majid et al., 2020)
Chinese honey	4-hydroxybenzoic acid, salicylic acid, *p*-coumaric acid, vanillic acid, gallic acid, caffeic acid, isoferulic acid, ferulic acid, syringic acid, erucic acid, catechinic acid, epicatechin, chlorogenic acid, guaijaverin, quercetin 3-O-β-xyloside, phlorizin, vincetoxicoside B, isoquercitrin, hyperoside, isorhamnetin 3-galactoside, isorhamnetin-3-O-β-D-glucoside, kaempferol 3-rutinoside, rutin, and narcissoside	Methanol	-	Oasis HLB and C18	HPLC–MS/MS	1 – 500 µg L^{-1}	0.99	0.2 – 1.0 µg kg^{-1}	0.6 – 3.0 µg kg^{-1}	71.5 – 115.2 %	(Wang et al., 2023)

PDA, photodiode array detector; MWCNTs, multiwalled carbon nanotubes.

1.10.3 Sugaring-out assisted liquid liquid extraction (SULLE)

The SULLE method was first introduced in 2008 by Wang et al. (2008) as an alternative to the LLE method, by adding a mass-separating agent (MSA) to a homogeneous solution to create biphasic immiscible systems. Wang et al. concluded that adding glucose, xylose, arabinose, fructose, maltose, sucrose, or their combinations to an ACN-water solution may cause the miscible acetonitrile (ACN) to form a two-phase system with water in all proportions, they named this new phenomenon " sugaring-out" (Wang, Ezejias, et al., 2008; Wang, Feng, et al., 2008).

The introduction of a monosaccharide or disaccharide as an MSA to ACN–water solution induces the phase separation of ACN, resulting in the formation of an immiscible layer (Dhamole et al., 2010b; Sadeghi & Coutinho, 2024). Sugars, being uncharged yet polar biomolecules, easily dissolve in water due to hydrogen bonding, where their hydroxyl or carbonyl oxygen interacts with water molecules. A similar phenomenon occurs in compounds containing N-H bonds, such as ACN (Dhamole et al., 2023; Wang, Ezejias, et al., 2008). It is likely that when sugars are introduced into an ACN-water mixture, the original hydrogen bonds in the solution are replaced by new bonds between sugar and water molecules (Dhamole et al., 2010a). This displacement causes ACN molecules to separate from water, creating a distinct phase that facilitates the extraction of target components into the ACN-rich layer (Moharkar et al., 2022; B. Wang, Ezejias, et al., 2008). As more sugar is added, the volume of the ACN layer increases, resulting in the distribution of ACN in the top phase and glucose in the bottom phase. A schematic diagram of the SULLE technique is shown in Figure 1.3. Given the exceptional properties of ACN for both organic and inorganic compounds, attributed to its high dielectric strength and dipole moment (INEOS, 2007; B. Wang, Ezejias, et al., 2008), this ACN–water two-phase system holds the potential