Compositional Profiling for the Quality Assessment of Canadian Honeys and Their Biotransformation into Functional Sweeteners

By

Mile Shao

Department of Food Science and Agricultural Chemistry

McGill University, Montréal

March 2025

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Table of Contents

ABSTRACT

Honey has been cherished for centuries as a natural sweetener and continues to hold a significant place in the food industry due to its unique taste, nutritional properties, and health benefits. With monofloral honeys attracting increased consumer attention, there is a growing need to develop novel methods for authenticating honeys. It is crucial not only to detect adulteration, such as the addition of syrups, but also to identify the botanical origins of honey, which has become an industrial demand. The most widely adopted technique for authenticating floral type is pollen analysis, which is highly sophisticated. Recent advances in honey authentication methods have primarily focused on identifying biomarkers, such as phenolic compounds. However, previous studies have suggested that both the carbohydrate composition and enzymes in honey have correlations with its botanical sources. Few studies have been conducted on the authentication of Canadian honeys, and no comprehensive profiling for sugars and enzymes were established. Therefore, this study aims to provide insight into the sugar and enzyme composition of four types of monofloral honey commonly found in the Canadian market.

The first objective was to conduct the carbohydrate and enzymatic profiling of 163 selected Canadian monofloral honeys, namely buckwheat, clover, blueberry, and goldenrod, and to establish a robust authentication method for botanical origin differentiation, while focusing on identifying potential biomarkers. The activities of five enzymes, namely diastase, invertase, acid phosphatase, glucose oxidase, and catalase, were examined. 2 monosaccharides (fructose and glucose), 6 disaccharides (trehalose, isomaltose, sucrose, maltose, nigerose, and gentiobiose), and 1 trisaccharide (erlose) were successfully identified and quantified among all honey samples using high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) and liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC/MS-QToF). Results showed the average enzymatic activity and sugar content varies across the four floral types. Multivariate analysis revealed the potentiality of acid phosphatase and catalase activities as markers for identifying botanical sources. Statistically significant ($p<0.0001$) negative correlations were observed between glucose content and Isomaltose, gentiobiose, or nigerose content, and between 5-hydroxymethylfurfural (HMF) content and diastase or invertase activities. Additionally, prediction models were generated based on the variables quantified with accuracy scores varing between 80-90%. Agreeing with the previous results, the model suggested

that acid phosphatase and catalase activities alongside electrical conductivity, peak area of HMF, invertase activity, pH and erlose content to be the most impactful features.

The second objective of this study aimed at lowering the caloric content of honey by optimizing the bioconversion of intrinsic D-fructose into D-allulose via D-allulose-3-epimerase (DAEase). The D-allulose-3-epimerase sequence from *Dorea sp.* was expressed in *Escherichia coli* and DAEase was produced. A three-variable central composite rotatable design was created to optimize the initial honey concentration, reaction time and quantity of enzyme addition for maximizing net allulose production as well as the bioconversion yield (%, w/w) using a response surface methodology (RSM). Initial honey concentration as well as the reaction time were found to have the greatest impact on the bioconversion yield of D-allulose. The optimized conditions were then applied in the bioconversion of D-allulose in honey from three selected monofloral origins (buckwheat, clover, blueberry). End-product D-allulose concentration, and bioconversion yield were assessed, and color differences, η_{50} apparent viscosity, and pH changes were measured. Significantly lower bioconversion rate of 9.55±5.55% (w/w) was observed with buckwheat honey, and a maximum bioconversion rate of 29.5% (w/w) was achieved in clover honey, providing a potential in producing fortified functional honey.

RÉSUMÉ

Depuis des siècles, le miel, comme édulcorant naturel, a été très apprécié par presque tout le monde y compris l'industrie alimentaire dû à son goût unique, ses propriétés nutritionnelles et ses bienfaits pour la santé. On constate un intérêt positif concernant des miels monofloraux par des consommateurs. Cet intérêt nous pousse à développer des méthodes avancées afin d'authentifier les miels. Il est essentiel non seulement de détecter les adultérations, tel que l'ajout de sirops, mais aussi d'identifier les origines botaniques du miel, qui est de plus en plus une exigence industrielle. La méthode d'authentification du miel type floral la plus utilisée fréquemment est basée sur l'analyse pollinique. Présentement les recherches sur les méthodes d'authentification du miel se concentrent principalement sur l'identification de biomarqueurs, tels que les composés phénoliques. Cependant, quelques recherches ont suggéré que la composition en carbohydrates et les enzymes présent dans le miel peuvent être corrélées avec l'origine botanique. Très peu d'études ont été menées sur l'authentification des miels Canadiens, et aucune n'a explorée le profilage des carbohydrates et des enzymes. Cette étude vise à caractériser les profiles des carbohydrates et des enzymes de quatre types de miel monofloral commun dans le marché canadien.

Le premier objectif consiste à réaliser le profilage des carbohydrates et des enzymes de 163 miels monofloraux canadiens, incluant le sarrasin, trèfle, bleuet et verge d'or, dans le but d'établir une méthode d'authentification robuste pour la différenciation de l'origine botanique, tout en appuyant plus sur l'identification de biomarqueurs potentiels. Les activités de cinq enzymes, incluant la diastase, l'invertase, l'acide phosphatase, le glucose oxydase et la catalase, ont été examinées. Deux monosaccharides (fructose et glucose), six disaccharides (trehalose, isomaltose, sucrose, maltose, nigerose et gentiobiose) et un trisaccharide (érlose) ont été identifiés et quantifiés avec succès dans tous les échantillons en utilisant la chromatographie en phase ionique haute performance avec détection par ampérométrie pulsée (HPAEC-PAD) et la chromatographie liquide à haute performance couplée à la spectrométrie de masse à temps de vol par quadrupole (LC/MS-QToF). Les résultats ont démontré que les activités enzymatiques et la teneur en sucre varient selon le type floral des miels. L'analyse multivariée a démontré que les activités de l'acide phosphatase et de la catalase ont le potentiel d'identifier le type floral des miels. Des corrélations négatives statistiquement significatives ($p<0,0001$) ont été observées entre la teneur en glucose et la teneur en isomaltose, gentiobiose, ou nigerose, ainsi qu'entre la teneur en 5-hydroxyméthylfurfural (HMF) et les activités diastase ou invertase. De plus, des modèles de

prédiction ont été générés selon les variables quantifiées, et un de ces modèles peut identifier le miel de trèfle et de sarrasin avec 100 % et 93 % de précision. En accord avec les résultats précédents, le modèle suggère que les activités de l'acide de phosphatase et la catalase, ainsi que l`électroconductivité, la surface maximale du HMF, l'activité de l'invertase, le pH et la teneur en érlose, sont les caractéristiques les plus marquantes.

Le deuxième objectif de cette étude porte sur le développement d'une approche de biotransformation pour réduire la teneur calorique du miel à travers la bioconversion du D-fructose intrinsèque en D-allulose via la D-allulose-3-épimérase. La séquence D-allulose-3-épimérase de *Dorea sp.*, a été exprimée dans *Escherichia coli* et l'enzyme a été produite. Un modèle de conception rotative central composite de surface de réponse à trois variables a été créé afin d'optimiser la concentration de miel, le temps de réaction et la quantité d'enzyme ajoutée, afin de maximiser la production nette d'allulose ainsi que le rendement de bioconversion (%, w/w). La concentration initiale du miel et le temps de réaction ont démontré l'effet le plus élevé sur le rendement de bioconversion du fructose en D-allulose. Les conditions optimisées ont ensuite été appliquées pour la biotransformation des échantillons du miel provenant de trois origines monoflorales sélectionnées (sarrasin, trèfle, bleuet). La concentration finale de D-allulose et le rendement de bioconversion de fructose ont été estimées, ainsi que les propriétés physico-chimiques des miels biotransformées, incluant les différences de couleur, la viscosité apparente ŋ50 et les variations du pH, ont été mesurées. Un taux de bioconversion significativement plus bas de 9,55±5,55 % (p/p) a été observé avec le miel de sarrasin, tandis qu'un taux de bioconversion maximal de 29,5 % (p/p) a été atteint avec le miel de trèfle, offrant un potentiel pour la production du miel fonctionnel enrichi.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Salwa Karboune, for her exceptional guidance, mentorship, and support throughout my research journey. Working under her supervision has been an inspiring experience; she has consistently opened my mind when facing challenges, and I will always cherish her insights. Moreover, her encouragement and feedback have been crucial in shaping my research. I would also like to express my appreciation to my supervisory committee member, Dr. Stéphane Bayen, for his invaluable advice, expertise, and support in completing the research. His contributions have greatly enriched my work, and I am grateful for his guidance throughout this process.

I am grateful to Dr. Lan Liu for her assistance with the LC/MS analysis, to Dr. Ali R. Taherian for his guidance during the rheology study, and to Dr. Asma Mdimagh for her expertise in data analysis with Python. I sincerely appreciate their patience in answering all my questions and their prompt responses whenever needed. Their support has been essential in the successful completion of this research.

To Parsley and Nandini, thank you for the training provided before I even started my master's study, which laid a solid foundation for my research. I am also grateful to Muriel for her help with the process of enzyme production and for always being there to answer my questions. To all my lab mates—Amalie, Amanda, Ann, Annas, Diana, Elham, Farnaz, Kelly, Mona, Najla, Omid, Rana, Rasha, Vanessa, Wansong, and Xinyi—I am deeply thankful for the time I spent and the amazing friendships I developed with them. Lastly, I wish to extend my gratitude to all the members of Dr. Bayen's lab for their help during the lockdown of our lab in 2023.

Last but not least, I would like to thank my mother for all her love and support. Also, to my friends Celeste, Lichen, Xutao and Shaojia, I am so fortunate to have at least one of you listening to my rumblings at the weirdest times possible and providing me with the mental support I needed to get through the toughest moments.

PREFACE AND CONTRIBUTION OF AUTHORS

This thesis includes five chapters.

Chapter I provides a short introduction on the honey with a focus on their bioactive compounds, heath benefits, and the potentiality of producing allulose enriched ones using D-allulose-3-epimerase (DAEase) to reduce the caloric content. In this chapter, the objectives of the current study were also outlined.

Chapter II presents a comprehensive literature review on the macro- and micro-components found in honey, with a focus on carbohydrates and enzymes. Additionally, the recent advances in the bioconversion of d-fructose, the major sugar present in honey, into d-allulose via d-allulose-3-epimerase were discussed, providing an insight into the production of a low-caloric functional honey.

Chapters III to IV are presented in the form of manuscripts and they will be submitted for publication. The connecting statements provide linking to the different parts of this study. Chapter III describes the result pertaining to the enzymatic and carbohydrate profiling of 163 Canadian monofloral honeys. Chemometric techniques were used to observe the correlations between the variables quantified, and prediction models were generated to identify potential biomarkers for honey authentication. Dr. Lan Liu contributed to the experiment related to the disaccharide and trisaccharide profiling by mass spectrometry, and Dr. Asma Mdimagh contributed towards the statistical analysis and prediction model generation of the compositional profiling results.

Chapter IV discusses the application and optimization of DAEase in honey for the endogenous biosynthesis of D-allulose. First, it describes the effects of temperature on the epimerization of fructose. Then, the bioconversion of intrinsic fructose in honey into allulose via DAEase is demonstrated. Finally, factors affecting the bioconversion yield were identified, and the optimized condition were tested in clover, buckwheat, and goldenrod monofloral honeys.

Chapter V covers the overall summary of the major findings in this study.

Mile Shao was responsible for the experimental work and the drafting of the current thesis.

Dr. Salwa Karboune, the supervisor of the current MSc. research project, guided throughout the entire research period and critically revised all chapters prior to the thesis submission.

LIST OF FIGURES

Figure 4.1 SDS-PAGE of the purified enzyme (Left: protein ladder, right: purified DAEase).

Figure 4.2 MS-MS fragmentation spectra of allulose, fructose, and glucose standards and in the bioconversion end-product (from top to bottom: allulose in honey and allulose standard (RT:1.53), fructose standards (RT: 1.88), glucose standards (RT: 2.32), fructose in honey, glucose in honey

Figure 4.3 D-allulose concentration (mg/ml) and relative activity (%) of DAEase in the reaction mixture containing 5% (w/v) initial fructose from pure fructose and honey at 70°C (a) and 40°C (b).

Figure 4.4 Allulose concentrations (mg/ml) produced in honey samples upon biotransformation catalyzed by D-allulose-3-epimerase (DAEase) from *Dorea sp* at (a) 70°C and (b) 40°C.(20% ( ), 70% (■) honey solution)

Figure 4.5 Contour plots of the absolute concentration of allulose (a1-3) and the bioconversion yield expressed as the final allulose concentration over the initial fructose concentration (b1-3). All reactions of biotransformation of honey by D-allulose-3-epimerase (DAEase) from *Dorea sp* were carried out at 70°C and pH 6.0.

Figure 4.6. Bioconversion yield of allulose in honey sample reactions under the optimum temperature (from left to right: B1-B4, Bw1-Bw4, C1-C4). (a) bioconversion yield after 10 min reaction; (b) bioconversion yield after 30 min reactions. Conversion yield was calculated as the final allulose concentration/ initial fructose concentration (%, w/w).

Figure 4.7 Correlation analysis between the bioconversion yield of allulose in honey samples after10 min or 30 min with the enzymatic activities (DN: diastase, IN: invertase, AP: acid phosphatase, GOX: glucose oxidase, CAT: catalase) and sugar content (Glu: glucose, Fru: fructose, F/G: fructose to glucose ratio, sucrose). (*: p-value <0.1; **: p-value <0.01)

Figure 4.8 The impact of the enzymatic biotransformation in the total color difference between the 100% (w/v) honey with mixture before the biotransformation (■) or with the reaction end-product ( ).

LIST OF TABLES

NOMENCLATURE/ LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AP	Acid phosphatase
CFIA	Canadian food inspection agency
DAEase	D-allulose-3-epimerase
DAGE	1-stearyl-2,3-dioleogyl glycerol
DN	Diastase number
DPEase	D-psicose-3-epimerase
DSC	Differential scanning calorimetry
DTEase	D-tagatose-3-epimerase
F/G	Fructose/glucose
FAO	Food and Agricultural Organization
FID	Flame ionization detector
FP	False positive
G/M	Glucose/moisture
GABA	g-aminobutyric acid
GC	Gas chromatography
GOX	Glucose oxidase
GRAS	Generally recognized as safe
HMF	5-hydroxymethylfurfural
HPAEC	High performance anion exchange chromatography
HPLC	High-performance liquid chromatography
IHC	International Honey Commission
IN	Invertase number
IPTG	Isopropyl-β-d-1-thiogalactopyranoside
IQR	Interquartile range
IRMS	Isotope-ratio mass spectrometry
LB	Luria-Bertani
LC	Liquid chromatography
LOF	Lack of fit
MRJP	Major royal jelly proteins

MS	Mass spectrometry
m/z	Mass to charge ratio
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NIR	Near-infrared spectroscopy
NMR	Nuclear magnetic resonance
PAD	Pulsed amperometric detector
PC	Principal component
PCA	Principal component analysis
p-NP	p-nitrophenol
pNPG	p-nitrophenyl-α-D-glucopyranoside
QToF	Quadrupole time-of-flight
RSM	Response surface methodology
RT	Retention times
SPME	Solid Phase Microextraction
TFC	Total flavonoid content
TPC	Total phenolic content
UV	Ultraviolet
WHO	World Health Organization

CHAPTER I. GENERAL INTRODUCTION

Honey has served as a sweetener for human consumption since the Stone Age. In many cultures its therapeutic usage was explored, such as wound healing, upper respiratory infection relieving, skin burn soothing and treating digestive tract ailments (Oryan, Alemzadeh, & Moshiri, 2016; Eteraf-Oskouei & Najafi, 2013; Ranneh, et al., 2021; Abuelgasim, Albury, & Lee, 2021). Recent studies have attributed the therapeutic applications of honey to its antioxidant, anti-inflammatory and anti-microbial activities, owing to the diverse phenolic and flavonoid composition.

Rising interest in honey consumption among the health-oriented populations as a substitute for conventional sugars is evidenced by a growing trend in its demand, especially with the organic ones (CBI, 2024). The global honey market size was valued at 8.94 billion USD in 2023 with a projected annual growth rate of 6.52% from 2024 to 2032 (Fortune Business Insight, 2024a). Within this sector, Manuka honey accounted for a market size of 381.09 million USD, with Europe merging as the dominant market, holding a share of 36.27% in 2022, and commanding a market price up to100 EUR per kg (Fortune Business Insight, 2024b; CBI, 2024). Manuka honey, a distinctive monofloral honey derived from *Leptospermum scoparium* in New Zealand and Eastern Australia, is renowned for its remarkable health benefits (Wang, Qiu, & Zhu, 2024). The determination of the chemical compositions, that are responsible for the health-promoting benefits, for the Manuka honey were brought into focus, with comprehensive studies reassuring the honey's exceptionally high market value. Consequently, this draws the attention of both consumers and researchers to other types of monofloral honeys.

Monofloral honeys refer to varieties of honey that are predominantly sourced from the nectar of a single botanical origin. Despite sharing large similarities between their polyfloral counterparts, monofloral honeys are generally more preferred by the consumers, as reflected in their higher market price (CBI, 2024). However, being one of the most easily and frequently adulterated food commodities, honey requires updated regulations not only to detect food fraud but also to protect the authenticity and reputation of the emerging monofloral market. Much research was conducted addressing this matter, as the traditional way of floral type identification, melissopalynology, is more or less seen as sophisticated and outdated (Islam, et al., 2022a; Tsagkaris, et al., 2021; Walker, Cowen, Gray, Hancock, & Burns, 2022).

Unfortunately, despite its various nutritive benefits, the high consumption of honey can lead to high caloric intake (Gillespie, Kemps, White, & Bartlett, 2023). To decrease caloric intake, the conversion of glucose or fructose into non-digestible carbohydrates can be a potential approach. D-allulose-3-epimerase (DAEase) can catalyze the bioconversion of D-fructose into D-allulose, a rare sugar with 70% relative sweetness of sucrose and neglectable caloric content (Xia, et al., 2021). In recent years, the application of DAEase in fructose-rich food showed promising possibilities in the production of D-allulose fortified produces, such as fruit juice (Patel, Kaushal, & Singh, 2020).

The first objective of this thesis was to perform the profiling of enzymatic activities and carbohydrates in order to identify potential biomarkers and to develop a robust honey authentication method. 163 honey samples from four monofloral types that are commonly found in the Canadian market, namely clover, buckwheat, blueberry and goldenrod honeys, were used in this study. With the goal of producing calorie-reduced functional honey, the investigation of the enzymatic conversion of D-fructose present in honeys into D-allulose was carried out using D-allulose-3-epimerase from *Dorea sp.* Clover, buckwheat and blueberry monofloral honeys were selected to investigate the feasibility of this approach and the effect of floral types on the bioconversion yield.

To accomplish this research, the following specific objectives were carried out:

1. Investigation of the enzymatic activity and carbohydrate profiling of Canadian monofloral honeys.
2. Multivariate analysis and generation of mathematical models to identify potential biomarkers for the prediction of the botanical origin of honeys.
3. Investigation of the endogenous biotransformation of D-fructose present in monofloral honeys into D-allulose.
4. Examination of the effect of botanical origins on the bioconversion of fructose in honeys into D-allulose by D-allulose-3-epimerase using optimal conditions.

CHATPER II. LITERATURE REVIEW

2.1 Introduction

The earliest evidence of human honey collection can be dated back to approximately 10,000 years ago (Barreiros, Cepeda, Franco, Nebot, & Vázquez, 2024). Remaining as a sweetener until the 19th century, the medicinal values of honey are more thoroughly explored in recent decades. Two types of floral honey, honeydew honey and nectar honey, exist. While the former is produced by honeybees (*Apis Mellifera*) from either the secretion of living plants (*Coniferous* and *Latifoliae* trees) or the excretion of plant-sucking insects (aphids and scale insects) on the living part of the plant, the latter comes from the nectar of blossoms. The major components of honey are sugars, which comprise over 75%-80% of its total mass, predominantly glucose and fructose. Other minor components found in honey include enzymes, proteins and amino acids, phenolic compounds, volatile compounds, organic acids, minerals, vitamins, and lipids (Nguyen, et al., 2024). The biological properties of honey include wound and sunburn healing, cardiovascular effect, weight management, anti-microbial, anti-viral, anti-inflammatory, antioxidant, anti-cancer, anti-hyperlipidemic, anti-parasitic, anti-diabetic, anti-mutagenic and anti-tumoral activities and serving as prebiotics (Spoială, Ilie, Ficai, Ficai, & Andronescu, 2023). Several studies have also pointed out the significance of honey for its health-promoting effect in ameliorating oxidative stress, anti-alcoholic effect, and antibacterial properties (Aliyu, et al., 2013; Guo, Deng, & Lu, 2019; McLoone, et al., 2021).

Honey as a diverse natural product has its composition affected by various factors including geographic locations, the botanical sources, as well as climate and seasons, status of the honeybees, treatments from the beekeepers, extraction methods and storage conditions (Alshareef, Al-Farhan, & Mohammed, 2022; Thrasyvoulou, et al., 2018). The preference of honey in the market can be determined by its claimed botanical sources as well as the color and organoleptic characteristics. Certain monofloral honeys are considered of higher quality due to their unique sensory features, and light-colored honeys are generally associated with higher market price (Lobos, Silva, Ulloa, & Pavez, 2022; Sekine, et al., 2019; Kivima, et al., 2021). Although the specie of honeybee specified by the CODEX is *A. Mellifera*, which is originated from Europe, Middle East and Africa, 3 other species of honeybee are native to Asia and tropical areas belonging to the genus *Apis*, namely *A. cerana*, *A. dorsata*, and *A. florea* (Nagma, Rashi, Netrapal, & Singh, 2021; Han,

Wallberg, & Webster, 2012). Besides various species of honeybees, honey can be produced by other types of bees, such as stingless bees, and bumblebees, as well as wasps and even honeypot ants across the world (Julika W. , et al., 2020; Chuttong, Chanbang, Sringarm, & Burgett, 2016; Islam, et al., 2022b; de Sousa, et al., 2016).

2.2 Honey botanical sources

Honey can be obtained by honeybees from the nectar of a wide range of floral plants, such as *Arbutus, Carduus, Castanea, Citrus, Erica, Eucalyptus, Helianthus, Rhododendron, Robinia, Rosmarinus, Taraxacum, Thymus, Tilia, Amorpha*, *Rubus*, *Ziziphus, Brassica,* and various species of the *Fabaceae* family, as well as honeydew from non-floral sources such as fir, and oak (Oddo, Piazza, & Pulcini, 1999; Tucak, Periškić, Škrivank, & Konjarević, 2007; Guo, Deng, & Lu, 2019; Rodríguez-Flore, Escuredo, Míguez, & Seijo, 2019b; Crăciun, Pârvulescu, Donise, Dobre, & Stanciu, 2020).

Avocado (*Persea americana*) honey is a rare type of artisanal honey with dark amber color and high pH (Dag, Afik, Yeselson, Schaffer, & Shafir, 2006). Avocado nectar contains high concentration of sucrose, D-mannoheptulose and its polyol form, perseitol. Perseitol is specific to avocado nectar during spring blooming season when other competing floral, such as citrus or wild mustard exists, hence can function as an indicator for avocado honey (Dvash, et al., 2002; Afik, Dag, Kerem, & Shafir, 2006).

Production of *Acacia* honey from several local *Acacia sp.* has been reported in several studies (Al-Khalifa & Al-Arify, 1999; Adgaba, et al., 2017; Ghramh , Khan, Zubair, & Ansari, 2020). The black locust (*Robinia pseudoacacia*) honey is often labled as *Acacia* honey, in this review all *R. pseudoacacia* honey will be referred to as black locust honey to differentiate the two (Despland, et al., 2017). Black locust honey has a very light color that falls into the water white catagory (Kasper-Szél, Amtmann, Takáts, & Kardos-Neumann, 2003). The black locust honey upon crystallization turns from light yellow hue with slight greenish tint into white with fine granules (Grujić, Grujić, Popov-Raljić, & Komić, 2011). Some exceptions applied when labeling monofloral honey based on pollen analysis, with black locust monofloral honey requiring only 20% of black locust pollen, instead of desired 45% (Uršulin-Trstenjak, et al., 2017).

Eucalyptus species are important sources of monofloral honey in the European market, which can also be found in Oceania, Africa, and Central and Southern America (Flores, Pérez, & Coello,

2014). The color of the *Eucalyptus* honey is reported to range from extra light amber to amber in various European and South America countries, and with certain Spanish origin Eucalyptus honey possesses a green undertone (Bobis, et al., 2020). The average F/G ratio range between 1.22 and 1.26, with exception for the *Eucalyptus* honey from northwest Spain, which has a relatively high F/G ratio of 1.5 (Rodríguez Flores, Escuredo Pérez, & Seijo Coello, 2014).

Heather (genus *Calluna* and *Erica*) honey from the *Ericaceae* family is considered more superior among Portuguese consumers (Martins, et al., 2008). French lavender (*Lavandula stoechas*) honey is produced in certain areas of the Ibrian penensula, experiencing shared habitat and flowering season with certain *Echium* species; so the pollen of French lavender honey is often a mix of the two with the *Echium plantagineum* pollen being the dominant type (Bonvehi & Coil , 1993). According to Bonvehi & Coil (1993), only mono- and di- saccharides were detected, with sucrose content remained low at an average of 0.26%. The differences between the furctose and glucose is less significant, and the average F/G ratio is 1.23, indicating a faster rate of cystallization during storage.

The oak and chestnut (*Aesculus hippocastanum*) trees are sharing the same habitat in the northwest of Spain, where the production of them both corresponding honeys could be found. Sensory-wise chestnut honey is relatively indistinguishable to that of oak honeydew honeys, and the patterns of their physiochemical attributes are similar (Rodríguez-Flore, Escuredo, Míguez, & Seijo, 2019; Rodríguez-Flores, Escuredo, & Seijo, 2016). The coloration of chestnut honey varies from light amber to dark amber, where in samples collected in France, the color intensity was lighter with a smaller variation between 71-99 mm Pfund (Yang, et al., 2012). Because chestnut pollen is often over-represented in honeys; thus in order for them to be identified as monofloral honey, the pollen sourced from chestnut flower should be greater than 80%, instead of the commonly recognized 45% (Uršulin-Trstenjak, Hrga, Stjepanović, Dragojlović, & Levanić, 2013). Over-representation of pollen has also been reported in other monofloral honeys including blong song (87%), *Eucalyptus* spp. (83%), *Mimosa scabrella* Benth. (92%), *Myracrodruon* (84%) (Nguyen, et al., 2024).

The nectar of rosemary blossoms (*Rosmarinus officinalis* L.) is the primary source of the production of rosemary honey being one of the most widely marketed honey in Spain (Perez-Arquillué, Conchello, Ariño, Juan, & Herrera, 1994). It is light-colored honey with low mineral

content. Commonly seen members of the Rubus genus including raspberries and blackberries (Vit, et al., 2010). The bramble honey produced in the northwest of Spain is sourced from the *Rubus* genus blossoms during early summer; though very few studies have analysed such type of honey (Escuredo, Seijo, & Fernández-González, 2011). According to Escuredo's team (2011), bramble honey possesses an amber to dark color which turns cream white or golden when granulating.

Among the genus *Ziziphus*, which belongs to the *Rhamnaceae* family, Sidr (*Ziziphus spp.*) honey as so called in Saudi Arabia and its nearby regions, is a popular type of honey with high market value in these areas. Most commonly seen botanical origins for zizphus honey include *Ziziphus jujuba*, *Ziziphus lotus*, and *Ziziphus spina-christi*, with the former two often refer to as jujube honey (Hegazi, et al., 2022). The average sugar production from the nectar of *Ziziphus spina-christi* tree blossome is 2.978 kg, hence with the estimation of honey production with 18% moisture content from one single tree could be as high as 3.6 kg (Nuru, Awad, Al-Ghamdi, Alqarni, & Radloff, 2012).

Honeydew honeys are produced mainly from the genus *Pinus*, *Abies*, *Picea*, and *Quercus* by the insects mainly from the family *Aphididae* (Mureșan, Cornea-Cipcigan, Suharoschi, Erler, & Mărgăoan, 2022). While possessing a stronger aroma, honeydew honey is generally less sweet than blossom honey, due to their low content of in both glucose and fructose. Honeydew honey has also higher oligosaccharide composition than blossom honey, for which it serves as prebiotics for human gut microbiomes (Seraglio, et al., 2019). However, honeydew contains high content of oligosaccharides, such as melezitose, making it difficult to digest compared to nectar, of which the sugar composition is sucrose, fructose and glucose (Liu, et al., 2024; Shaaban, Seeburger, Schroeder, & Lohaus, 2020). Bees consuming melezitose will intentionally increase their food intake, a behavior linked to the poor assimilation of this trisaccharide (Seeburger, et al., 2020). Overfeeding the bees with honeydew may cause malnutrition, intestinal cramp, and even death. Honeydew flow disease, a regional phenomenon observed with bee farms in Europe, is generally associated with the insufficiency of nectar during long-winter (Seeburger, et al., 2020). This scarcity forces bees to be fed on honeydew collected from firs and spruces, leading to the production of honeydew honey (Seeburger, et al., 2021). With an increasing demand for honeydew honey in the European market and a relatively low production yield, adulteration has become an unavoidable issue (Seraglio, et al., 2019). Consequently, detection methods are required to prevent fraud by the addition of cheaper blossom honey to honeydew honey, though with wide variability

in authentication parameters measured from the same plant origin honey, differentiation of the two is difficult.

Stingless bee honey has been sourced from different stingless bee honeys from Australia, Africa, Southeast Asia, and Latin America, and in total there are over 500 species of stingless bees in these subtropical regions (Nordina, Sainika, Chowdhury, Saim, & Idrus, 2018). Similar to *A. mellifera*, stingless bees which dwell in colonies where worker bees collect pollen and nectar to store them as honey in the hives. Like honeybee honey, stingless bee honey possesses a long history of traditional use and has likewise been reported to exhibit therapeutical properties such as wound healing, and antidiabetic and antioxidant activities (Rao, Krishnan, Salleh, & Gan, 2016).

2.3 Honey classification and production

From the market's point of view, honey can be divided into monofloral honey and multifloral honey, for which the former constitutes a predominant type of honey and the latter combines nectars collected from different botanic sources for which none is predominant. The pollen spectrum would refer to the plant pollen grains that are over 45% of the total pollen counts as the dominant, between 15% and 45% as secondary, between 3%-15% as minor and those that are less than 3% as trace pollen (Ozkok, et al., 2023; Song, Yao, & Yang, 2012). In general, when over 45% of the pollens are confirmed to be from the same floral type through a melissopalynology analysis, the honey is defined as monofloral. However, exceptions could be applied when the nectar is naturally low in pollen content such as avocado, citrus, lavender, rosemary, and thyme, in which 10%-45% pollen grain would be sufficient for them to be claimed as dominant pollen, though additional analysis would be required to complete the authentication (Felsner, et al., 2004; Serra Bonvehi, Ventura Coll, & Orantes Bermejo, 2019). In contrast, when the nectar is naturally high in pollen content such as chestnut, 90% pollen grain is required for it to be claimed as the dominant type (Ozkok, et al., 2023). Despite the previously mentioned two categorizations, honey can also be classified by the harvesting and processing practices, such as comb honey, chunk honey or cut comb in honey, drained honey, extracted honey, pressed honey, and filtered honey (FAO, 2015).

Regulations are set based on several components to ensure the authenticity of honey when sold on the market for human consumption. The CODEX and EU have defined and established international standards for all types of honey that are applicable for the global market. Although

specific thresholds are different for honey authentication criteria established by each regulatory body from each country, the basic components of interest are fructose and glucose content, sucrose content, moisture content, water-insoluble content, electrical conductivity, free acid, diastase activity and 5-Hydroxymethylfurfural (HMF) content (FAO, 2022). Some research also suggests the implementation of invertase activity, coupled with diastase and HMF, as an indicator for honey freshness, and has been adapted by several European countries as a recommended authentication measurement (Kamboj, Sandhu, & Nanda, 2024; Alaerjani & Mohammed, 2024). Noticing that when regulating honey quality, especially when considering the diastase activity and HMF content, baker's honey is mentioned as exceptions (FAO, 2022). This type of honey is excluded from the aforementioned honey classifications and is referred to as being suitable for industrial usage but may have a foreign taste or odor, or has begun or already fermented, or has been overheated.

Table 2.1. USDA color classification for honey

color intensity	Pfund scale (mm)
Water white	<8
Extra white	9-17
White	18-34
Extra light amber	35-50
Light amber	51-85
Amber	86-114
Dark amber	>114

The color of honey from different botanical sources is affected by its mineral, phenolic compound and protein content. HMF concentration, antioxidative capacity and enzymatic activity have also been found to be correlated with the color intensity of honey (de Almeida-Muradian, Sousa, Barth, & Gallmann, 2014; Starowicz, Ostaszyk, & Zieliński, 2021; Flanjak, Strelec, Kenjerić, & Primorac, 2016). **Table 2.1** presents the color scale for common honeys, of which the basic yellow amber tone comes from the condensation of caramelized sugar. However, there are exceptions, such as sunflower honey, which has a bright yellow color; chestnut honey, which features a red undertone; *Eucalyptus* honey which exhibits a greyish color; and honeydew honey which often shows a greenish tint (Krell, 1996). Water content has been found to be negatively correlated with the color intensity of honey (Mădaș, et al., 2019). The aroma and taste of honey are largely dependent on the botanical-source-dependent volatile organic compounds and other components such as sugar, acid, amino acid, tannins, and phenolics (Bobis, et al., 2020). *Apis mellifera* produces honey with

less sucrose content compared to that produced by *A. dorsata* (Fahim, Dasti, Ali, Ahmed, & Nadeem, 2014).

On average the consumption of honey per capita can be grouped into four rates, based on annually consumption volume, with China and Argentina having a small consumption rate at 0.1-0.2 kg per capita, followed by Italy, France, Great Britain, Denmark, and Portugal at 0.3-0.4 kg per capita (Alvarez-Suarez, Tulipani, Romandini, Bertoli, & Battino, 2009). The US, Canada and Australia have a medium consumption rate at 0.6-0.8 kg per capita, and high consumption rate is found in countries like Germany, Austria, Switzerland, Hungary, and Greece (Covaci, Brejea, & Covaci, 2023). With honey's high popularity in European countries, the EU has the second-largest honey market globally, attributing to significant production in Romania, Spain, and Hungary. Major honey production regions and countries include China, Turkey, Iran, Argentina, Brazail, India, Mexico, Ethiopia, Yemen, Southeast Asia, Oceania and various countries from the EU (Shahbandeh, 2024). With rising consumer awareness over high-quality authentic honey, the competition and challenges faced by honey producing companies globally also increases. Shahbandeh (2024) summarized the global honey production in 2022 with China being the main honey producing country (461 kilotons of honey), then followed by Turkey, Iran, Argentina, Ukraine, India, Russia, Brazil, Canada, and Republic of Korea.

2.4 Chemical composition of honey

2.4.1 Sugar composition of honey from different botanical source

Sugar is the major component of honey (70-85%) with monosaccharide, di-saccharide, and tri-/oligosaccharides constituting 75-95%, 10-15%, and 2% of the total sugar profile, respectively (Crăciun, Pârvulescu, Donise, Dobre, & Stanciu, 2020). Monosaccharides include fructose, glucose, arabinose, and rhamnose, among which the total quantity of the former two reducing sugars needs to reach at least 60% of the honey according to most regulations for honey authenticity. Disaccharides detected in honey include sucrose, maltose, isomaltose, turanose, trehalose, nigerose, maltulose, palatinose, melibiose, gentiobiose, kojibiose, cellobiose, sophorose, laminaribiose, trehalulose and inulobiose; and trisaccharides include erlose, maltotriose, raffinose, isomaltotriose, melezitose, panose, 1-kestose (or 6-kestose), nigerotriose, theanderose and cellotriose (EU, 2001; Schievano, Sbrizza, Zuccato, Piana, & Tessari, 2020; Silva, et al., 2019; Ouchemoukh, Schweitzer, Bey , Djoudad-Kadji , & Louaileche, 2010; Belitz, Grosch, &

Schieberle, 2009; de la Fuente, Sanz, Martínez-Castro, Sanz, & Ruiz-Matute, 2007). The percentage of sugar and the ratio between fructose and glucose determine the physicochemical properties of honey such as viscosity, hygroscopicity, and crystallization. While strongly dependent on the botanical and geographical origin of the honey, weather, processing, and storage conditions will also affect the sugar content of honey to a lesser extent. Among all the di-, tri- and oligosaccharides, which collectively constitute approximately 3.65% (w/w) of the honey, maltose is the most abundant, accounting for around 29.4% of these sugars (Belitz, Grosch, & Schieberle, 2009).

Sucrose is a major sweetener that serves as natural energy storage in plants. Upon consumption, sucrose is hydrolyzed into glucose and fructose by sucrase in the small intestine and can be easily absorbed for energy generation in human bodies. It is a non-reducing disaccharide with low hygroscopicity, less browning, and possesses mild sweetness. In the case of honey saccharides composition, maltose is quantified in higher percentage than sucrose, making it the most abundant disaccharide in this complex sugar mixture. The isomers of sucrose, namely trehalulose, turanose, maltulose, leucrose, palatinose (isomaltulose), are all naturally present in honey (Yuan, et al., 2024). Trehalulose (1-O-α-D-glucopyranosyl-D-fructose) can be found in honey and pollen, especially in the stingless bee honeys (Fletcher, et al., 2020). The disaccharide trehalulose has low insulinemic index and glycaemic index due to its unique and stable 1,1-glycosidic linkage against digestion (Zulkifli, Sivakumar, Maulidiani, & Ismail, 2023). Its aldose analogue, trehalose, is a disaccharide of which two glucose molecules are linked with α-1,1-glycosidic bond, and its sweetness is equivalent to 45% of that of sucrose (Côté, 2007; Aidoo, Depypere, Afoakwa, & Dewettinck, 2013). It has been reported to prevent obesity by inducing white adipose tissue browning coupled by the suppression of white adipose hypertrophy (Chen & Gibney, 2023; Ohtake & Wang, 2011). Turanose (3-O-α-D-glucopyranosyl-1,3-D-fructose) is a reducing disaccharide with 50% sweetness of sucrose (Choi, Seo, Hamaker, & Yoo, 2024). It has low calorigenic, α-glucosidase inhibitory and anti-inflammatory effects, and has been produced via amylosucrase isomerization of sucrose (Han, Lee, & Yoo, 2021). The presence of Leucrose in the sugar profile of honey is only reported in a few studies, and it can improve blood glucose level and reduce hepatic lipid accumulation (Zulkifli, Radzi, Saludes, Dalisay, & Ismail, 2022). Palatinose, also known as isomaltulose, is a disaccharide that exists in small quantities in honey and possesses 30-40% the sweetness of sucrose (Sawale, Shendurse, Mohan, & Patil, 2017). Palatinose has the more

stable α-1,6 glycosidic linkage that reduces the hydrolysis rate to 26-45% in the small intestine, hence it delays the glycemic and insulinemic responses and reduces the metabolic rate (Seibel & Buchholz, 2010). Nigerose was first discovered in Japanese sake; it has two glucose molecules linked with α-1,3 glycosidic bond, and this specific linkage contributes to its indigestibility and hence its inert caloric content. It also serves as a potential prebiotic by promoting the growth of gut microbiota and possesses other health benefits such as preventing dental caries, regulating intestinal flora, and regulating lipid metabolism (Hodoniczky, Morris, & Rae, 2012). Melibiose is D-galactopyranosyl disaccharide composed of galactose and glucose linked with α-1,6 glycosidic linkage. It is non-digestible by humans but can be fermented by gut microflora (Tanaka, Shinoki, & Hara, 2016). Melibiose, while possessing nutraceutical and therapeutic properties such as promoting calcium absorption and inhibiting aggregation-mediated neurodegenerative disorders, was also reported to stabilize proteins, making it a potential candidate for pharmaceutical applications (Mineo, Hara, Shigematsu, Okuhara, & Tomita, 2002; Chen, et al., 2020; Palomäki, Lipiäinen, Strachan, & Yliruusi, 2020). It can also serve as a prebiotic, as evidence shows the proper intake of melibiose promotes the gut health (Adamberg, et al., 2018). Kojibiose (2-O-α-D-glucopyranosyl-D-glucopyranoside) and the oligosaccharides derived from it can selectively also promotes the beneficial gut microbiota growth. In addition to its potential prebiotic properties, kojibiose is also undigestible by common oral bacteria (Onyango, et al., 2020). Therefore, kojibiose can potentially serve as a low-calorie sweetener with prebiotic properties that may help prevent tooth decay. However, its application at industrial level has been hindered by the challenges in isolating it from natural source and scaling up its synthesis, both chemically and enzymatically.

The trisaccharide melezitose (α-d-glucopyranose-1,3-β-d-fructose-2,1-α-d-glucopyranose) is derived from turanose and sucrose. It is a fast-crystalizing sugar found in honey. Some applications of melezitose include stabilizing the native state of proteins in pharmaceutical and therapeutic formulations, acting as a masking agent to reduce the bitterness of high potency sweeteners like steviol glycosides, substituting sugar for a lower caloric content, and serving as a metabolic biomarker in the prenatal screening of Down Syndrome (Behera & Balaji, 2021). Isomelezitose and trehalose are two trisaccharides naturally present in trace amounts in honey. Their presence results from transglycosylation mediated by invertase, an α-glucosidase present in honey (Garcia-Gonzalez, Minguet-Lobato, Plou, & Fernandez-Lobato, 2020). Erlose (α-d-glucopyranosyl-

(1→4)-α-d-glucopyranosyl-(1→2)-β-d-Fructose) and panose (α-d-glucopyranosyl-(1→6)-α-d-glucopyranosyl-(1→4)-α-d-Glucose) are two other trisaccharides present in honey, both of which possess low-caloric and a cariogenic properties (Vergès, et al., 2016). Maltotriose, another trisaccharide, is composed of three glucose units linked by α-(1, 4) glycosidic bonds. (Aquinas, Chithra, & Bhat, 2024) Additionally, other commonly found trisaccharides in honey include raffinose, isomaltotriose, kestose, nigerotriose, theanderose and cellotriose (Lane, Calonne, Slattery, & Hickey, 2019; Silva, et al., 2019).

The analysis of sugar for different honey are heterogeneous, depends on the purpose of the study as well as detection methods. Besides fructose, glucose, and sucrose, the detection and quantification of other oligosaccharides in different honeys vary. In contrary to sucrose which breaks down rapidly during storage, most of the oligosaccharide present in the honey are relatively stable and hence can serve as fingerprints indicating the botanical origins of different types of honey (Schievano, Sbrizza, Zuccato, Piana, & Tessari, 2020). As multifloral honeys are lacking distinct physical or chemical features to set up standards for authentication purposes (Zerrouk & Bahloul, 2020), only monofloral honey will be reviewed for their sugar profiles. The sugar profiling of twenty monofloral honeys, summarized in **Table 2.2**, were chosen considering a minimum requirement of two studies quantifying sugars beyond fructose, glucose, and sucrose.

Table 2.2 Sugar profiling of different monofloral honeys and honeydew honeys

Botanical origin	Monosaccharide (Min-Max, %)	Disaccharide (Min-Max, %)	Trisaccharide and other oligosaccharide (Min-Max, %)	F/G ratio	Reference
Apiaceae/ Umbelliferae	Fructose:38.0-42.1 Glucose:24.6-35.0	Sucrose: 0.0-3.3 Maltose: 0.9-5.6 Gentiobiose: 0.0-0.1 Isomaltose: 0.1-4.7 Kojibiose: 0.1-0.3 Maltulose: 0.5-1.4 Turanose: 1.0-2.3	Erlose: 0.0-0.5 Raffinose: 0.0-0.3 Melezitose: 0.0-0.6	Mean: 1.3 Min-Max:1.1-1.6	(Terrab, et al.; 2003; Ouchemoukh, et al., 2010, Homrani, et al., 2020)
Avocado	Fructose:30.7-41.3 Glucose:18.4-31.7	Sucrose: 0.1-2.3 Maltose: 1.3-6.4 Cellobiose: 0.1-0.1 Gentiobiose: 0.0-0.1 Isomaltose: 0.9-4.0 Melibiose: 0.0-0.04 Kojibiose: 1.0-2.8 Laminaribiose: 0.1-0.3 Maltulose: 2.4-4.3 Turanose: 1.2-3.8 Trehalose: 0.4-0.8	Erlose: 0.1-1.3 Maltotriose: 0.1-0.2 Raffinose: 0.01-0.04 Melezitose: 0.1-2.3 Panose: 0.3-0.5 Theanderose: 0.1-0.2	Mean: 1.3 Min-Max: 1.2-1.3	(De la Fuente, et al., 2007; Manzanares, et al., 2014; Serra Bonvehi, et al., 2019)
Buckwheat	Fructose:30.6-48.0 Glucose:27.3-47.0	Sucrose: 0.1-0.9 Maltose: 1.6-6.8 Gentiobiose: 0.0-0.3 Isomaltose: 0.4-2.1 Trehalose: 0.0-1.5	Erlose: 0.0-1.7 Maltotriose: 0.0-1.3 Raffinose: 0.0-1.1 Melezitose: 0.0-1.6 Panose: 0.0-1.1	Mean: 1.2-1.3 Min-Max:1.1-1.4	(Pasini, et al., 2013; Kowalski, et al., 2013; Deng, et al., 2018)
Chestnut	Fructose:32.7-50.2 Glucose:20.1-31.5	Sucrose: 0.0-5.5 Maltose: 0.0-6.7 Gentiobiose: 0.16-0.2 Isomaltose: 0.3-3.4 Turanose: 0.7-3.5 Trehalose: 0.0-2.5	Erlose: 0.1-0.7 Maltotriose: 0.0-0.1 Isomaltotriose: 0.01-0.04 Raffinose: 0.0-0.2 Melezitose: 0.0-1.0 Panose: 0.1-0.2	Mean: 1.5 Min-Max: 1.3-1.8	(Šarić, et al., 2008; Primorac, et al., 2011; Escuredo, et al., 2013; Manzanares, et al., 2017; Rodríguez-Flores, et al., 2019b;

			Maltotetraose: 0.0-0.01		
Citrus	Fructose:31.9-55.0 Glucose:23.7-34.8	Sucrose: 0.0-12.0 Maltose:1.0-9.8 Cellobiose:0.1-0.3 Gentiobiose: 0.0-0.1 Isomaltose: 0.0-2.2 Kojibiose: 0.0-2.6 Laminaribiose: 0.1-0.6 Maltulose: 0.3-2.7 Turanose: 1.4-2.9 Trehalose: 0.0-0.8 Trehalulose:0.5-2.0 Palatinose: 0.0-0.5 Nigerose: 0.3-1.2	Erlose: 0.0-1.1 Maltotriose: 0.01-0.02 Isomaltotriose: 0.0-0.1 Raffinose: 0.0-0.6 Melezitose: 0.1-0.4 Kestose: 0.0-0.1 Planteose: 0.0-0.1 Neokestose: 0.0-0.1 Panose: 0.0-0.2 Theanderose:0.0-0.1	Mean: 1.21-1.58 Min-Max: 1.12-1.86	(Mateo & Bosch-Reig, 1993; Terrab, et al., 2003; De la Fuente et al., 2011; Khalafi, et al., 2016; Chakir, et al., 2016; Bouhlali, et al., 2019; Karabagias, 2019, Homrani, et al., 2020)
Clover	Fructose:37.1-42.8 Glucose:28.9-37.7	Sucrose: 0.2-1.7 Maltose: 0.6-5.1 Gentiobiose: 0.1-0.1 Isomaltose: 0.6-1.4 Trehalose: 0.06-0.07	Erlose: 0.7-0.8 Maltotriose: 0.09-0.1 Isomaltotriose: 0.01-0.01 Raffinose: 0.03-0.1 Melezitose: 0.06-0.1 Panose: 0.2-0.4	NA	(Ciappini, et al., 2016; Pascual-Maté, et al., 2018)
Echium	Fructose:33.2-43.9 Glucose:28.0-36.8	Sucrose: 0.1-3.9 Maltose: 0.9-7.1 Cellobiose: 0.1-0.2 Gentiobiose: 0.0-0.01 Isomaltose: 0.2-2.1 Kojibiose: 0.5-2.6 Laminaribiose: 0.0-0.5 Maltulose: 0.8-2.4 Turanose: 0.9-3.1 Trehalose: 0.3.-2.4 Trehalulose: 0.7-1.6	Erlose: 0.2-0.5 Maltotriose: 0.01-0.03 Isomaltotriose: 0.00-0.01 Melezitose: 0.1-1.6 Kestose: 0.0-0.1 Planteose: 0.0-0.1 Neokestose: 0.0-0.1 Panose: 0.0-0.2 Theanderose: 0.01-0.04	Mean: 1.13 Min-Max: 1.04-1.22	(De la Fuente et al., 2011; Manzanares, et al., 2017)

		Palatinose: 0.1-0.3 Nigerose: 0.4-1.7			
Eucalyptus	Fructose:27.8-45.6 Glucose:22.0-33.9	Sucrose: 0.0-4.5 Maltose: 0.0-5.9 Cellobiose: 0.0-0.2 Gentiobiose: 0.0-0.04 Isomaltose: 0.0-2.4 Kojibiose: 0.0-2.5 Laminaribiose: 0.1-0.3 Maltulose: 0.1-3.9 Turanose: 0.0-1.8 Trehalose: 0.0-0.9 Trehalulose: 0.6-2.2 Palatinose: 0.0-0.5 Nigerose: 0.2-1.6	Erlose: 0.0-0.5 Maltotriose: 0.0-0.03 Isomaltotriose: 0.0-0.3 Raffinose: 0.0-0.4 Melezitose: 0.0-0.6 Kestose: 0.01-0.05 Planteose: 0.0-0.05 Neokestose: 0.0-0.1 Panose: 0.0-0.3 Theanderose: 0.0-0.1	Mean: 1.22-1.5 Min-Max: 1.15-1.8	(Mateo & Bosch-Reig, 1993; Terrab, et al., 2003; Ouchemoukh, et al., 2010; De la Fuente et al., 2011; Escuredo, et al., 2013; Kamboj, et al., 2013; Rodríguez Flores, et al., 2014; Ciappini, et al., 2016; Chakir, et al., 2016; Belay et al., 2017; Bouhlali, et al., 2019)
Erica/ Heather	Fructose:33.4-52.7 Glucose:21.0-39.7	Sucrose: 0.0-4.5 Maltose: 0.4-6.0 Cellobiose: 0.1-0.3 Gentiobiose: 0.0-0.4 Isomaltose: 0.2-7.4 Kojibiose: 0.0-3.1 Laminaribiose: 0.1-0.3 Maltulose: 0.3-2.6 Turanose: 0.0-2.8 Trehalose: 0.0-2.1 Trehalulose: 0.7-1.6 Palatinose: 0.1-0.4 Nigerose: 0.5-1.6	Erlose: 0.0-0.9 Maltotriose: 0.0-0.3 Isomaltotriose: 0.0-0.2 Raffinose: 0.0-2.6 Melezitose: 0.0-3.9 Kestose: 0.0-0.1 Planteose: 0.0-0.1 Neokestose: 0.0-0.1 Panose: 0.0-0.4 Theanderose: 0.0-0.1 Maltotetraose: 0.0-0.3	Mean: 1.13-1.32 Min-Max: 1.12-1.46	(Mateo & Bosch-Reig, 1993; Terrab, et al., 2003; Martins, et al., 2008; Ouchemoukh et al., 2010; De la Fuente, et al., 2011; Waś, et al., 2011; Escuredo, et al., 2013; Pascual-Maté, et al., 2018; Rodríguez-Flores, et al., 2019a)
Lavender	Fructose:34.0-48.8 Glucose:21.3-36.8	Sucrose: 0.0-5.2 Maltose: 1.2-7.8 Gentiobiose: 0.1-0.2 Isomaltose: 0.1-2.0 Melibiose: 0.0-0.5	Erlose: 0.0-1.4 Maltotriose: 0.1-0.2 Isomaltose: 0.0-0.02 Raffinose: 0.0-1.3 Melezitose: 0.0-0.3	Mean: 1.22 Min-Max: 1-1.43	(Mateo & Bosch-Reig, 1993; Martins, et al., 2008; Chakir, et al., 2016; Pascual-Maté, et al., 2018; Bouhlali, et al., 2019)

		Kojibiose: 0.0-3.0 Maltulose: 1.0-2.6 Turanose: 0.2-0.7 Trehalose: 0.1-0.9	Panose: 0.1-0.2 Maltotetraose: 0.0-0.01		
Rape	Fructose:28.6-38.1 Glucose:26.5-42.6	Sucrose: 0.0-5.6 Maltose: 0.0-3.3 Turanose: 0.0-1.9 Trehalose: 0.0-5.6	Erlose: 0.0-0.1 Raffinose: 0.0-0.4 Melezitose: 0.0-1.8	Mean: 1.04-1.11 Min-Max: 0.94-1.22	(Tomczyk, et al., 2019; Scripcă, et al., 2019; Pauliuc, et al., 2020; Zhang, et al., 2021)
Rubus	Fructose:33.1-42.0 Glucose:24.4-35.2	Sucrose: 0.0-1.6 Maltose: 0.8-6.5 Turanose: 0.2-0.4 Trehalose: 0.0-2.2	Raffinose: 0.2-0.6 Melezitose: 0.0-1.2	Mean: 1.26-1.3 Min-Max: 0.9-1.7	(Escuredo, et al., 2011; Escuredo, et al., 2013; Pauliuc, et al., 2020)
Rosemary	Fructose:31.0-45.6 Glucose:22.7-37.0	Sucrose: 0.0-5.9 Maltose: 0.6-9.0 Cellobiose: 0.0-0.1 Isomaltose: 0.6-2.0 Kojibiose: 1.0-3.2 Laminaribiose: 0.1-0.4 Matulose: 0.9-2.6 Turanose: 1.3-2.8 Trehalose: 0.3-0.5 Trehalulose: 0.5-1.6 Palatinose: 0.1-0.3 Nigerose: 0.6-1.1	Erlose: 0.0-2.1 Maltotriose: 0.0-0.04 Isomaltoriose: 0.0-0.04 Raffinose: 0.1-0.5 Melezitose: 0.0-0.3 Kestose: 0.0-0.04 Planteose: 0.0-0.1 Neokestose: 0.0-0.1 Panose: 0.0-0.4 Theanderose: 0.0-0.1	Mean: 1.17 Min-Max: 0.99-1.4	(Mateo & Bosch-Reig, 1993; De la Fuente, et al., 2011; Chakir, et al., 2016; Bouhlali, et al., 2019;
Robinia/ black locust	Fructose:33.8-53.3 Glucose:22.3-43.7 Rhamnose:0.0-0.01	Sucrose: 0.0-9.9 Maltose: 0.0-5.4 Gentiobiose: 0.0-0.03 Isomaltose: 0.0-1.7 Kojibiose: 0.0-1.2 Maltulose: 0.0-2.2 Turanose: 0.0-5.5 Trehalose: 0.0-3.7 Palatinose: 0.0-0.6	Erlose: 0.0-4.3 Maltotriose: 0.0-1.4 Isomaltotriose: 0.0-0.1 Raffinose: 0.0-0.3 Melezitose: 0.0-0.4 Kestose: 0.0-0.2 Panose: 0.0-0.1	Mean: 1.5-1.7 Min-Max: 0.44-1.91	(Kasper-Szél, et al., 2003; Šarić, et al., 2008; Marghitas, et al., 2010; Grujićc, et al., 2011; Primorac, et al., 2011; Marc, et al., 2012; Belay et al., 2017; Uršulin-Trstenjak, et al., 2017; Juan-Borrás, et al., 2014; Mohamed,

		Nigerose: 0.0-0.5			et al., 2018; Bouhlali, et al., 2019; Mădas, et al., 2019; She, et al., 2019; Scripcă, et al., 2019; Tomczyk, et al., 2019; Vranic, et al., 2019; Schievano, et al., 2020; Khan, et al., 2021)
Sage	Fructose:38.2-49.9 Glucose:10.9-41.4 Arabinose:0.0-0.04	Sucrose: 0.0-7.7 Maltose: 0.0-5.2 Isomaltose: 0.3-1.3 Turanose: 0.0-0.1 Trehalose: 0.0-0.6	Maltotriose: 0.0-0.01 Isomaltotriose: 0.0-0.6 Raffinose: 0.0-0.4	Mean: 1.3 Min-Max: 1.0-1.9	(Šarić, et al., 2008; Primorac, et al., 2011; Gašić, et al., 2015)
Sunflower	Fructose:32.4-49.9 Glucose:32.3-45.5	Sucrose: 0.0-3.7 Maltose: 0.7-3.4 Gentiobiose: 0.0-0.04 Isomaltose: 0.0-0.7 Kojibiose: 0.3-2.5 Maltulose: 0.4-1.5 Turanose: 0.1-0.7 Trehalose: 0.0-3.0	Raffinose: 0.0-0.7 Melezitose: 0.6-1.5	Mean: 1-1.33 Min-Max: 0.9-1.62	(Mateo & Bosch-Reig, 1993; Terrab, et al., 2003; Kamboj, et al., 2013; Juan-Borrás, et al., 2014; Pauliuc, et al., 2020)
Thyme	Fructose:32.9-50.3 Glucose:26.1-39.3	Sucrose: 0.0-2.6 Maltose: 0.0-2.3 Turanose: 0.1-0.6 Trehalose: 1.3-2.8	Raffinose: 0.1-0.7 Melezitose: 0.5-0.2	Mean: 1.30-1.38 Min-Max: 1.06-1.62	(Khalafi, et al., 2016; Chakir, et al., 2016; Bouhlali, et al., 2019; Karabagias, 2019; Pauliuc, et al., 2020)
Tilia spp./ Lime/ Linden	Fructose:33.9-44.4 Glucose:23.1-34.5	Sucrose: 0.1-9.9 Maltose: 0.0-3.3 Isomaltose: 0.0-2.7 Turanose: 0.0-2.9 Trehalose: 0.1-3.1	Erlose: 0.0-0.7 Maltotriose: 0.0-0.1 Isomaltotriose: 0.1-1.0 Raffinose: 0.0-0.4 Melezitose: 0.3-2.6 Panose: 0.1-0.8	Mean: 1.19-1.30 Min-Max: 1.01-1.33	(Waś, et al., 2011; Marc, et al., 2012; Juan-Borrás, et al., 2014; Gašić, et al., 2014; Scripcă, et al., 2019; Tomczyk, et al., 2019)
Ziziphus	Fructose:33.2-46.8 Glucose:21.6-35.7	Sucrose: 0.0-4.0 Maltose: 1.2-5.6	Erlose: 0.0-1.9 Maltotriose: 0.1-0.2	Mean: 1.33-1.50 Min-Max: 1.30-1.54	(Mekious, et al., 2015; Chakir, et al.,

		Gentiobiose: 0.2-0.5 Isomaltose: 0.9-1.1 Turanose: 1.3-2.4 Trehalose: 1.2-1.4	Raffinose: 0.0-0.3 Melezitose: 0.1-0.5		2016; Khalafi, et al., 2016; Zerrouk, et al., 2017; Bouhlali, et al., 2019; Taha, et al., 2020)
Oak honeydew	Fructose:32.6-39.4 Glucose:22.7-28.8	Sucrose: 0.0-1.2 Maltose: 0.3-6.6 Cellobiose: 0.1-0.2 Gentiobiose: 0.0-0.3 Isomaltose: 0.5-4.5 Melibiose: 0.02 Kojibiose: 1.7-5.8 Laminaribiose: 0.1-0.12 Maltulose: 1.5-5.3 Turanose: 1.9-3.9 Trehalose: 0.0-0.6 Trehalulose: 0.7-1.0 Palatinose: 0.2-0.3 Nigerose: 1.0-1.3	Erlose: 0.0-1.4 Maltotriose: 0.1-0.2 Isomaltotriose: 0.0-0.1 Raffinose: 0.0-1.3 Melezitose: 0.1-1.7 Panose: 0.1-0.3 Theanderose: 0.0-0.1 Maltotetraose: 0.0-0.03	Mean: 1.33 Min-Max: 1.22-1.77	(Mateo & Bosch-Reig, 1993; De la Fuente, et al., 2007; Pascual-Maté, et al., 2018; Rodríguez-Flores, et al., 2019b)

Honeys from the *Apiaceae* or Umbelliferae family are generally characterized by high fructose content, with multiple studies from Morocco and Algeria reporting a minimum fructose content reported was as high as 38.0% (Terrab, González, Díez, & Heredia, 2003; Ouchemoukh, Schweitzer, Bey , Djoudad-Kadji , & Louaileche, 2010; Homrani, et al., 2020). Manzanares et al. (2014) performed sugar profiling for avocado honey and detected 1.54-2.14% trehalose, and 0.67-2.33% melezitose in samples collected from 2007-2009 in Tenerife. However, Serra Bonvehi (2019) failed in detecting trehalose, raffinose and melezitose from their avocado honey samples collected between 2012-2013 in the provinces of Granada and Málaga.

Buckwheat honey collected form Italian beekeepers generally has a low F/G ratio range from 1.1-1.4. Maltose content was higher at a range between 2.4% to 6.8%. Low sucrose content is suggesting a sufficient ripening process before harvesting from the hive (Pasini, Gardini, Marcazzan, & Caboni, 2013). The buckwheat honey sample collected from the northeast of China has no maltose detected (Deng, et al., 2018). The sucrose and glucose content as well as the low sucrose content and low F/G ratio agreed with the result obtained by Pasini et al. (2013). The highest fructose (48.03%, w/w), glucose (47.02%, w/w), and lowest maltose (1.57%) concentrations were reported from Poland buckwheat samples (Kowalski, Łukasiewicz, & Berski, 2013). High concentrations of trehalose (1.37±0.37%) was found in chestnut honey obtained from the Tenerife region of Spain, which does not agree with the result obtained from other studies at 0.06%-0.14% (Escuredo, et al., 2013; Rodríguez-Flore, et al., 2019b; Rodríguez-Flores, et al., 2016; Manzanares, et al., 2017; Pascual-Maté, et al., 2018).

It was suggested by that heather honey generally has a low concentration of oligosaccharide (Pascual-Maté, et al., 2018). According to Martins et al (2008), sucrose was absent in the Portuguese heather honey sample collected between 1991-1993. Raffinose and melezitose have been quantified in honey sample collected from certain localities, with the presence of melezitose attributed to the presence of honeydew during the production process. Rodríguez-Flores et al. (2019a) analyzed the sugar profile of heather honeys and found that the presence of melezitose is location-dependent. This trisaccharide was absent from all nine heather honey samples collected from the Atlantic region and present in the 31 samples collected from the Mediterranean region. They hypothesized the possible cause being the differences between *Erica* species availability for honey production at different localities (Rodríguez-Flores, Escuredo, Seijo-Rodríguez, & Seijo,

2019a). The research conducted by Waś et al., (2011) found that the average F/G ratio in heather honey is 1.32, with average glucose and fructose contents of 29.9% and 39.3% respectively, and the sucrose content remains low (<0.6%) for all sample tested. Although not present in all sample, the raffinose content detected in Martins' study was significantly higher than that in other studies by Mateo & Rosch-Reig (1993), Ouchemoukh el al (2010), and Pascual-Maté et al. (2018). Kaškonienė et al (2010) assessed the carbohydrate composition of willow, winter rape and spring rape honey from Lithuania, for which all three types of honey has their average F/G ratio below 1. The PCA results from Pauliliuc's team (2020) suggest high correlations between the turanose content and rape honey identification.

Glucose, fructose, sucrose, maltose, isomaltose, turanose, and erlose were quantified for black locust honey with HPLC by Mădas's team (2019), and positive correlations were observed between sucrose and erlose or turanose and maltose concentrations. Nine oligosaccharides have been extracted and quantified for 71 black locust honey samples collected from 6 different geographic locations in China (She, et al., 2019). According to She's team (2019), the highest concentration of all nine oligosaccharides were observed with sample collected from two adjacent provinces in the northwest of China, with turanose being the most abundant oligosaccharide (3.41-4.4% (w/w), 4.38- 5.28% (w/w), respectively). During the ripening process of the honey, significant decrease in sucrose content coupled with elevation of turanose content, which were initially absent in the nectar (Yan, et al., 2023).

Lower concentrations of monosaccharides are found in honeydew honeys when compared to nectar honeys, while higher amounts of both disaccharides and trisaccharide, such as raffinose, erlose, melezitose, trehalose, or isomaltose, are often found in honeydew honeys (Šarić, Matković, Hruškar, & Vahčić, 2008; Victorita, et al., 2008; Pascual-Maté , et al., 2018). Sucrose, trehalose, maltose, turanose and panose, on the other hand, characterize nectar honey (Bogdanov, Jurendic, Sieber, & Gallmann, 2008). The presence of melezitose, originating from insect sources such as aphids, along with erlose and raffinose, is often associated with the usage of honeydew by the bee for honey production and maturation (Escuredo, Seijo, & Fernández-González, 2011; Pascual-Maté, et al., 2018). The content of melezitose in honeydew honey is approximately 1.06-2.61% (w/w), whereas in floral honeys, it is usually between 0.01-0.29% (Quirantes-Piné, et al., 2024).

2.4.2 Microcomponents

2.4.2.1 Amino acids, proteins

Honey contains around 0.5% protein, mainly enzymes and free amino acids. The main source of protein detected in honey are from pollen, and the amino acid profile, but not the protein composition; it is often dependent on the botanical sources. However, the authenticity of the honey cannot be determined solely from amino acid composition analysis (Lobos, Silva, Ulloa, & Pavez, 2022; Cott, et al., 2004). The total amount of free amino acids in honey is approximately 1% with proline being the major amino acid (50-80% w/w). The identified amino acids from honey include Glu, Asp, Asn, Ser, Gln, His, Gly, Thr, β-Ala, Arg, α-Ala, Pro, Tyr, Val, Met, Cys, Ile, Leu, Trp, Phe, Lys, g-aminobutyric acid (GABA) and ornithine (Kuś, 2020). Proline mainly comes from honeybees and can serve as a marker for honey ripening as well as sugar adulteration detection, as its level will be significantly lower in adulterated honeys (Nisbet, Kazak, & Ardalı, 2018). Higher proline content is often found in honeydew honeys; however, wide variabilities among different types of honey make it less significant in differentiating the botanical origins (Seraglio, et al., 2019). In addition, high variabilities observed in amino acid profile of honey from the same plant source further limit the discriminatory effort (Kivima, et al., 2021).

Various studies have been conducted evaluating the amino acid profile of different botanical sourced honey form various locations. The predominant amino acid in *Eucalyptus* honey is Pro which has also been found in higher concentration in heather honey; the lavender honey has significantly higher Tyr and Phe content when compared to eucalyptus honey; significant differences exist between the Val contents in eucalyptus, thyme, orange blossom and rosemary honeys; honeydew honey has higher Pro and Phe content in general; sunflower honey has relatively high concentrations of Ala, Arg, Asn, Gln, Gly, His, Leu, Lys, Phe, Pro, Ser, Tyr, and Val; basil honey is lacking Gln, Gly, Lys, while having significantly high Phe concentration; and high Gln, Lys, Glu, Arg and His are often observed with rape honey (Bobis, et al., 2020; Kivima, et al., 2021; Cotte, et al., 2004; Chen, et al., 2017; Kečkeš, et al., 2013). Amino acid may also affect the aroma features of the honey (Hermosín, Chicón, & Cabezudo, 2003).

The final protein content present in the ripened honey is derived from honeybees rather than the direct plant origin (Bobis, et al., 2020). Depends on sampling location and beekeeping methods the eucalyptus honey, for example, has a protein content that varies between 0.2-1.24 mg/g honey;

chestnut, black locust and sunflower honey has a protein content of 0.59-0.71 mg/g honey, 0.1 mg/g honey, and 0.4 mg/g honey, respectively (Rossano, et al., 2012; Erban, Shcherbachenko, Talacko, & Harant, 2019). In other studies, higher protein content was obtained for chestnut honey (94.8mg/100g) followed by sage honey (79.2 mg/100g), honeydew honey (59.4 mg/100g), and *Robinia* honey (30.4 mg/100g). Higher protein content doesn't always imply higher proline concentration nor higher enzymatic activity, though high correlation between protein content, proline content, invertase, diastase and glucose-oxidase activity has been found (Flanjak, Strelec, Kenjerić, & Primorac, 2016). The Major Royal Jelly Proteins (MRJPs) composed of over 90% of the total protein content in honey. Over 67 MRJPs have been identified together with a-glucosidase, glucose oxidase, and profilin in orange honey (Rossano, et al., 2012). Apalbumin1, denoted as MRJP1, is the most abundant protein component of royal jelly proteins, which serves as a marker for freshness as its degradation is correlated with storage time and condition (Shen, et al., 2015)

2.4.2.2 Phenolic compounds

Phenolic compounds found in honey are originated from nectar, as they are the secondary metabolites synthesized via the phenylpropanoid pathway by plants to reduce oxidative stress (Liu, et al., 2021). Their existence in honey varies depends on multiple factors including botanical and geographic origin, as well as climatic conditions (Ayoub, et al., 2023). For instance, Kavanagh et al. (2019) found higher total phenolic content (TPC) in Irish multifloral honey obtained from urban area when compared to that obtained from rural area ; and the author hypothesized that the density of the blossome may contribute to this difference. Additionally, it is suggested that higher TPC is correlated with darker-colored honeys (García-Tenesaca, et al., 2017; Becerril-Sánchez, Quintero-Salazar, Dublán-García, & Escalona-Buendía, 2021).

Flavonoids is the most diverse group of polyphenolic compounds identified in honey. Some major flavonoids, such as catechin, myricetin, quercetin, apigenin, kaempferol, luteolin, rutin, isorhamnetin, pinocembrin, chrysin, pinobanksin, galangin, hesperetin and gallochatechin are detected in various types of honeys (Bobis, et al., 2020; Guo, Deng, & Lu, 2019; Kivima, et al., 2021). Another major group of phenolic compounds detected in honey is phenolic acid (Wilczyńska & Żak, 2024). The most common phenolic acids found in various honeys, are gallic acid, benzoic acid, vanillic acid, protocatechuic acid, caffeic acid, p-coumaric acid, *p*-hydroxybenzoic acid, ferulic acid and cinnamic acid. For example, ferulic acid is a common type

of phenolic acid detected in the black locust honey collected from the Transylvania region, Romania, with a concentration range from 0.72ppm to 8.66 ppm, composing 29% of the total phenolic acid (12.11 ppm) (Marghitas, et al., 2010).

Beneficial effects of honey on human health have been described in various ancient cultures (Eteraf-Oskouei & Najafi, 2013). Recent studies have confirmed its antioxidant, antimicrobial, antiviral, anticancer, antidiabetic properties, with protective activities on the nervous, cardiovascular, gastrointestinal, and respiratory systems, which were attributed to the phenolic acids and flavonoid compounds (Cianciosi, et al., 2018). For example, the anti-obesity effect has been linked to phenolic acids such as vanillic acid, syringic acid, gallic acid, ferulic acid, chlorogenic acid, and ellagic acid, and flavonoids such as quercetin, myricetin, galangin, kaempferol, chrysin, apigenin, and hesperetin (Olas, 2020).

2.4.2.3 Volatile compounds

Volatile compounds are the major contributors to the unique aromatic profiles of different types of honeys. In general, the volatile compounds can be classified into several classes: ketone, ester, aldehyde, alcohol, acids, ethers, hydrocarbons, terpenes and its derivatives, benzene and its derivatives, furan and pyran compounds, norisoprenoids, nitrogen compounds, and sulphur compounds (Bobis, et al., 2020; Machado, Miguel, Vilas-Boas, & Figueiredo, 2020).

Factors influencing the profile of volatile compounds identified in honeys include their botanical origin characteristics, the processing and storage conditions of honey, and geographic locations (Manyi-Loh, Ndip, & Clarke, 2011; Stanimirova, et al., 2010). Ethanol, 1-propanol, butanol, pentanol and other primary alcohols, as well as carbon dioxide, glycerol and several acids are naturally present in honey in small quantities as a result of microbial fermentation (Martins, et al., 2008). Mădas et al (2019) performed the volatile compound profiling on black locust honey from different regions of Romania using SPME/GC-MS. In total, 79 compounds detected, and only 56 are common to all samples, suggesting a significant impact of locality on the generation of volatile compounds

In the study of volatile compounds in *Eucalyptus* honey, more abundant vomifoliol is present than when compared to other types of honey (Bobis, et al., 2020). Acetone is considered as an indicator of pine honey, but other study also found acetone in large quantities in black locust honey and

rosemary honey (Mădaș, et al., 2019). Kortesniemi et al (2018) analyzed the volatile compounds in five types of monofloral honey from Finland. They noted a high detection frequency of p-cresol and butyric acid in buckwheat honey, two compounds responsible for the unpleasant aroma associated with this honey. The unique woody, herbal and chemical scent of cloudberry-bog honey was associated with the high concentrations of 1-propanol, p-cymene, isophorone and citral. Vanillin, 3-phynylpropanoate, and 3-hydroxy-4,5-dimethyl-2(5H)-furanone contribute to the pleasant caramel and floral smell of lingonberry honey. Additionally, 3-methylbutanal which gives malty aroma is absent from sweet clover honey. Finally, hexyl hexanoate, and (E)-β-damascenone that has apple aroma are found in willowherb honey (Kortesniemi, et al., 2018).

2.4.2.4 Organic acids

Various organic acids are present in honey at minor concentrations of 0.57-0.6%, including citric acid, fumaric acid, butyric acid, tartaric acid, α-ketoglutaric acid, pyroglutamic acid, glycolic acid, maleic acid, malic acid, succinic acid, d-gluconic acid, acetic acid, formic acid, lactic acid, oxalic acid and pyruvic acid (Bobis, et al., 2020; Guo, Deng, & Lu, 2019). Gluconic acid is produced by glucose oxidase, and it contributes to the acidic property of honey and aids with shelf-life extension (Ma, et al., 2019; Lichtenberg-Kraag, 2015). The presence of acetic acid and butyric acid was reported to be the result of bee metabolism, and acetic acid was found at high concentration (12.26%-24.73%) in the Romanian black locust honey, especially for the ones collected from the southern part of the country (Mădaș, et al., 2019). Marghitas et al (2010) reported a high concentration (12.01 ppm) of abscisic acids in the black locust honey from the Transylvania region, Romania. This plant hormone, present in floral nectars, helps protect the plant against environmental stressors.

2.4.2.5 Minerals

The minerals found in the honey mainly come from the soil sources, entering the plant via the root system, then transported into the nectar (Marghitas, et al., 2010). Mineral content affects the color and the flavour of honey, with higher mineral content resulting in darker colors and stronger flavour (Crăciun, Pârvulescu, Donise, Dobre, & Stanciu, 2020). Various studies have evaluated the mineral content of different types of honey from different geographic locations, and reported the dependence of the mineral content on botanical and geographic origins as well as environmental conditions (Tuzen, Silici, Mendil, & Soylak, 2007; Lachman, et al., 2007; Perna,

Intaglietta, Simonetti, & Gambacorta, 2014). In general, the mineral content in honeydew honey is higher than that in blossom honey. The mineral content of honey varies between 0.02 and 0.3% with potassium, calcium, sodium and magnesium being the relatively more abundant ones, and iron, copper, manganese and chlorine being the rarer elements (Bobis, et al., 2020; Marghitas, et al., 2010; Vorlová & Čelechovská, 2002). The mineral content were assessed for monofloral, and multifloral honey produced in the central-southern regions of Chile and confirmed the abundancy of potassium followed by sodium and calcium (Lobos, Silva, Ulloa, & Pavez, 2022; Rodríguez-Flores M. , Escuredo, Seijo-Rodríguez, & Seijo, 2019a). Similarly, Belay et al (2017) indicated that potassium was more abundant in 8 monofloral *Eucalyptus* honey from Ethiopia than sodium. However, Deng et al (2018) reported different mineral profile for buckwheat honey from northeast China, where the calcium content exceeds the potassium content by almost 4 times (Deng, et al., 2018).

Increased interested has been drawn over the assessment of heavy metal content in honey to characterize their safety. Heavy metals such as molybdenum, nickel, lead, vanadium are occasionally detected in trace amount in certain honey samples (Karabagias, et al., 2017). High aluminum and copper content in honey might be the result from volcanic origin soil or pollution caused by human activity (Bogdanov, Haldimann, Luginbühl, & Gallmann, 2007). Pollutants collected by bees during foraging, apicultural practices and honey processing may also introduce hazardous metal into the final product (Pohl, 2009). Vorlová & Čelechovská (2002) found higher zinc, iron, manganese, copper content in honeydew when compared with rape honey and multifloral honey from Czech Republic. This agreed with the results obtained by Vit et al (2010) confirming higher calcium, copper, magnesium, manganese, iron, zinc concentrations in fir honeydew honey when comparing with seven other types of blossom honeys (black locust, buckwheat, heather, linden, rape, raspberry, and sunflower).

2.4.2.6 Lipid

There exist approximately 0.04% (w/w) lipid in honey, which constitutes of glyceride, sterols, phospholipids, and fatty acids and their esters. With such low quantity, the lipid portion of the honey neither contributes to its sensory properties nor towards its nutritive values. However, lipid may reveal the entomological origin of honey by means of the species of bees that produces the honey. Schievano et al. (2021), conducted a study using nuclear magnetic resonance (NMR)

spectra to investigate the entomological lipid marker for a variety of monofloral honeys. Bee waxes, (E)-2-decenedioic acid, (E)-2-nonendioic acid, and a diacylglyceryl ether (1-stearyl-2,3-dioleogyl glycerol, DAGE) are found in genuine honey produced by *A. mellifera*. They confirmed the production of DAGE from the salivary gland of the foraging bees, and same as enzymes of the same source, its content may vary depending on the age of the bee and the nectar collection period. Moreover, the DAGE content, when compared to enzymatic activities, is found to be more stable in honey for an extended period and is unaffected by heating, hence making it a potential candidate in tracing the botanical origin of the honey more accurately.

2.5 Protein composition: enzyme

Several enzymes were detected in honey including invertase (EC3.2.1.20), glucose oxidase (EC1.1.3.4) and diastase (EC3.2.1.1) being identified as the most abundant ones in addition to β-glucosidase, catalase (EC 1.11.1.6), glucosylceramidase, and acid phosphatase (Babacan & Rand, 2005; Borutinskaite, Treigyte, Čeksteryte, Kurtinaitiene, & Navakauskiene, 2018; Sahin, Kolayli, & Beykaya, 2020; Julika W. , et al., 2020; Abd-Elaziz, Karam, Ghanem, Moharam, & Kansoh, 2020; Zhang, et al., 2021). Enzymes are added via the cephalic (post-cerebral) or thoracic salivary gland, and the hypopharyngeal glands of bees, and consequently, the enzymatic activity profiles of honey before and after ripening depends on several factors (Al-Sherif, et al., 2017; Hu, et al., 2019). These factors include the species, age, diet, seasonal physiological changes, population density, genetic predisposition, and colony strength of the bees, as well as extrinsic factors such as hive conditions (e.g., space), temperature, and moisture as well as geographic and botanical origins of the honey (Oddo, Piazza, & Pulcini, 1999; Babacan & Rand, 2005; Al-Sherif, et al., 2017; Lichtenberg-Kraag, 2015). Indeed, a correlation between nectar viscosity and salivary enzyme secretion has been suggested, and the effect of life stage of the bee was also reported, as younger bees produce less enzyme (Julika W. , et al., 2020; Horn & Böhm, 2004; Flanjak, Strelec, Kenjerić, & Primorac, 2016).

When the nectar flow is abundant, such as with black locust, it has been reported that it can be transferred faster to the cell, hence the honey is less elaborated, with lower enzyme released. In summer when the foraging bee is the dominant population, honey with higher enzymatic activities is produced (Vorlová & Čelechovská, 2002). Seasonal changes in the digestive enzyme, amylase, activity in bees were also observed with two peaks shown in spring and summer, suggesting higher

diastase activity in the honey matured during these two seasons (Kodrík, Krištůfek, & Svobodová, 2022). Honeydew however, unlike nectar, upon collection are already abundant in enzymes, especially invertase, hence the enzymatic activities of invertase, diastase, glucose-oxidase, and acid phosphatase in honeydew honeys are generally higher (Flanjak, Strelec, Kenjerić, & Primorac, 2016). Although botanical origins affect the enzymatic activities of different types of honey, using enzymatic activities to identify botanical sources are only applicable with fresh raw honeys where the processing is minimum, as both storage and heat processing cause reduction in enzymatic activity (Flanjak, Strelec, Kenjerić, & Primorac, 2016).

2.5.1 Invertase

The presence of invertase in the honey mainly comes from foraging bees, that collects nectar and mix with secretions from the salivary and hypopharyngeal glands during the returning flight and when passing nectar from bees to bees in the hive (Oddo, Piazza, & Pulcini, 1999; Babacan & Rand, 2005; Al-Sherif, et al., 2017) . Invertase activity facilitates the condensation of the sugar solution, preventing the honey to be fermented, leaving the energy-dense food for bee consumption in the honeycomb (Sánchez, Huidobro, Mato, Muniategui, & Sancho, 2001). Studies have also shown the contribution of invertase in the formation of oligosaccharides during honey formation and storage via translglucosylation. Invertase activities are commonly expressed in Hadorn units as invertase number (**IN**) which indicates the quantity of sucrose (in grams) hydrolyzed in one hr by the enzyme from 100 g of honey. U/kg refers to the enzymatic activity that transforms 1μM substrate in 1 min under ideal conditions, and in the case of invertase, it is equal to 7.344732 times the result in IN (Julika W. , et al., 2020). The general recommendation from the International Honey Commission on invertase activity in general types of honey are ≥10 IN or 73.45 IU (International Honey Commission, 2009). While longer extraction time and high negatively correlates to the invertase activity, the optimal condition for invertase extraction would be at 24ºC for 1h, and invertase activity would reduce after 24 hrs, even at temperatures as low as 35°C (Sahin, Kolayli, & Beykaya, 2020; Kamboj, Sandhu, & Nanda, 2024).

Higher flow of nectar will lead to lower enzyme content because less secretion from young bees is injected into the nectar before the ripening processes occurs in the closed cells, and high sucrose content in the sap is correlated with low invertase activity in the final honey while diastase activity is independent from the sugar composition (Lichtenberg-Kraag, 2015; Flanjak, Strelec, Kenjerić,

& Primorac, 2016). The existence of plant derived invertase has been confirmed by (Heil, 2011), which might be responsible for the sucrose conversion prior to honey ripening. Raw honey has superior invertase activity when compared to processed ones. Although not generally recognized and required as a quality criterion, invertase activity has been assessed by beekeeper associations in certain European countries (e.g. Germany, Spain, and Belgium) as a supplementary element for determining the freshness and storage condition of honeys (Flanjak, Strelec, Kenjerić, & Primorac, 2016).

2.5.2 Diastase

The breaking down of starch into maltose and dextrin contributes to the presence of diastase. Diastase activities have been widely applied in Europe as a measurement for honey freshness, as the activity decrease with improper storage and extensive heat treatment, while invertase shows higher sensitivities towards deterioration related to the above two factors, the activity of both enzymes varies in a wide range between different botanical sources (Bogdanov, et al., 1999; Oddo, Piazza, & Pulcini, 1999; Julika W. , et al., 2020; Özcan & Ölmez, 2014). Besides, the enzymatic activity is an indicator for degree of ripening of honey (Lichtenberg-Kraag, 2015). Diastase activity is expressed in diastase number (**DN**), or Schade units, or Gothe scale, which represents the quantity of starch (in 10mg) hydrolyzed by the diastase in 1 g of honey in one hour at 40ºC (Rodríguez-Flores, Escuredo, & Seijo, 2016). According to the EU regulation, diastase activity should be higher than 8 DN, with exception for honey naturally low in enzymatic activity, such as *Citrus* and *Robinia* honey, which need to have a diastase activity of no less than 3 while exhibiting an HMF content of no more than 15ppm (EU, 2001; Lichtenberg-Kraag, 2015).

Differences between the enzymatic activity of honey produced stingless bees and honeybees (*A. mellifera*) has been examined in several studies (Julika W. , et al., 2020; Chuttong, Chanbang, Sringarm, & Burgett, 2016; Vit, Pedro, & Roubik, 2013). Stingless bee honeys from Southeast Asia have significant weaker diastase activities when compared to honeybee honeys. Study has reported diastase activity in Malaysia honey bee honey to be 12.14 ± 0.78 DN, whereas for the stingless bee honey to be 1.53 - 3.89DN from Malaysia or 1.5 ± 1.6 DN in Thailand samples (Chuttong, Chanbang, Sringarm, & Burgett, 2016; Julika W. , et al., 2020; Seow, Tan, & Mat Easa, 2021). However, the enzymatic activity tested with South America stingless bee honey shows a much wider range of 2.4-21 DN (Vit, Pedro, & Roubik, 2013).

2.5.3 Glucose oxidase

While mainly responsible for the antibacterial property of honey, the presence of hydrogen peroxide in honey is mainly regulated by the bee enzyme glucose oxidase, and the plant derived catalase. Glucose oxidase, secreted from the hypopharyngeal glands of honeybees is the main microbial defense mechanism employed, which produces the antiseptic agents, the hydrogen peroxide and the gluconic acid using β-D-glucose in the honey as substrates (Oddo, Piazza, & Pulcini, 1999; Sahin, Kolayli, & Beykaya, 2020). It's a holoenzyme with two subunits, each at 85 kDa (Borutinskaite, Treigyte, Čeksteryte, Kurtinaitiene, & Navakauskiene, 2018). The enzyme is heat and light sensitive and loses activity more easily than diastase during long-term storage (Guttentag, Krishnakumar, Cokcetin, Harry, & Carter, 2021; Alshareef, Al-Farhan, & Mohammed, 2022; Ghoniemy, Esmail, Mahmoud, & Mohamed, 2022). The utilization of molecular oxygen to generate hydrogen peroxide serves as a potential antibacterial mechanism in improving the stability of honey during storage (Sahin, Kolayli, & Beykaya, 2020). While the status of the foraging bee playes a role in determining the glucose oxidase concentration found in honey, the diversity of their diet has also been found to correlate with such. Glucose oxidase performs higher activity in less dehydrated honey, where the enzyme has easy access to the substrate, and too high of a glucose concentration will partially inhibit its activity. (Sun, et al., 2021; Alshareef, Al-Farhan, & Mohammed, 2022).

2.5.4 Catalase

Catalases, originating from the pollen of the blossom, decompose hydrogen peroxide into oxygen and water to protect cells and tissues from oxidative stress, hence preventing the toxic effect of excess peroxide in honey on bees. Hence its abundance in the honey is largely affected by the quantity of pollen grains present. Catalase in honey is composed of four subunits with each having a molecular weight of 60 kDa (Borutinskaite, Treigyte, Čeksteryte, Kurtinaitiene, & Navakauskiene, 2018). Recent studies also highlighted its contribution to the antibacterial effects of honey, showing positive correlations between catalase activity and the growth inhibition of common foodborne pathogens (Dżugan, et al., 2020; Osés, Rodríguezk, Valencia, Fernández-Muiño, & Sancho, 2024).

2.5.5 Acid phosphatase

Acid phosphatase, a hydrolase that generates inorganic phosphate from organic phosphates, is associated with the fermentation processes of honey, which is mainly sourced from plant origins (pollen and nectar) other than bees (Flanjak, Strelec, Kenjerić, & Primorac, 2016; Alonso-Torre, et al., 2006). Compared to invertase and diastase, such enzyme is more prone to high temperature and undesired storage condition, and its activity will decrease in the first year of storage then followed by a slight increase starting at around 20-month of storage when yeast fermentation takes place, and another decrease after 30 months of storage (Alonso-Torre, et al., 2006). Alonso-Torre's team (2006) also demonstrated negative correlation between the pH and the acid phosphate activity, with higher pH, lower enzymatic activity was detected. Glucosylceramidase is another bee sourced enzyme that are more correlated with the health condition of honeybees, with a deficiency in such enzyme lead to the accumulation of glucosylceramide causing abnormalities in the behaviour of insects. Its presence has been detected in buckwheat honey using gel-free MS (Borutinskaite, Treigyte, Čeksteryte, Kurtinaitiene, & Navakauskiene, 2018).

2.5.6 Protease

Proteolytic activity has been detected with 2D-zymography in orange, eucalyptus, chestnut, and sulla honey. This confirms the potential existence of proteolytic enzymes in honeys, which may come from the midgut of honeybees in order to digest plant protein (Kodrík, Krištůfek, & Svobodová, 2022). Their presence can largely influence the protein profile of the honey, hence altering its quality and nutritional value of honey or royal jelly. Although the sources of the proteolytic enzymes are not from the plants, diet do exhibit impact on the metabolic activities of honeybees in general, hence the evaluation of proteolytic enzymes in honey may serve as a future approach in identifying the botanical sources of honeys (Rossano, et al., 2012).

2.6 Honey identification and authentication

In general, monofloral honeys are more appreciated by the consumer and are being perceived as higher quality in terms of nutrient values and distinct sensory attributes, which in turns brings them higher market values. Hence the authentication process became necessary for the market to gain higher profit (Mădas ̧ et al., 2019). Due to the higher demand for more valuable honeys, especially the region specific and rare botanical origin sourced monofloral honey, as well as its by-product,

food fraud has become inevitable. Typical honey adulterants include sugar and sugar syrup. On the other hand, some adulteration methods involve feeding of bees with various sugar syrup, blending lower- value honey with higher-value varieties, or harvest honey before it fully matures and artificially accelerating the evaporation by heating (Crăciun, Pârvulescu, Donise, Dobre, & Stanciu, 2020). Although the major focus for the current regulation is on the former type of adulteration, various research starts looking for different fingerprints to differentiate honeys by their botanical origins.

Traditional adulteration detection methods including analyzing relevant physicochemical parameters such as moisture content, ash, proline, HMF, pH, reducing and non-reducing sugar content, electrical conductivity, diastase activity, free acidity, and rheological parameters (Oroian & Sorina, 2017). High selectivity and sensitivity are associated with these methods, and they are often used during honey trading. Isotope ratio mass spectrometry (IRMS) using stable carbon isotopes and melissopalynology are two of the most reliable methods in honey adulteration detection and botanical identification (Tsagkaris, et al., 2021; Boruah, et al., 2024). However, these methods require sophisticated sample preparation procedures and highly experienced personnel. In the recent decades more focus were put on the development of simpler methods on detecting non-targeted honey adulteration, including electrochemical analysis, flow injection analysis, biosensor, differential scanning calorimetry (DSC), GC-FID, HPLC, GC-MS, LC-MS, IRMS, NMR, NIR, and Raman spectroscopy (Biswas, Borse, & Chaudhari, 2025).Other components of honey such as phenolic compounds or the physicochemical properties of honey are viewed as potential biomarkers to facilitate the inspection procedure. Sugar profiling often serves as supplemental measurements for honey source classification. While the contents of sucrose, glucose and fructose have greater significance from the legislation point of view, correlations between the oligosaccharide composition and the botanical origin of the honey are suggested in a few studies (Pascual-Maté, et al., 2018). Minor oligosaccharide composition is relatively stable under ambient conditions compared to sucrose; hence sugar profiling can act as an effective tool in confirming the authenticity of pure monofloral honey (Schievano, Sbrizza, Zuccato, Piana, & Tessari, 2020). However, their heterogeneity due to locality and climatic condition differences make them less reliable as a standalone criterion for honey source authentication (Pascual-Maté, et al., 2018; Pauliuc, Dranca, & Oroian, 2020). Since no single parameter is sufficient in identifying the

botanical or geographic origin of honeys, chemometric analysis is necessary for providing a more comprehensive understanding of different biomarkers.

The carbohydrate composition of honey is influenced by various factors including the botanical sources, geographic origins, bee species, weather, harvest and storage condition, and ripening time (Crăciun, Pârvulescu, Donise, Dobre, & Stanciu, 2020). However, as the impact of botanical and geographic origins on the sugar profiling of honeys has been previously discussed in **Section 2.4.1**, it will not be further discussed in the current section. In terms of bee species, higher turanose, erlose, and maltose but lower trehalose and isomaltose content were seen with *A. mellifera* produced ziziphus honey when compared to *A. florea* ones (Taha, Al-Kahtani, & Taha, 2020). Different from honeybee honey, studies on stingless bee honeys across the world report lower quantities of fructose and glucose, which are commonly found to be below the threshold regulated for the former, and higher sucrose and maltose content (Ávila, Beux, Ribani, & Zambiazi, 2018; Esa, et al., 2022). Some stingless bees from Brazil produces honey with significantly different sugar profile, with glucose content range from 37.1%-45.7% and fructose content range from 50% to 59.2% depending on botanical origin and bee species, while maltose is missing in all four floral-source honey produced by two different local stingless bee species (de Sousa, et al., 2016). Additionally, the trehalulose content (13-44g/100g) is often higher in stingless bee honeys when compared to honeybee honey, thus, is often considered as a biomarker for the authentication oof stingless bee honeys (Fletcher, et al., 2020).

While some sugar composition helps with honey authentication regarding botanical origin identification, other endogenous sugars may help with honey adulteration detection as both direct and indirect adulteration may change the sugar profile of the honey. Endogenous sugar are saccharides naturally present in genuine honeys but found only in trace amounts in commercial syrups. Examples include maltulose, turanose, nigerose, kojibiose, erlose, isomaltose, and palatinose. Mannose can be found in chestnut, and linden floral honey, as well as honeydew honeys. Therefore, its detection in honey from other botanical sources may imply adulteration with mannose-containing C3 plant-derived sugar syrup or overfeeding of honeybees with commercial syrup (Schievano, Sbrizza, Zuccato, Piana, & Tessari, 2020). Blastose is not identified as a honey disaccharide, however, its presence was detected in a model solution containing sucrose and glucose after prolong incubation at 35°C. Hence, such disaccharide may serve as a potential indicator for sucrose addition in honey (Silva, et al., 2019). Fructose inulin syrup adulteration can

be detected with inulotriose as adulterant marker. Šarić's team (2008) has also found up to 10.7% sucrose in a multifloral honey sample collected from mountain meadow in Croatia. Based on the regulation the sucrose content in honey should not exceed 5%, except for sage honey (8%) and honeydew honey (10%). The absence of blastose is confirmed in Portuguese multifloral honey ripened for 2, 4, 12 months, but is detected in incubated honey-mimicking sugar solutions, hence it may serve as an indicator for adulteration. By far there is no reported evidence of the presence of inulotriose in natural honey, and such trisaccharide has been nominated to be a marker for honey adulterated with high-fructose inulin syrup (Silva, et al., 2019). Silva's (2019) experiment also reported the formation of dehydrated oligosaccharides which are absent in pure honey sample, suggesting another indicator for direct adulteration.

2.7 Honey formation and compositional change during processing and storage

The transformation of bee-collected nectar to honey involves specific substances, such as the enzymes discussed in the previous section. Upon collecting nectar, foraging bees transfer it back to house bees, initiating the ripening process that transforms nectar into honey (Ministry of Agriculture, Food and Fisheries, 2024). During this process, enzymes secreted from the bees' hypopharyngeal glands are added to the aqueous sugar solution (Klose, Rolke, & Baumann, 2017). Once deposited into the honeycomb, moisture is further removed as bees fan the cells to promote evaporation, facilitating the final stages of ripening. During ripening, the moisture and disaccharide content decrease with an increase in glucose and fructose content as well as enzymatic activities (Eyer, Neumann, & Dietemann, 2016). In general, it takes up to 3 days for the nectar to be converted to honey, and during such process, fresher nectar will be continuously added into each individual honeycomb cell. The cells will be capped with beeswax once the chamber is full (Wen, et al., 2017). In addition, honey production and honeybee activities are affected by the environment at high altitude with low temperature, low pressure, low oxygen level, and high UV radiation (Khan, et al., 2021).

Moisture content, influenced by seasonal and climatic changes, is a parameter associated with honey maturation and durability, for which a higher water content will lead to fermentation (Zerrouk & Bahloul, 2020). Moisture content analysis as part of the physicochemical analysis is a relevant parameter that can affect the viscosity, density, tase, flavor, color, crystallization, and fermentation of the honey, as the high water-content facilitates the granulation processing during

honey ripening and storage. During ripening, the moisture and dissacharide content decrease with the increase in both glucose and fructose, and in particular the latter (Gašić, et al., 2014; Zhang, et al., 2021). It has been observed that the disaccharide content is affected by year differences, while geographic location has a larger impact the glucsoe and fructose conetent via carbon isotop fractionation for rape honey ripening (Li, et al., 2022)

The formation of various saccharide compounds, including glucose, fructose, maltose, and isomaltose, results from enzymatic reactions catalyzed by diastase, invertase, β- glucosidase, β-amylase and β-fructosidase from the honeybees (Schievano, Sbrizza, Zuccato, Piana, & Tessari, 2020; Silva, et al., 2019; Alaerjani, et al., 2022). During ripening, these enzyamatic activity along with glucose oxidase activity increased significantly (Gašić, et al., 2014). Interestingly, black locust honey ripened for 7 to 10 days exhibited lower glucose oxidase activity compared to the immature samples (Ma, et al., 2019). Extensive studies on the activity of invertase demonstrate its transglycosylation activities in both bees and honey moieties, with the enzyme being capable of transferring the a-glucosyl residues to other glucose parts (White Jr & Maher, 1953). Other oligosaccharide may be produced via non-enzymatic transglycosylation, where glycosyl units are transferred to the hydroxyl groups of another glycoside. Non-enzymatic transglycosylation facilitates the polymerization of saccharide molecules under conditions of high acidity, high sugar content, low moisture, and thermal processing. This process contributes to the formation of oligosaccharides in honey, where all criteria are met except for high temperature (Silva, et al., 2019). According to the study conducted by Silva's team (2019), maltose and isomaltose, along with other di- and trisaccharides such as gentiobiose, inulobiose, sophorose, 1-kestose, and panose, can be associated with the non-enzymatic transglycosylation pathway.

Crystallization is a natural phenomenon commonly observed with honey and has received some controversy among consumers. As honey being a concentrated aqueous solution, frequently supersaturated, of the two major monosaccharide, glucose and fructose, their concentrations play a critical role in the occurrence of crystallization. Research indicates that the ratio of fructose to glucose is the key factor, for the lower the F/G ratio, the quicker the honey will crystalize (Juan-Borrás , Domenech, Hellebrandova, & Escriche , 2014). Besides F/G ratio, the ratio between glucose and moisture content also affects the rate of crystallization, with a slower crystallization when G/M is less than 1.7, and more rapid crystallization when G/M is greater than 2. Although in-depth explanation is available for elucidating the crystallization mechanism at molecular level,

higher glucose content of the honey indicates a higher prevalence to crystallization, as glucose is less water-soluble than fructose (Martins, et al., 2008; Gašić, et al., 2015).

The degradation of fructose generates 5-hydroxymethylfurfural (HMF) in acidic environment, which are not commonly present in fresh honey, and hence increase with storage in a temperature and pH value-dependent manner (Oddo, Piazza, & Pulcini, 1999; Fallico, Arena, Verzera, & Zappalà, 2006). Acidity is another variable associated with storage, as increased free acid can be seen over time when gluconic acid, formic acid, and levulinic acid is formed from glucose or HMF (Crăciun, Pârvulescu, Donise, Dobre, & Stanciu, 2020). The change in acidity may induce microbial growth (Khan, et al., 2021). HMF as an intermediate product for Maillard reaction, although its exact formation pathway within honey is still unclear (Yang, Zhang, Li, Huang, & Miao, 2019). It can serve as an indicator for the freshness of honey, as its presence implies long-term poor storage or excessive heating during processing. The CODEX limit HMF level in honey to be below 40mg/kg, with exceptions of 80mg/kg for specifically declared honey from tropical regions (Fallico, Arena, Verzera, & Zappalà, 2006). Various botanical-origin related chemical factors such as acid content, lactone content and mineral content affect the formation of such compound (Shapla, Solayman, Alam, Khalil, & Gan, 2018). When heated under high temperature in acidic conditions, HMF can also be produced from oligo- or poly- saccharide. Among the most abundant reducing sugars in honey, fructose has a higher affinity in forming HMF, and fructose has a reactivity five times higher than that of glucose at pH 4.6 (Shapla, Solayman, Alam, Khalil, & Gan, 2018). Honeybee species may also affect the prevalence of the chemical composition of the honey produced by changing with storage conditions, as *A. florea* honey has a lower generation of HMF during heating when compared with *A. mellifera* honey (Al-Ghamdi, Mohammed, Ansari, & Adgaba, 2019).

2.8 Biotransformation of saccharides in honey

Undoubtingly, sugars are one of the most widely consumed goods among humans, and sweeteners have become the most consumed product worldwide during the last decade. They are present either as preservatives, flavoring agents or bulk products due to their general association with pleasant when opioid and dopamine are triggered to be released (Yin, et al., 2020). It has been a rising concern for the public for being prone to developing obesity, type 2 diabetes, and cardiovascular diseases from high sugar intake, especially fructose, sucrose, and maltose, form daily diet (Witek,

Wydra, & Filip, 2022). Nevertheless, monosaccharide such as fructose has been suggested not only for providing empty calorie but also for possessing negative impact on insulin sensitivity, blood lipid profile, and hepatic and visceral lipid accumulation (Softic, et al., 2020). The trend for the food industry to reduce the usage of caloric sweeteners, such as glucose, fructose, and sucrose, is growing as a response to this increasing public awareness. Hence, low-calorie sweeteners are the popular solution in replacing traditional addition sugar in foods and beverages, which in turn generates a huge market based on the enormous demand. Both naturally existing low-calorie sweeteners and synthetic ones, such as aspartame, saccharin, acesulfame-K, sucralose, and neotame, are of interest for industry, with the latter being less favorable for exhibiting artificial tastes and the potency to cause undesired side effects for consumers (Chattopadhyay, Raychaudhuri, & Chakraborty, 2014). Therefore, over the last two decades the demand for naturally sourced, low-calorie sweeteners has increased significantly.

To correctly define low calorie sweeteners, the substance needs to provide less energy while delivering the same sweetness when comparing to sucrose, hence they can be divided into two categories, bulk low-calories sweeteners, and high-potency sweeteners (Fry, 2012). Some examples of the former that has wide applications in the food industry includes tagatose, and sugar alcohols such as erythritol, xylitol, sorbitol, etc., while the latter includes plant extract such as steviol glycosides, monk fruit extract, etc. However, agricultural sustainability, quality stability, and commercial viability might be some factors retard the growth of the application for the plant-based natural sweeteners. Understanding the synthesis of these sweeteners can not only resolve the scalability limitation but also contribute to the discovery of novel sweeteners and the amelioration of the natural yield of the plants.

Although di- and oligsaccharides with health benefits are naturally found in honey, the majority of its dry content is still glucose and fructose which comprise over 60% of its total mass (Lane, Calonne, Slattery, & Hickey, 2019). According to the WHO's recommendations on daily sugar intake, 'free sugars' include both added sugars in food and beverages, as well as mono- and disaccharides that are naturally unbound in foods like fruit juice, syrup, and purees (WHO, 2015). As such, the sugars found in honey are categorized as free sugars. Hence to further improve the nutritive value of the honey, conversions of its endogenous monosaccharides into their healthier derivatives are worth exploring. Some of the potential applications of saccharide biotransformation that may contribute to the calorie reduction of honeys include the bioconversion of fructose into

mannose or allulose (Wu, Chen, Guang, Zhang, & Mu, 2020; Izumori, 2006). However, although mannose is a natural monosaccharide that can serve as potential prebiotic to exert some health benefits towards human gut microbiota, its presence in honey is often seen as an indicator for sugar syrup adulteration, hence to the authenticity point of view, such biotransformation is less suitable in the case of honey.

2.8.1 Polyols

Various naturally occurring hexose derived polyols such as erythritol, xylitol, sorbitol, and mannitol, although being slightly less sweet than sucrose, possesses less calories. These sugar alcohols have no effect on the releasing of insulin, for which all the aforementioned characters make them suitable as a sugar replacement, especially for diabetics (Mazi & Stanhope, 2023). Although naturally present in food products such as fruit, their abundance is relatively low. Therefore, to meet the demands of industrial applications, chemical or biological interventions are required for bulk synthesis and purification. As an example, the production of erythritol from glucose undergoes a complicated enzymatic cascade that is usually performed with yeast (Li, Li, Liu, & Luo, 2023). Using both glucose and fructose as substrate, the one-step enzymatic synthetic method of D-sorbitol, a sugar alcohol that is 60% sweeter than sucrose, was discovered in the last century with glucose-fructose oxidoreductase purified from *Zymomonas mobilis* (Braga, et al., 2024). Conversion of the monosaccharides into sorbitol can also be conducted with free or immobilized *Z. mobilis* cells. D-Mannitol is a hexitol that has approximately 50-70% the sweetness of sucrose and a low caloric value of 1.6 kcal/g (Bhatt, Mohan, & Srivastava, 2012). It was initially extracted from plants; however, such a process was replaced due to its low economic efficiency. The biosynthesis of D-mannitol from D-fructose can be achieved through the action of NADH- or NADPH-dependent mannitol dehydrogenase, which uses fructose as a substrate. Alternatively, it can be produced via microbial fermentation using yeasts, fungi, and lactic acid bacteria, with both glucose and fructose serving as substrates (Dai, Meng, Mu, & Zhang, 2017).

2.8.2 Rare sugar

Although alternative sweeteners such as xylitol have been industrially manufactured, their consumption is often linked to gastrointestinal discomfort, hence replacing traditional added sugar in food commodities with rare sugar has become a more promising solution (Mäkinen, 2016).

Because the structures of monosaccharides and type of glycosidic linkages between disaccharides strongly affect their digestibility and health properties, various research have demonstrated the potential health benefits in multiple rare sugars with the unusual structures found among them. Rare sugars defined by the International Society of Rare Sugar are "monosaccharides and their derivatives that are present in limited quantities in nature" (Hayashi, et al., 2014). Rare sugar often possesses similar texture, taste, and bulk properties as sucrose, which makes them better candidates in replacing free sugar than artificial sweeteners especially in food applications. With increasing interest in rare sugar application in the food industry for the reduced calories in traditionally calorie-dense commodities, their production routes have been explored (Kim, Kim, & Yeom, 2020).

Technological advances in the recent decades, especially with bioconversion of rare sugar from the more abundant monosaccharides, enables the industrial level application in food manufacturing. Over 40 types of monosaccharides are either naturally present or synthesizable, among which, only seven are non-rare, namely glucose, fructose, galactose, mannose, ribose, xylose, and arabinose (Mijailovic, Nesler, Perazzolli, Aït Barka, & Aziz, 2021). In the early 1990s, a research team in Japan established a strategy called izumoring that enables the production of 34 hexoses and their polyol derivatives from glucose or fructose under a system of enzymatic and microbiological reactions (Itoh, et al., 1994). Three key biocatalysts, namely D-tagatose-3-epimerase, polyol dehydrogenase, and aldose isomerase are involved in these biochemical reactions that can be arranged and connected into a symmetric ring model. And this new concept contributes to some later development and improvement of the biosynthesis of rare sugars.

D-allulose (D-psicose), a C3 epimer of D-fructose, is a type of rare sugar that has 70% sweetness of sucrose, and inert caloric content of 0.2 Kcal/g (Jiang, et al., 2020). It has been listed as GRAS (Generally Recognized as Safe) by the USDA in 2014. Being a rare sugar, allulose are scarcely found in nature, especially terrestrial plants. However, besides being a food ingredient to replace sugar, it also has high-economical values for its nutraceutical properties (Bayu, Warsito, Putra, Karnjanakom, & Guan, 2021). Here, not only allulose has been shown to exhibit anti-diabetic, anti-obesity, anti-hyperlipidemic, anti-atherosclerosis, anti-inflammatory and neuroprotective effects, its abilities in improving gelling properties, reducing oxidation during Maillard reaction, acting as prebiotics, and improving the flavor profile also demonstrated its potential applications in the food industry (Chen, Gao, & Li, 2022; Xia, et al., 2021; Choi, Kwon, Kim, & Choi, 2018).

Its presence in confectionery food products suggests the formation of allulose via non-enzymatic route when heat is applied (Oshima, Kimura, & Izumori, 2006).

In order to synthesize allulose on a large-scale, multiple methods have been studied in the past few decades, which can be divided into two categories: chemical synthesis, and biosynthesis. The chemical synthesize of D-allulose can be achieved by using molybdate ion as catalyst in acidic aqueous solution for the conversion of glucose or fructose into allulose, or the redox reactions with 1,2:4,5-di-O-isopropylidene-B-D-fructopyranose being the reagent and 1,2;4,5-di-O-isopropylidene-B-D-allulose as the product followed by acid hydrolysis, or isomerization of D-fructose in base solutions (Doner, 1979; Cree & Perlin, 1968; Bilik & Tihlarik, 1974). However, some of the major disadvantages are associated with the chemocatalysis methods, such as high labor intensity for product purification, complex reaction conditions, low conversion yield, and the production of hazardous by-products. Hence, these chemical synthesis methods are not applicable for industrial scale allulose production and are later replaced by biosynthetic methods (Wang, et al., 2020). Biosynthesize of D-allulose is achieved via enzymatic epimerization reactions, with D-tagatose-3-epimerase (DTEase) discovered to be able to use for the conversion of D-fructose into D-allulose in the last century. In the recent decades, the utilization of D-allulose-3-epimerase (DAEase) or D-psicose-3-epimerase (DPEase) from various bacteria sources for allulose mass production has drawn much attention. Based on the enzymatic conversion, the synthesize of D-allulose using DAEase carrying bacteria via microbiological fermentation has also been developed. **Table 2.3** displays the microbial sources of DAEase found in various researcheds.

.

Table 2.3 Summary of microbial sources of D-allulose-3-epimerase (DAEase) and their optimum reaction conditions and allulose production yield reported in literature.

Strain (DAEase)	Metal dependence	Co-factor	Allulose: Fru eqn.	Optimum pH	Optimum temp.	Reference
Pseudomonas sp. ST-24				7-9	60	Itoh, et al., 1994
Agrobacterium tumefaciens		Mn2+, Co2+	33:67	7.5	60	Kim, et al., 2006
Rhodobacter sphaeroides SK011		Mn2+	23:77	9	40	Zhang, Mu, Jiang & Zhang, 2009
Clostridium cellulolyticum H10	Yes	Co2+	32:68	8	55	Mu, et al., 2011;
Ruminococcus sp.		Mn2+		7.5-8	60	Zhu, et al., 2012
Clostridium scindens 35704	Yes	Mn2+	28:72	7.5	60	Zhang, et al., 2013a
Clostridium sp. BNL1100	Yes	Co2+	28:72	8	65	Mu, et al., 2013
Desmosporasp. 8437	Yes	Co2+	30:70	7.5	55-65	Zhang, et al., 2013b
Clostridium bolteae		Co2+	31:69	7	55	Jia, et al., 2013
Dorea sp. CAG317	Yes	Co2+	30:70	6	70	Zhang, et al., 2015
Treponema primitia ZAS-1	Yes	Co2+	28:72	8	70	Zhang, Zhang, Jiang & Mu, 2015
Arthrobacter globiformis M30	Yes	Mg2+		7.0-8.0	70	Yoshihara, et al., 2017
Agrobacterium sp. ATCC 31749	Yes	Co2+	30:70	7.5-8	55-60	Tseng, et al., 2019
Staphylococcus aureus		Mg2+		8	70	Zhu, et al., 2019
Christensenella minuta DSM 22607 (DTE-CM)	Yes	Ni2+		6		Wang, et al., 2020
Bacillus sp. KCTC 13219	Yes	Mn2+, Co2+		8	55	Patel, et al., 2021
Pirellula sp. SH-Sr6A		Co2+	30:70	7.5	60	Li, et al., 2021
Novibacillus thermophilus		Co2+		7	70	Jia, et al., 2021
Arthrobacter psychrolactophilus		Mg2+		8.5	70	Laksmi, et al., 2022
Blautia produca	Yes	Mn2+	30:70	8	55	Tang, 2022
Ruminiclostridium papyrosolvens		Co2+	39.7:70.1	7.5	60	Yang, et. al, 2022

2.9 Conclusion

With a growing recognition of its health promoting properties, the value of honey is now more valued by health-oriented population, not only for its renowned and unique sensory profiles but also for its potential health benefits. Attributed to their complex yet distinct physicochemical properties, including sugar composition, phenolic compounds and volatile compounds, different types of monofloral honeys gradually build their reputation among consumers. Therefore, there is a growing demand in the agri-food industry to adopt standardized guidelines, not only for regulating adulteration but also for more accurate and robust authentication of honey's botanical and geographical origins. In recent years, much attention has been drawn to this initiative and numerous studies have been conducted to support these efforts. Given the high cost and low yield to the traditional authentication methods, ongoing research focuses on establishing new authentication criteria. Currently, enzymes, being the major contributor to the formation of honey, are widely adopted as a regulation parameter for quality assessment, but are less considered in terms of honey authentication, despite some were indeed plant originated. Coupled with chemometric techniques, some of the main approaches for the researchers to find new methods of honey authentication include phenolic compound profiling, volatile compound profiling and sugar profiling, in particular di-, tri-, and oligosaccharides, all of which have shown promises in revealing the identity of honeys. Despite having various health benefits, it is still inevitable that their consumption should be constrained, as the major component of honey is in fact fructose and glucose. Hence, to further improve the nutritional profile of honey and reduce its caloric content, biotransformation of intrinsic sugars into functional sweeteners were investigated. DAEase was found to perform the biogeneration of D-allulose, a GRAS, low-caloric and health-promoting sweetener, from D-fructose, making it a potential candidate for the fortification and production of functional honeys.

Reference

Abd-Elaziz, A., Karam, E., Ghanem, M., Moharam, M., & Kansoh, A. (2020). Production of a novel α-amylase by Bacillus atrophaeus NRC1 isolated from honey: Purification and characterization. *International Journal of Biological Macromolecules*, *148*, 292-301.

Adamberg, K., Adamberg, S., Ernits, K., Larionova, A., Voor, T., Jaagura, M., . Alamäe, T. (2018). Composition and metabolism of fecal microbiota from normal and overweight children are differentially affected by melibiose, raffinose and raffinose-derived fructans. *Anaerobe*, *52*, 100-110.

Adgaba, N., Al-Ghamdi, A., Tadesse, Y., Getachew, A., Awad, A., Ansari, M., Alqarni, A. (2017). Nectar secretion dynamics and honey production potentials of some major honey plants in Saudi Arabia. *Saudi Journal of Biological Sciences*, *24*(1), 180–191.

Afik, O., Dag, A., Kerem, Z., & Shafir, S. (2006). Analyses of Avocado (Persea americana) Nectar Properties and their Perception by Honey bees (Apis mellifera). *Journal of chemical ecology*, *32*(9), 1949-1963.

Aidoo, R. P., Depypere, F., Afoakwa, E. O., & Dewettinck, K. (2013). Industrial manufacture of sugar-free chocolates – Applicability of alternative sweeteners and carbohydrate polymers as raw materials in product development. *Trends in Food Science & Technology*, *32*(2), 84-96.

Alaerjani, W. M., & Mohammed, M. E. (2024). Impact of floral and geographical origins on honey quality parameters in Saudi Arabian regions. *Scientific Reports*, *14*, 8720.

Alaerjani, W., Abu-Melha, S., Alshareef, R., Al-Farhan, B., Ghramh, H., Al-Shehri, B., Mohammed, M. E. (2022). Biochemical Reactions and Their Biological Contributions in Honey. *molecules*, *27*(15), 4719.

Al-Ghamdi, A., Mohammed, S. E., Ansari, M. J., & Adgaba, N. (2019). Comparison of physicochemical properties and effects of heating regimes on stored Apis mellifera and Apis florea honey. *Saudi Journal of Biological Sciences*, *26*(4), 845-848.

Aliyu, M., Ibrahim, S., Inuwa, H., Sallau, A., Abbas, O., Aimola, I., Uche, N. (2013). Ameliorative Effects of Acacia Honey against Sodium Arsenite-Induced Oxidative Stress in Some Viscera of Male Wistar Albino Rats. *Biochemistry Research International*, *2013*, 502438.

Al-Khalifa, A., & Al-Arify, I. (1999). Physicochemical characteristics and pollen spectrum of some Saudi honeys. *Food Chemistry*, *67*(1), 21-25.

Alonso-Torre, S. R., Cavia, M. M., Fernández-Muiño, M. A., Moreno, G., Huidobro, J. F., & Sancho, M. T. (2006). Evolution of acid phosphatase activity of honeys from different climates. *Food Chemistry*, *97*(4)750-755.

Alshareef, R., Al-Farhan, B., & Mohammed, M. (2022). Glucose Oxidase and Catalase Activities in Honey Samples from the Southwestern Region of Saudi Arabia. *Applied Sciences*, *12*(15), 7584.

Al-Sherif, A., Mazeed, A., Ewis, M., Nafea, E., Hagag, E.-S., & Kamel, A. (2017). Activity of salivary glands in secreting honey-elaborating enzymes in two subspecies of honeybee (Apis mellifera L). *Physiological Entomology*, *42*(4), 397-403.

Alvarez-Suarez, J. M., Tulipani, S., Romandini, S., Bertoli, E., & Battino, M. (2009). Contribution of honey in nutrition and human health: a review. *Mediterranean Journal of Nutrition and Metabolism* , *3*, 15-23.

Aquinas, N., Chithra, C. H., & Bhat, M. R. (2024). Progress in bioproduction, characterization and applications of pullulan: a review. *Polymer Bulletin*, *81*, 12347–12382.

Ávila, S., Beux, M. R., Ribani, R. H., & Zambiazi, R. C. (2018). Stingless bee honey: Quality parameters, bioactive compounds, health-promotion properties and modification detection strategies. *Trends in Food Science & Technology*, *81*, 37-50.

Ayoub, W. S., Ritu, Zahoor, I., Dar, A. H., Farooq, S., Mir, T. A., Ganaie, T. A., Srivastava, S., Pandey, V. K., & Altaf, A. (2023). Exploiting the polyphenolic potential of honey in the prevention of chronic diseases. *Food Chemistry Advances*, *3*, 100373.

Babacan, S., & Rand, A. (2005). Purification of Amylase from Honey. *Food Chemistry and Toxicology*, *70*(6), 413-418.

Bayu, A., Warsito, M. F., Putra, M. Y., Karnjanakom, S., & Guan, G. (2021). Macroalgae-derived rare sugars: Applications and catalytic synthesis. *Carbon Resources Conversion*, *4*, 150-163.

Becerril-Sánchez, A. L., Quintero-Salazar, B., Dublán-García, O., & Escalona-Buendía, H. B. (2021). Phenolic Compounds in Honey and Their Relationship with Antioxidant Activity, Botanical Origin, and Color. *Antioxidants*, *10*(11), 1700.

Behera, P., & Balaji, S. (2021). The forgotten sugar: A review on multifarious applications of melezitose. *Carbohydrate Research*, *500*, 108248.

Belay, A., Haki, G., Birringer, M., Borck, H., Lee, Y.-C., Cho, C.-W., Melaku, S. (2017). Sugar profile and physicochemical properties of Ethiopian monofloral honey. *International Journal of Food Properties*, *20*(11), 2855-2866.

Belitz, H.-D., Grosch, W., & Schieberle, P. (2009). Sugars, Sugar Alcohols and Honey. In H.-D. Belitz, W. Grosch, & P. Schieberle, *Food Chemistry* (pp. 862–891). Berlin: Springer.

Bhatt, S. M., Mohan, A., & Srivastava, S. K. (2012). Challenges in Enzymatic Route of Mannitol Production. *ISRN Biotechnology*, *2013*, 914187.

Bilik, V., & Tihlarik, K. (1974). Reactions of saccharides catalyzed by molybdate ions. IX. Epimerization of Hexose. *Chemical Papers*, *28*(1), 106-109.

Biswas, A., Borse, B. B., & Chaudhari, S. R. (2025). Quantitative NMR analysis of sugars in natural sweeteners: Profiling in honey, jaggery, date syrup, and coconut sugar. *Food Research International*, *199*, 115358.

Bobis, O., Moise, A., Ballesteros, I., Reyes, E., Durán, S., Sánchez-Sánchez, J., Cruz-Quintana, S., Giampieri, F., Battino, M., & Alvarez-Suarez, J. M. (2020). Eucalyptus honey: Quality parameters, chemical composition and health-promoting properties. *Food Chemistry*, *325*, 126870. Advance online publication.

Bogdanov, S., Haldimann, M., Luginbühl, W., & Gallmann, P. (2007). Minerals in honey: Environmental, geographical and botanical aspects. *Journal of Apicultural Research*, *46*(4), 269-275.

Bogdanov, S., Jurendic, T., Sieber, R., & Gallmann, P. (2008). Honey for Nutrition and Health: A Review. *Journal of the American College of Nutrition*, *27*(6), 677-689.

Bogdanov, S., Lüllmann, C., Martin, P., von der Ohe, W., Russmann, H., Vorwohl, G., … Vit, P. (1999). Honey quality and international regulatory standards: review by the International Honey Commission. *Bee World*, *80*(2), 61-69.

Bonvehi, J. S., & Coil , F. V. (1993). Physico-chemical properties,composition and pollen spectrum of french lavender (Lavandula stoechas L.) honey produced in Spain. *Zeitschrift für Lebensmittel-Untersuchung und Forschung*, *196*, 511-517.

Boruah, T., Devi, H., Dulal, K., Das, P. N., Devi, B., Nayik, G. A., & Singh, R. (2024). Chapter two - Botanical (melissopalynological) and geographical analysis of honey. In G. A. Nayik, J. Uddin, & V. Nanda, *Advanced Techniques of Honey Analysis: Characterization, Authentication, and Adulteration* (pp. 39-62). Academic Press.

Borutinskaite, V., Treigyte, G., Čeksteryte, V., Kurtinaitiene, B., & Navakauskiene, R. (2018). Proteomic identification and enzymatic activity of buckwheat (Fagopyrum esculentum) honey based on different assays. *Journal of Food and Nutrition Research*, *57*(1), 57-69.

Bouhlali, E. D., Bammou, M., Sellam, K., El Midaoui, A., Bourkhis, B., Ennassir, J., . . . Filali-Zegzouti, Y. (2019). Physicochemical properties of eleven monofloral honey samples produced in Morocco. *Arab Journal of Basic and Applied Sciences*, *26*(1), 476–487.

Braga, A., Maia, A. B., Gomes, D., Rodrigues, J. L., Rainha, J., & Rodrigues, L. R. (2024). Improving Fructooligosaccharide Production via sacC Gene Deletion in Zymomonas mobilis: A Novel Approach for Enhanced Prebiotic Production. *Food Bioprocess Technol*, *18*, 899-915.

Chakir, A., Romane, A., Marcazzan, G. L., & Ferrazzi, P. (2016). Physicochemical properties of some honeys produced from different plants in Morocco. *Arabian Journal of Chemistry*, S946-S954.

Chattopadhyay, S., Raychaudhuri, U., & Chakraborty, R. (2014). Artificial sweeteners – a review. *Journal of food science and technology*, 611-621.

Chen, A., & Gibney, P. A. (2023). Dietary Trehalose as a Bioactive Nutrient. *Nutrients*.

Chen, C. M., Lin, C. H., Wu, Y. R., Yen, C. Y., Huang, Y. T., Lin, J. L., Lin, C. Y., Chen, W. L., Chao, C. Y., Lee-Chen, G. J., Su, M. T., & Chang, K. H.(2020). Lactulose and Melibiose Inhibit α-Synuclein Aggregation and Up-Regulate Autophagy to Reduce Neuronal Vulnerability. *Cells*, *9*(5), 1230.

Chen, H., Jin, L., Chang, Q., Peng, T., Hu, X., Fan, C., Wang, W. (2017). Discrimination of botanical origins for Chinese honey according to free amino acids content by high-performance liquid chromatography with fluorescence detection with chemometric approaches. *Journal of the science of food and agriculture*, 2042-2049.

Chen, Z., Gao, X.-D., & Li, Z. (2022). Recent Advances Regarding the Physiological Functions and Biosynthesis of D-Allulose. *Frontiers in Microbiology*, *13*, 881037.

Choi, B.-R., Kwon, E.-Y., Kim, H.-J., & Choi, M.-S. (2018). Role of Synbiotics Containing d-Allulose in the Alteration of Body Fat and Hepatic Lipids in Diet-Induced Obese Mice. *Nutrients*, *10*(11), 1797.

Choi, B.-Y., Seo, D.-H., Hamaker, B. R., & Yoo, S.-H. (2024). Enhanced production of turanose using a mutant amylosucrase from Bifidobacterium thermophilum immobilized on silica carrier. *International Journal of Biological Macromolecules*, *283*(3), 136981.

Chuttong, B., Chanbang, Y., Sringarm, K., & Burgett, M. (2016). Physicochemical profiles of stingless bee (Apidae: Meliponini) honey from South East Asia (Thailand). *Food Chemistry*, 149-155.

Cianciosi, D., Forbes-Hernández, T. Y., Afrin, S., Gasparrini, M., Reboredo-Rodriguez, P., Manna, P. P., Battino, M. (2018). Phenolic Compounds in Honey and Their Associated Health Benefits: A Review. *Molecules*, *23*(9), 2322.

Ciappini, M., Vitelleschi, M., & Calvinõ, A. (2016). Chemometrics Classification of Argentine Clover and Eucalyptus Honeys According to Palynological, Physicochemical, and Sensory Properties. *International Journal of Food Properties*, 111 - 123.

Côté, G. L. (2007). 12 - Flavorings and other value-added products from sucrose. In R. Robert, *Novel Enzyme Technology for Food Applications* (pp. 243-269). Woodhead Publishing.

Cott, J., Casabianca, H., Giroud, B., Albert, M., Lheritier, J., & Grenier-Loustalot, M. (2004). Characterization of honey amino acid profiles using high-pressure liquid chromatography to control authenticity. *Analytical and Bioanalytical Chemistry*, 1342-1350.

Cotte, J.-F., Casabianca, H., Giroud, B., Lhéritier, J., Albert, M., & Grenier-Loustalot, M.-F. (2004). Characterization of honey amino acid profiles using high-pressure liquid chromatography to control authenticity. *Analytical and Bioanalytical Chemistry*, 1342-1350.

Covaci, B., Brejea, R., & Covaci, M. (2023). Sweeteners World Trade and Behaviour in the Pandemic. Evidence from Honey Remedies Nexus Mountain Apis Mellifera Product. *Sugar tech : an international journal of sugar crops & related industries*, 1–12.

Crăciun, M. E., Pârvulescu, O. C., Donise, A. C., Dobre, T., & Stanciu, D. R. (2020). Characterization and classification of Romanian acacia honey based on its physicochemical parameters and chemometrics. *Scientific reports*, *10*(1), 20690.

Cree, G., & Perlin, A. (1968). O-isopropylidene Derivatives of D-allulose (D-psicose) and D-erythro-hexopyranos-2,3-diulose. *Canadian Joural of Biochemistry*, 765-770.

Dag, A., Afik, O., Yeselson, Y., Schaffer, A., & Shafir, S. (2006). Physical, chemical and palynological characterization of avocado (Persea americana Mill.) honey in Israel. *International Journal of Food Science and Technology*, 387-394.

Dai, Y., Meng, Q., Mu, W., & Zhang, T. (2017). Recent advances in the applications and biotechnological production of mannitol. *Journal of Functional Foods*, 404-409.

de Almeida-Muradian, L., Sousa, R., Barth, O., & Gallmann, P. (2014). Preliminary data on Brazilian monofloral honey from the northeast region using FT-IR ATR spectroscopic, palynological, and color analysis. *Química Nova*, 716-719.

de la Fuente, E., Ruiz-Matute, A., Valencia-Barrera, R. M., Sanz, J., & Martínez Castro, I. (2011). Carbohydrate composition of Spanish unifloral honeys. *Food Chemistry*, 1483–1489.

de la Fuente, E., Sanz, M., Martínez-Castro, I., Sanz, J., & Ruiz-Matute, A. (2007). Volatile and carbohydrate composition of rare unifloral honeys from Spain. *Food Chemistry*, 84-93.

de Sousa, J., de Souza, E., Marques, G., de Toledo Benassi, M., Beatriz, Pintado, M., & Magnani, M. (2016). Sugar profile, physicochemical and sensory aspects of monofloral honeys produced by different stingless bee species in Brazilian semi-arid region. *LWT - Food Science and Technology*, 645-651.

Deng, J., Liu, R., Lu, Q., Hao, P., Xu, A., Zhang, J., & Tan, J. (2018). Biochemical properties, antibacterial and cellular antioxidant activities of buckwheat honey in comparison to manuka honey. *Food Chemistry*, 243-249.

Despland, C., Walther, B., Kast, C., Campos, V., Rey, V., Stefanoni, N., & Tappy, L. (2017). A randomized-controlled clinical trial of high fructose diets from either Robinia honey or free fructose and glucose in healthy normal weight males. *Clinical Nutrition ESPEN*, 16-22.

Doner, L. (1979). Isomerization of D-fructose by Base: Liquid-Chromatographic Evaluation and the Ioslation of D-Psicose. *Carbohydrate Research*, 209-216.

Dvash, L., Afik, O., Shafir, S., Schaffer, A., Yeselson, Y., Dag, A., & Landau, S. (2002). Analyses of Avocado (Persea americana) Nectar Properties and their Perception by Honey bees (Apis mellifera). *Journal of Agricultural and Food Chemistry*, 5283-5287.

Dżugan, M., Grabek-Lejko, D., Swacha, S., Tomczyk, M., Bednarska, S., & Kapusta, I. (2020). Physicochemical quality parameters, antibacterial properties and cellular antioxidant activity of Polish buckwheat honey. *Food Bioscience*, *34*, 100538.

Erban, T., Shcherbachenko, E., Talacko, P., & Harant, K. (2019). The unique protein composition of honey revealed by comprehensive proteomic analysis: Allergens, venom-like proteins, antibacterial properties, royal jelly proteins, serine proteases, and their inhibitors. *Journal of Natural Products*, 1217-1226.

Esa, N. E. F., Ansari, M. N. M., Razak, S. I. A., Ismail, N. I., Jusoh, N., Zawawi, N. A., Jamaludin, M. I., Sagadevan, S., & Nayan, N. H. M (2022). A Review on Recent Progress of Stingless Bee Honey and Its Hydrogel-Based Compound for Wound Care Management. *Molecules*, *27*(10), 3080.

Escuredo, O., Míguez, M., Fernández-González, M., & Seijo, M. C. (2013). Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. *Food Chemistry*, 851-856.

Escuredo, O., Seijo, M. C., & Fernández-González, M. (2011). Descriptive analysis of Rubus honey from the north-west of Spain. *International Journal of Food Science and Technology*, 2329-2336.

Eteraf-Oskouei, T., & Najafi, M. (2013). Traditional and Modern Uses of Natural Honey in Human Diseases: A Review. *Iranian journal of basic medical sciences*, 731-742.

EU. (2001). COUNCIL DIRECTIVE 2001/110/EC relating to honey. *Official Journal of the European Communities*, 47-52.

Eyer, M., Neumann, P., & Dietemann, V. (2016). A Look into the Cell: Honey Storage in Honey Bees, Apis mellifera. *PLoS One*, *11*(8), e0161059.

Fahim, H., Dasti, J., Ali, I., Ahmed, S., & Nadeem, M. (2014). Physico-chemical analysis and antimicrobial potential of Apis dorsata, Apis mellifera and Ziziphus jujube honey samples from Pakistan. *Asian Pacific Journal of Tropical Biomedicine*, 633-641.

Fallico, B., Arena, E., Verzera, A., & Zappalà, M. (2006). The European Food Legislation and its impact on honey sector. *Accreditation and Quality Assurance*, 49–54.

FAO. (2015). *Composition and quality requirements for honey and requirements for the provision of food information.* Minister of Agriculture.

FAO. (2022). *STANDARD FOR HONEY.* Codex Alimentarius.

Felsner, M. L., Cano, C. B., Bruns, R. E., Watanabe, H. M., Almeida-Muradian, L. B., & Matos, J. R. (2004). Characterization of monofloral honeys by ash contents through a hierarchical design. *Journal of Food Composition and Analysis*, 737-747.

Flanjak, I., Strelec, I., Kenjerić, D., & Primorac, L. (2016). Croatian produced unifloral honey characterized according to the protein and proline content and enzyme activities. *Journal of Apicultural Science*, 39-48.

Fletcher, M. T., Hungerford, N. L., Webber, D., Carpinelli de Jesus, M., Zhang, J., Stone, I. S., ... Zawawi, N. (2020). Stingless bee honey, a novel source of trehalulose: a biologically active disaccharide with health benefits. *Scientific Reports*, *10*, 12128.

Flores, M., Pérez, O., & Coello, M. (2014). Characterization of eucalyptus globulus honeys produced in the eurosiberian area of the Iberian Peninsula. *International Journal of Food Properties*, 2177–2191.

Fry, J. (2012). 3 - Natural low-calorie sweeteners. In D. Baines, & R. Seal, *Natural Food Additives, Ingredients and Flavourings* (pp. 41-75). Woodhead Publishing.

Garcia-Gonzalez, M., Minguet-Lobato, M., Plou, F. J., & Fernandez-Lobato, M. (2020). Molecular characterization and heterologous expression of two α-glucosidases from Metschnikowia spp, both producers of honey sugars. *Microbial Cell Factories*, *19*(1), 140.

García-Tenesaca, M., Navarrete, E. S., Iturralde, G. A., Villacrés Granda, I. M., Tejera, E., Beltrán-Ayala, P., Giampieri, F., Battino, M., & Alvarez-Suarez, J. M. (2017). Influence of Botanical Origin and Chemical Composition on the Protective Effect against Oxidative Damage and the Capacity to Reduce In Vitro Bacterial Biofilms of Monofloral Honeys from the Andean Region of Ecuador. *International Journal of Molecular Sciences*, *19*(1), 45.

Gašić, U. M., Natić, M. M., Mišić, D. M., Lušić, D. V., Milojković-Opsenica, D. M., Tešić, Ž. L., & Lušić, D. (2015). Chemical markers for the authentication of unifloral Salvia officinalis L. honey. *Journal of Food Composition and Analysis*, 128–138.

Gašić, U., Šikoparija, B., Tosti, T., Trifković, J., Milojković-Opsenica, D., Natić, M., & Tešić, Ž. (2014). Phytochemical Fingerprints of Lime Honey Collected in Serbia. *Journal of AOAC International*, 1259-1267.

Ghoniemy, H. A., Esmail, A. H., Mahmoud, A. A.-T., & Mohamed, A. M. (2022). Evaluation of vitamin C, proline, enzymes and hydroxymethylfurfural levels in clover honey at different storage conditions. *Egyptian Journal of Chemistry*, 427-434.

Ghramh , H., Khan, K., Zubair, A., & Ansari, M. (2020). Quality evaluation of Saudi honey harvested from the Asir province by using high-performance liquid chromatography (HPLC). *Saudi Journal of Biological Sciences*, 2097–2105.

Grujić, S., Grujić, R., Popov-Raljić, J., & Komić, J. (2011). Characterization of Black Locust (Robinia Pseudoacacia) Honey from Three Geographical Regions of North-West Bosnia and Herzegovina. *7th International Congress of Food Technologist, Biotechnologists and Nutritionists*, (pp. 274-278). Opatija.

Guo, P., Deng, Q., & Lu, Q. (2019). Anti-alcoholic effects of honeys from different floral origins and their correlation with honey chemical compositions. *Food Chemistry*, 608-615.

Guttentag, A., Krishnakumar, K., Cokcetin, N., Harry, E., & Carter, D. (2021). Factors affecting the production and measurement of hydrogen peroxide in honey samples. *Access Microbiology*, *3*(3), 000198.

Han, D.-J., Lee, B.-H., & Yoo, S.-H. (2021). Physicochemical properties of turanose and its potential applications as a sucrose substitute. *Food Science and Biotechnology*, 433-441.

Han, F., Wallberg, A., & Webster, M. T. (2012). From where did the Western honeybee (Apis mellifera) originated? *Ecology and Evolution*, 1949–1957.

Hayashi, N., Yamada, T., Takamine, S., Iida, T., Okuma, K., & Tokuda, M. (2014). Weight reducing effect and safety evaluation of rare sugar syrup by a randomized double-blind, parallel-group study in human. *Journal of Functional Foods*, 152-159.

Hegazi, A. G., Al Guthami, F. M., Ramadan, M. F., Al Gethami, A. F., Craig, A., & Serrano, S. (2022). Characterization of Sidr (Ziziphus spp.) Honey from Different Geographical Origins. *Applied Sciences*, *12*(18), 9295;

Heil, M. (2011). Nectar: generation, regulation, and ecological functions. *Trends In Plant Science*, 191-200.

Hermosín, I., Chicón, R., & Cabezudo, M. (2003). Free amino acid composition and botanical origin of honey. *Food Chemistry*, 263-268.

Hodoniczky, J., Morris, C. A., & Rae, A. L. (2012). Oral and intestinal digestion of oligosaccharides as potential sweeteners: A systematic evaluation. *Food Chemistry*, 1954-1958.

Homrani, M., Escuredo, O., Rodríguez-Flores, M. S., Fatiha, D., Mohammed, B., Homrani, A., & Seijo, M. C. (2020). Botanical Origin, Pollen Profile, and Physicochemical Properties of Algerian Honey from Different Bioclimatic Areas. *Foods*, *9*(7), 938.

Horn, H., & Böhm, D. (2004). The relationship between the yield, moisture, proline and the enzyme activities invertase and diastase in honey. *Deutsche Lebensmittel-Rundschau*, 88.

Hu, H., Bezabih, G., Feng, M., Wei, Q., Zhang, X., Wu, F., Li, J. (2019). In-depth Proteome of the Hypopharyngeal Glands of Honeybee Workers Reveals Highly Activated Protein and

Energy Metabolism in Priming the Secretion of Royal Jelly. *Molecular & Cellular Proteomics*, 606-621.

International Honey Commission. (2009). *Harmonised methods of the international honey commission.* Retrieved from International Honey Commission: https://www.ihc-platform.net/ihcmethods2009.pdf

Islam, M. K., Lozada Lawag, I., Green, K. J., Sostaric, T., Hammer, K. A., Lim, L. Y., & Locher, C. (2022a). An investigation of the suitability of melissopalynology to authenticate Jarrah honey. *Current research in food science*, 506–514.

Islam, M., Lawag, I., Sostaric , T., Ulrich, E., Ulrich, D., Dewar, T., Locher, C. (2022b). Australian Honeypot Ant (Camponotus inflatus) Honey—A Comprehensive Analysis of the Physiochemical Characteristics, Bioactivity, and HPTLC Profile of a Traditional Indigenous Australian Food. *molecules*, *27*(7), 2154.

Itoh, H., Okaya, H., Khan, A. R., Tajima, S., Hayakawa, S., & Izumori, K. (1994). Purification and Characterization of D-Tagatose 3-Epimerase from Pseudomonas sp. ST-24. *Bioscience, Biotechnology, and Biochemistry*, 2168–2171.

Izumori, K. (2006). Izumoring: A strategy for bioproduction of all hexoses. *Journal of Biotechnology*, 717-722.

Jia, D.-X., Sun, C.-Y., Jin, Y.-T., Liu, Z.-Q., Zheng, Y.-G., Li, M., Chen, D.-S. (2021). Properties of d-allulose 3-epimerase mined from Novibacillus thermophilus and its application to synthesis of d-allulose. *Enzyme and microbial technology*, *148*, 109816.

Jia, M., Mu, W., Chu, F., Zhang, X., Jiang, B., Zhou, L. L., & Zhang, T. (2014). A d-psicose 3-epimerase with neutral pH optimum from Clostridium bolteae for d-psicose production: cloning, expression, purification, and characterization. *Biotechnologically relevant enzymes and proteins*, 717–72.

Jiang, S., Xiao, W., Zhu, X., Yang, P., Zheng, Z., Lu, S., Jiang, S., Zhang, G., & Liu, J. (2020). Review on D-Allulose: In vivo Metabolism, Catalytic Mechanism, Engineering Strain Construction, Bio-Production Technology. *Frontiers in Bioengineering and Biotechnology*, *8*, 26.

Juan-Borrás , M., Domenech, E., Hellebrandova, M., & Escriche , I. (2014). Effect of country origin on physicochemical, sugar and volatile composition of acacia, sunflower and tilia honeys. *Food Research International*, 86–94.

Julika, W., Ajit, A., Ismail, N., Aqilah, N., Naila, A., & Sulaiman, A. (2020). Sugar profile and enzymatic analysis of stingless bee honey collected from local market in Malaysia. *Energy Security and Chemical Engineering Congress*, *736*, 062001.

Kamboj, R., Sandhu, R. S., & Nanda, V. (2024). Effect of heating and pH on hydroxymethylfurfural content, diastase and invertase activity of Dalbergia honey using response surface methodology. *Food Chemistry Advances*, 5, 100766.

Karabagias, I. (2019). Seeking of reliable markers related to Greek nectar honey geographical and botanical origin identification based on sugar profile by HPLC-RI and electro-chemical

parameters using multivariate statistics. *European Food Research and Technology*, *245*, 805–816

Karabagias, I., Louppis, A., Karabournioti, S., Kontakos, S., Papastephanou, C., & Kontominas, M. (2017). Characterization and classification of commercial thyme honeys produced in specific Mediterranean countries according to geographical origin, using physicochemical parameter values and mineral content in combination with chemometrics. *European food research and technology*, 889-900.

Kasper-Szél, Z., Amtmann, M., Takáts, A., & Kardos-Neumann, A. (2003). A Comparative Analysis of Hungarian Robinia and Milkweed Honeys Based on Their Chemical and Physical Characteristics. *Acta Alimentaria*, 395-403.

Kečkeš, J., Trifković, J., Andrić, F., Jovetić, M., Tešić, Ž., & Milojković-Opsenica, D. (2013). Amino acids profile of Serbian unifloral honeys. *Journal of the Science of Food and Agriculture*, 3368-3376.

Khalafi, R., Goli, S. A., & Behjatian, M. (2016). Characterization and Classification of Several Monofloral Iranian Honeys Based on Physicochemical Properties and Antioxidant Activity. *International Journal of Food Properties*, 1065–1079.

Khan, K. A., Ghramh , H., Babiker, M., Ahmad, Z., El-Niweiri, M., Ibrahim, E., Brima, E. I., & Mohammed, M. E. A. (2021). Tolerance of Ziziphus and Acacia honeys to one year storage conditions and altitude. *Journal of King Saud University – Science*., *33*, 101577.

Kim, H.-J., Hyun, E.-K., Kim, Y.-S., Lee, Y.-J., & Oh, D.-K. (2006). Characterization of an Agrobacterium tumefaciensd-Psicose 3-Epimerase That Converts d-Fructose to d-Psicose. *Applied and Environmental Microbiology*, *72*(2), 981–985.

Kim, S.-J., Kim, Y.-S., & Yeom, S.-J. (2020). Phosphate sugar isomerases and their potential for rare sugar bioconversion. *Journal of microbiology*, 725-733.

Kivima, E., Tanilas, K., Martverk, K., Rosenvald, S., Timberg, L., & Laos, K. (2021). The Composition, Physicochemical Properties, Antioxidant Activity, and Sensory Properties of Estonian Honeys. *Foods*, *10*(3), 511.

Klose, S. P., Rolke, D., & Baumann, O. (2017). Morphogenesis of honeybee hypopharyngeal gland during pupal development. *Frontiers in Zoology volume*, *14*, 22.

Kodrík, D., Krištůfek, V., & Svobodová, Z. (2022). Bee year: Basic physiological strategies to cope with seasonality. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *264*, 111115.

Kortesniemi, M., Rosenvald, S., Laaksonen, O., Vanag, A., Ollikka, T., Vene, K., & Yang, B. (2018). Sensory and chemical profiles of Finnish honeys of different botanical origins and consumer preferences. *Food Chemistry*, 351–359.

Kowalski, S., Łukasiewicz, M., & Berski, W. (2013). Applicability of physico-chemical parameters of honey for identification of the botanical origin. *Acta Scientiarum Polonorum, Technologia Alimentaria*, 51 - 59.

Krell, R. (1996). *Value Added Products from Beekeeping.* Rome: FAO.

Kuś, P. M. (2020). Honey as Source of Nitrogen Compounds: Aromatic Amino Acids, Free Nucleosides and Their Derivatives. *Molecules*, *25*(4), 847.

Lachman, J., Kolihová, D., Miholová, D., Košata, J., Titěra, D., & Kult, K. (2007). Analysis of minority honey components: possible use for the evaluation of honey quality. *Food Chemistry*, 973-979.

Laksmi, F. A., Nirwantono, R., Nuryana, I., & Agustriana, E. (2022). Expression and characterization of thermostable D-allulose 3-epimerase from Arthrobacter psychrolactophilus (Ap DAEase) with potential catalytic activity for bioconversion of D-allulose from d-fructose. *International journal of biological macromolecules*, 426–438.

Lane, J., Calonne, J., Slattery, H., & Hickey, R. M. (2019). Oligosaccharides Isolated from MGO™ Manuka Honey Inhibit the Adhesion of Pseudomonas aeruginosa, Escherichia Coli O157:H7 and Staphylococcus Aureus to Human HT-29 cells. *Foods*, *8*(10), 446.

Li, C., Li, L., Feng, Z., Guan, L., Lu, F., & Qin, H.-M. (2021). Two-step biosynthesis of d-allulose via a multienzyme cascade for the bioconversion of fruit juices. *Food Chemistry*, *357*, 129746.

Li, H., Wu, M., She, S., Lin, G., Zhou, J., & Chen, L. (2022). Study on stable carbon isotope fractionation of rape honey from rape flowers (Brassica napus L.) to its unifloral ripe honey. *Food Chemistry*, *386*, 132754.

Li, J., Li, H., Liu, H., & Luo, Y. (2023). Recent Advances in the Biosynthesis of Natural Sugar Substitutes in Yeast. *Journal of fungi*, *9*(9), 907.

Lichtenberg-Kraag, B. (2015). Evidence for correlation between invertase activity and sucrose content during the ripening process of honey. *Journal of Apicultural Research*, 365-373.

Liu, W., Feng, Y., Yu, S., Fan, Z., Li, X., Li, J., & Yin, H. (2021). The Flavonoid Biosynthesis Network in Plants. *International journal of molecular sciences*, *22*(23), 12824

Liu, Y., Dunker, S., Durka, W., Dominik, C., Heuschele, J. M., Honchar, H., Hoffmann, P., Musche, M., Paxton., Settele., & Schweiger, O. (2024). Eco-evolutionary processes shaping floral nectar sugar composition. *Scientific Reports*, *14*, 13856.

Lobos, I., Silva, M., Ulloa, P., & Pavez, P. (2022). Mineral and Botanical Composition of Honey Produced in Chile's Central-Southern Region. *Foods*, *11*(3), 251.

Ma, T., Zhao, H., Liu, C., Zhu, M., Gao, H., Cheng, N., & Cao, W. (2019). Discrimination of Natural Mature Acacia Honey Based on Multi-Physicochemical Parameters Combined with Chemometric Analysis. *Molecules*, *24*(14), 2674.

Machado, A. M., Miguel, M. G., Vilas-Boas, M., & Figueiredo, A. C. (2020). Honey Volatiles as a Fingerprint for Botanical Origin—A Review on their Occurrence on Monofloral Honeys. *Molecules*, *25*(2), 374.

Mădaş, N. M., Mărghitaş, L. A., Dezmirean, D. S., Bonta, V., Bobiş, O., Fauconnier, M. L., Francis, F., Haubruge, E., & Nguyen, K. B. (2019). Volatile Profile and Physico-Chemical Analysis of Acacia Honey for Geographical Origin and Nutritional Value Determination. *Foods*, *8*(10), 445.

Mäkinen, K. K. (2016). Gastrointestinal Disturbances Associated with the Consumption of Sugar Alcohols with Special Consideration of Xylitol: Scientific Review and Instructions for Dentists and Other Health-Care Professionals. *International journal of dentistry*.

Manyi-Loh, C. E., Ndip, R. N., & Clarke, A. M. (2011). Volatile Compounds in Honey: A Review on Their Involvement in Aroma, Botanical Origin Determination and Potential Biomedical Activities. *International journal of molecular sciences*, *2016*, 5967907.

Manzanares, A., García, Z., Galdón, B., Rodríguez, E., & Romero, C. (2014). Physicochemical characteristics of minor monofloral honeys from Tenerife, Spain. *LWT - Food Science and Technology*, 572-578.

Marghitas, L., Dezmirean, D., Pocol, C., Ilea, M., Bobis, O., & Gergen, I. (2010). The Development of a Biochemical Profile of Acacia Honey by Identifying Biochemical Determinants of its Quality. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 84-90.

Martins, R. C., Lopes, V. V., Valentão, P., Carvalho, J. C., Isabel, P., Amaral, M. T., Batista, M. T., Andrade, P. B., & Silva, B. M. (2008). Relevant principal component analysis applied to the characterisation of Portuguese heather honey. *Natural Product Research*, *22*(17), 1560–1582.

Mateo, R., & Bosch-Reig, F. (1997). Sugar profiles of Spanish unifloral honeys. *Food Chemistry*, 33-41.

Mazi, T. A., & Stanhope, K. L. (2023). Erythritol: An In-Depth Discussion of Its Potential to Be a Beneficial Dietary Component. *Nutrients*, *15*(1), 204.

McLoone, P., Zhumbayeva, A., Yunussova, S., Kaliyev, Y., Yevstafeva, L., Verrall, S., Sungurtas, J., Austin, C., Allwood, J. W., & McDougall, G. J. (2021). Identification of components in Kazakhstan honeys that correlate with antimicrobial activity against wound and skin infecting microorganisms. *BMC Complementary Medicine and Therapies*, *21*(1), 300.

Mekious, S., Houmani, Z., Bruneau, É., Masseaux, C., Guillet, A., & Hance, T. (2015). Characterization of honeys produced in the region of Djelfa steppe land in Algeria. *Biotechnology, Agronomy and Society and Environment*, 221-231.

Mijailovic, N., Nesler, A., Perazzolli, M., Aït Barka, E., & Aziz, A. (2021). Rare Sugars: Recent Advances and Their Potential Role in Sustainable Crop Protection. *Molecules*, *26*(6), 1720.

Mineo, H., Hara, H., Shigematsu, N., Okuhara, Y., & Tomita, F. (2002). Melibiose, difructose anhydride III and difructose anhydride IV enhance net calcium absorption in rat small and large intestinal epithelium by increasing the passage of tight junctions in vitro. *The Journal of nutrition*, 3394–3399.

Ministry of Agriculture, Food and Fisheries. (2024, January 9). *Bee behaviour during foraging, bulletin 111.* Retrieved from British Columbia: https://www2.gov.bc.ca/assets/gov/farming-natural-resources-and-industry/agriculture-and-seafood/animal-and-crops/animal-production/bee-assets/api_fs111.pdf

Mohamed, A., Abdah, M., Ramasamy, K., Hasan, M., Hamimi, I., & Zolkapli, E. (2018). Physicochemical analysis and sugar profiling of Acacia honey. *Physicochemical analysis and sugar profiling of Acacia honey*, 157-164.

Mu, W., Chu, F., Xing, Q., Yu, S., Zhou, L., & Jiang, B. (2011). Cloning, Expression, and Characterization of a d-Psicose 3-Epimerase from Clostridium cellulolyticum H10. *Journal of Agricultural and Food Chemistry*, *59*(14), 7785–7792.

Mu, W., Zhang, W., Fang, D., Zhou, L., Jiang, B., & Zhang, T. (2013). Characterization of a d-psicose-producing enzyme, d-psicose 3-epimerase, from Clostridium sp. *Biotechnology Letters* , 1481–1486.

Mureșan, C., Cornea-Cipcigan, M., Suharoschi, R., Erler, S., & Mărgăoan, R. (2022). Honey botanical origin and honey-specific protein pattern: Characterization of some European honeys. *LWT*, *154*, 112883.

Nagma, P., Rashi, M., Netrapal, S., & Singh, B. S. (2021). Socio-Economic Analysis of Traditional and Modern Beekeeping in Western Himalayan Region Uttarakhand, India. *International Journal of Zoological Investigations*, 713-722.

Nguyen, T. Q., Kružík, V., Škorpilová, T., Javůrková, Z., Pospiech, M., Anh, L. T., ... Čížková, H. (2024). Physicochemical, sugar, and volatile profile characterization of blong song, bidens, coffee, and mint honeys originating from Vietnam. *Journal of Apicultural Research*, *63*(5), 946–958

Nisbet, C., Kazak, F., & Ardalı, Y. (2018). Determination of Quality Criteria that Allow Differentiation Between Honey Adulterated with Sugar and Pure Honey. *Biological trace element research*, 288–293.

Nordina, A., Sainika, N. Q., Chowdhury, S. R., Saim, A. B., & Idrus, R. B. (2018). Physicochemical properties of stingless bee honey from around the globe: A comprehensive review. *Journal of Food Composition and Analysis*, *73*, 91-102.

Nuru, A., Awad, A., Al-Ghamdi, A., Alqarni, A., & Radloff, S. (2012). Nectar of Ziziphus spina-christi (L.) Willd (Rhamnaceae): Dynamics of Secretion and Potential for Honey Production. *Journal of Apicultural Science*, 49-59.

Oddo, L., Piazza, M., & Pulcini, P. (1999). Invertase Activity in Honey. *Apidologie*, 57-65.

Ohtake, S., & Wang, Y. J. (2011). Trehalose: Current use and future applications. *Journal of Pharmaceutical Sciences*, *100*(6), 2020-2053.

Olas, B. (2020). Honey and Its Phenolic Compounds as an Effective Natural Medicine for Cardiovascular Diseases in Humans? *Nutrients*, *12*(2), 283

Onyango, S. O., De Clercq, N., Beerens, K., Van Camp, J., Desmet, T., & Van de Wiele, T. (2020). Oral Microbiota Display Profound Differential Metabolic Kinetics and Community Shifts upon Incubation with Sucrose, Trehalose, Kojibiose, and Xylitol. *Applied and Environmental Microbiology*, *86*(16), e01170-20.

Oroian, M., & Sorina, R. (2017). Honey authentication based on physicochemical parameters and phenolic compounds. *Computers and Electronics in Agriculture*, 148-156.

Osés, S. M., Rodríguezk, C., Valencia, O., Fernández-Muiño, M. A., & Sancho, T. (2024). Relationships among Hydrogen Peroxide Concentration, Catalase, Glucose Oxidase, and Antimicrobial Activities of Honeys. *Foods*, *13*(9), 1344

Oshima, H., Kimura, I., & Izumori, K. (2006). Psicose Contents in Various Food Products and its Origin. *Food Science and Technology Research*, 137-143.

Ouchemoukh, S., Schweitzer, P., Bey , M., Djoudad-Kadji , H., & Louaileche, H. (2010). HPLC sugar profiles of Algerian honeys. *Food Chemistry*, 561–568.

Özcan, M. M., & Ölmez, Ç. (2014). Some qualitative properties of different monofloral honeys. *Food Chemistry*, 212-218.

Ozkok, A., Bilgic, H. A., Kosukcu, C., Arik, G., Canli, D., & Yet, I. (2023). Comparing the melissopalynological and next generation sequencing (NGS) methods for the determining of botanical origin of honey. *Food Control*, *148*, 109630.

Palomäki, E. A., Lipiäinen, T., Strachan, C. J., & Yliruusi, J. K. (2020). Effect of trehalose and melibiose on crystallization of amorphous paracetamol. *International Journal of Pharmaceutics*, *590*, 119878.

Pascual-Maté, A., Osés, S., Marcazzan, G., Gardini, S., Fernández Muiño, M., & Sancho, M. (2018). Sugar composition and sugar-related parameters of honeys from the northern Iberian Plateau. *Journal of Food Composition and Analysis*, 34-43.

Pasini, F., Gardini, S., Marcazzan, G. L., & Caboni, M. F. (2013). Buckwheat honeys: Screening of composition and properties. *Food Chemistry*, 2802-2811.

Patel, S. N., Kaushal, G., & Singh, S. P. (2021). D-Allulose 3-epimerase of Bacillus sp. origin manifests profuse heat-stability and noteworthy potential of D-fructose epimerization. *Microbial cell factories*, *20*(1), 60.

Pauliuc, D., Dranca, F., & Oroian, M. (2020). Article Antioxidant Activity, Total Phenolic Content, Individual Phenolics and Physicochemical Parameters Suitability for Romanian Honey Authentication. *foods*, *9*(3), 306.

Perez-Arquillué, C., Conchello, P., Ariño, A., Juan, T., & Herrera, A. (1994). Quality evaluation of Spanish rosemary (Rosmarinus officinalis) honey. *Food Chemistry*, 207-210.

Perna, A., Intaglietta, I., Simonetti, A., & Gambacorta, E. (2014). Metals in Honeys from Different Areas of Southern Italy. *Bulletin of Environmental Contamination and Toxicology*, 253-258.

Pohl, P. (2009). Determination of metal content in honey by atomic absorption and emission spectrometries. *TrAC Trends in Analytical Chemistry*, 117-128.

Primorac, L., Flanjak, I., Kenjerić, D., Bubalo, D., & Topolnjak, Z. (2011). Specific rotation and carbohydrate profile of Croatian unifloral honeys. *Czech Journal of Food Sciences*, 515-519.

Quirantes-Piné, R., Sanna, G., Mara, A., Borrás-Linares, I., Mainente, F., Picó, Y., Zoccatelli, G., Lozano-Sánchez, J., & Ciulu, M. (2024). Mass Spectrometry Characterization of Honeydew Honey: A Critical Review. *Foods*, *13*(14), 2229.

Rao, P. V., Krishnan, K. T., Salleh, N., & Gan, S. H. (2016). Biological and therapeutic effects of honey produced by honey bees and stingless bees: a comparative review. *Revista Brasileira de Farmacognosia*, 657-664.

Rodríguez Flores, M., Escuredo Pérez, O., & Seijo Coello, M. (2014). Characterization of eucalyptus globulus honeys produced in the eurosiberian area of the Iberian Peninsula. *International Journal of Food Properties*, 2177–2191.

Rodríguez-Flore, M. S., Escuredo, O., Míguez, M., & Seijo, M. C. (2019b). Differentiation of oak honeydew and chestnut honeys from the same geographical origin using chemometric methods. *Food Chemistry*, *297*, 124979.

Rodríguez-Flores, M., Escuredo, O., Seijo-Rodríguez, A., & Seijo, M. C. (2019a). Characterization of the honey produced in heather communities (NW Spain). *Journal of Apicultural Research*, *58*(1), 84–91.

Rodríguez-Flores, S., Escuredo, O., & Seijo, M. C. (2016). Characterization and antioxidant capacity of sweet chestnut honey produced in North-West Spain. *Journal of Apicultural Science*, 19-30.

Rossano, R., Larocca, M., Polito, T., Perna, A., Padula, M., Martelli, G., & Riccio, P. (2012). What Are the Proteolytic Enzymes of Honey and What They Do Tell Us? A Fingerprint Analysis by 2-D Zymography of Unifloral Honeys. *PLoS ONE*, e49164.

Sahin, H., Kolayli, S., & Beykaya, M. (2020). Investigation of Variations of Invertase and Glucose Oxidase Degrees against Heating and Timing Options in Raw Honeys. *Journal of Chemistry*, *2020*, 5398062,.

Sánchez, M., Huidobro, J., Mato, I., Muniategui, S., & Sancho, M. (2001). Evolution of Invertase Activity in Honey over Two Years. *Journal of Agricultural and Food Chemistry*, 416-422.

Šarić, G., Matković, D., Hruškar, M., & Vahčić, N. (2008). Characterisation and Classification of Croatian Honey by Physicochemical Parameters. *Food Technology and Biotechnology*, 355-367.

Sawale, P. D., Shendurse, A. M., Mohan, M. S., & Patil, G. R. (2017). Isomaltulose (Palatinose) – An emerging carbohydrate. *Food Bioscience*, 46-52.

Schievano, E., Dettori, A., Piana, L., & Tessari, M. (2021). Floral origin modulates the content of a lipid marker in Apis mellifera honey. *Food Chemistry*, *361*, 130050.

Schievano, E., Piana, L., & Tessari, M. (2023). Automatic NMR-based protocol for assessment of honey authenticity. *Food Chemistry*, *420*, 136094.

Schievano, E., Sbrizza, M., Zuccato, V., Piana, L., & Tessari, M. (2020). NMR carbohydrate profile in tracing acacia honey authenticity. *Food Chemistry*, *309*, 125788.

Seeburger, V. C., D'Alvise, P., Shaaban, B., Schweikert, K., Lohaus, G., Schroeder, A., & Hasselmann, M. (2020). The trisaccharide melezitose impacts honey bees and their intestinal microbiota. *PloS one*, *15*(4), e0230871.

Seeburger, V. C., Shaaban, B., Schweikert, K., Lohaus, G., Schroeder, A., & Hasselmann, M. (2021). Environmental factors affect melezitose production in honeydew from aphids and scale insects of the order Hemiptera. *Journal of Apicultural Research*, 127–137.

Seibel, J., & Buchholz, K. (2010). Tools in Oligosaccharide Synthesis: Current Research and Application. *Advances in Carbohydrate Chemistry and Biochemistry*, 101-138.

Sekine, E. S., Takashiba, E. H., Bueno, R. O., Bueno, P. A., Caxambu, M. G., Sereia, M. J., . . . Toledo, V. A. (2019). Floral Origin and Physical and Chemical Characteristics of Honey from Africanized Bees in Apiaries of Ubiratã and Nova Aurora, State of Paraná. *Sociobiology*, 126-135.

Seow, E.-K., Tan, T.-C., & Mat Easa, A. (2021). Role of honey diastase on textural, thermal, microstructural, chemical, and sensory properties of different dodols. *LWT*, *148*, 111715.

Seraglio, S. K., Silva, B., Bergamo, G., Brugnerotto, P., Gonzaga, L. V., Fett, R., & Costa, A. C. (2019). An overview of physicochemical characteristics and health-promoting properties of honeydew honey. *Food Research International*, 44-66.

Serra Bonvehi, J., Ventura Coll, F., & Orantes Bermejo, J. F. (2019). Characterization of avocado honey (Persea americana Mill.) produced in Southern Spain. *Food Chemistry*, 214-221.

Shaaban, B., Seeburger, V., Schroeder, A., & Lohaus, G. (2020). Sugar, amino acid and inorganic ion profiling of the honeydew from different hemipteran species feeding on Abies alba and Picea abies. *PloS one*, *15*(1), e0228171.

Shahbandeh, M. (2024, May 22). *Major producers of honey worldwide 2022*. Retrieved from Statista: https://www.statista.com/statistics/812172/global-top-producers-of-honey/

Shapla, U., Solayman, M., Alam, N., Khalil, M., & Gan, S. (2018). 5-Hydroxymethylfurfural (HMF) levels in honey and other food products: effects on bees and human health. *Chemistry Central Journal*, *12*, 35.

She, S., Chen, L., Song, H., Lin, G., Li, Y., Zhou, J., & Liu, C. (2019). Discrimination of geographical origins of Chinese acacia honey using T complex 13C/12C, oligosaccharides and polyphenols. *Food Chemistry*, 580–585.

Shen, L. R., Wang, Y. R., Zhai, L., Zhou, W. X., Tan, L. L., Li, M. L., Liu, D. D., & Xiao, F. (2015). Determination of royal jelly freshness by ELISA with a highly specific anti-apalbumin 1, major royal jelly protein 1 antibody. *Journal of Zhejiang University. Science. B*, *16*(2), 155–166.

Silva, S., Moreira, A., Domingues, M., Evtuguin, D., Coelho, E., & Coimbra, M. (2019). Contribution of non-enzymatic transglycosylation reactions to the honey oligosaccharides origin and diversity. *Pure and Applied Chemistry*, 1231-1242.

Softic, S., Stanhope, K. L., Boucher, J., Divanovic, S., Lanaspa, M. A., Johnson, R. J., & Kahn, C. R. (2020). Fructose and Hepatic Insulin Resistance. *Critical reviews in clinical laboratory sciences*, *57*(5), 308–322.

Song, X.-Y., Yao, Y.-F., & Yang, W.-D. (2012). Pollen Analysis of Natural Honeys from the Central Region of Shanxi, North China. *PloS one*, *7*(11), e49545.

Spoială, A., Ilie, C.-I., Ficai, D., Ficai, A., & Andronescu, E. (2023). Synergic Effect of Honey with Other Natural Agents in Developing Efficient Wound Dressings. *Antioxidants*, *12*(1), 34.

Stanimirova, I., Üstün, B., Cajka, T., Riddelova, K., Hajslova, J., Buydens, L. M., & Walczak, B. (2010). Tracing the geographical origin of honeys based on volatile compounds profiles assessment using pattern recognition techniques. *Food Chemistry*, 171-176.

Starowicz, M., Ostaszyk, A., & Zieliński, H. (2021). The Relationship between the Browning Index, Total Phenolics, Color, and Antioxidant Activity of Polish-Originated Honey Samples. *Foods*, *10*(5), 967.

Sun, J., Zhao, H., Wu, F., Zhu, M., Zhang, Y., Cheng, N., Xue, X., Wu, L., & Cao, W. (2021). Molecular Mechanism of Mature Honey Formation by GC-MS- and LC-MS-Based Metabolomics. *Journal of agricultural and food chemistry*, *69*(11), 3362–3370.

Taha, E.-K., Al-Kahtani, S., & Taha, R. (2020). Comparison of the physicochemical characteristics of sidr (Ziziphus spp.) honey produced by Apis florea F. and Apis mellifera L. *Journal of Apicultural Research*, *60*(3), 470-477.

Tanaka, S., Shinoki, A., & Hara, H. (2016). Melibiose, a Nondigestible Disaccharide, Promotes Absorption of Quercetin Glycosides in Rat Small Intestine. *Journal of agricultural and food chemistry*, 9335–9341.

Tang, X., An, Y., Iqbal, M. W., Cong, H., Zhang, G., Zhang, Y., Ravikumar, Y., Zabed, H. M., Zhao, M., Zhou, H., & Qi, X. (2022). The Characterization of a Novel D-allulose 3-Epimerase from Blautia produca and Its Application in D-allulose Production. *Foods*, *11*(20), 3225.

Terrab, A., González, A. G., Díez, M. J., & Heredia, F. J. (2003). Characterisation of Moroccan unifloral honeys using multivariate analysis. *European Food Research and Technology*, *218*, 88–95.

Thrasyvoulou, A., Tananaki, C., Goras, G., Karazafiris, E., Dimou, M., Liolios, V., ... Gounari, S. (2018). Legislation of honey criteria and standards. *Journal of Apicultural Research*, 88–96.

Tomczyk, M., Bocian, A., Sidor, E., Miłek, M., Zaguła, G., & Dżugan, M. (2022). The Use of HPTLC and SDS-PAGE Methods for Coniferous Honeydew Honey Fingerprinting Compiled with Mineral Content and Antioxidant Activity. *Molecules*, 720.

Tsagkaris, A. S., Koulis, G. A., Danezis, G. P., Martakos, I., Dasenaki, M., Georgiou, C. A., & Thomaidis, N. S. (2021). Honey authenticity: analytical techniques, state of the art and challenges. *RSC Advances*, 11273–11294.

Tseng, W.-C., Chen, C.-N., Hsu, C.-T., Lee, H.-C., Fang, H.-Y., Wang, M.-J., ... Fang, T.-Y. (2018). Characterization of a recombinant d-allulose 3-epimerase from Agrobacterium sp. ATCC 31749 and identification of an important interfacial residue. *International journal of biological macromolecules*, 767-774.

Tucak, Z., Periškić, M., Škrivank, M., & Konjarević, A. (2007). The Influence of the Botanic Origin of Honey Plants on the Quality of Honey. *Poljoprivreda*, 234-236.

Tuzen, M., Silici, S., Mendil, D., & Soylak, M. (2007). Trace element levels in honeys from different regions of Turkey. *Food Chemistry*, 325-330.

Uršulin-Trstenjak, N., Hrga, I., Stjepanović, B., Dragojlović, D., & Levanić, D. (2013). Determination of botanic origin of the Croatian black locust honey (Istria region) using melissopalynological analysis. *Journal of Hygienic Engineering and Design*, 122-126.

Uršulin-Trstenjak, N., Puntarić, D., Levanić, D., Gvozdić, V., Željka Pavlek, Puntarić, A., ... Vidosavljević, M. (2017). Pollen, Physicochemical, and Mineral Analysis of Croatian Acacia Honey Samples: Applicability for Identification of Botanical and Geographical Origin. *Journal of Food Quality*, 2017, 8538693.

Vergès, A., Cambon, E., Barbe, S., Moulis, C., Remaud-Siméon, M., & André, I. (2016). Novel product specificity toward erlose and panose exhibited by multisite engineered mutants of amylosucrase. *Protein Science*, *26*(3), 566–577.

Vit, P., Pedro, S. R., & Roubik, D. (2013). *Pot-Honey : a legacy of stingless bees.* New York: Springer.

Vit, P., Rodríguez-Malaver, A., Rondón, C., González, I., Bernardo, M., & García, M. (2010). Bioactive indicators related to bioelements of eight unifloral honeys. *Archivos Latinoamericanos de Nutrición*, 405-410.

Vorlová, L., & Čelechovská, O. (2002). Activity of Enzymes and Trace Element Content in Bee Honey. *Acta Veterinaria Brno*, 375-378.

Wang, Y., Ravikumar, Y., Zhang, G., Yun, J., Zhang, Y., Parvez, A., . . . Sun, W. (2020). Biocatalytic Synthesis of d-Allulose Using Novel d-Tagatose 3-Epimerase From Christensenella minuta. *Frontiers in Chemistry*, *8*, 622325.

Waś, E., Rybak-Chmielewska, H., Szczęsna, T., Kachaniuk, K., & Teper, D. (2011). Characteristics of Polish unifloral honeys. III. Heather honey (Calluna vulgaris L.). *Journal of Apicultural Science*, 129-137.

Wen, Y., Wang, L., Jin, Y., Zhang, J., Su, L., Zhang, X., Zhou, J., & Li, Y. (2017). The microbial community dynamics during the vitex honey ripening process in the honeycomb. *Frontiers in Microbiology*, *8*, 1649.

White Jr, J. W., & Maher, J. (1953). Transglucosidation by honey invertase. *Archives of Biochemistry and Biophysics*, 360-367.

WHO. (2015, March 4). *WHO calls on countries to reduce sugars intake among adults and children*. Retrieved from World Health Organization: https://www.who.int/news/item/04-03-2015-who-calls-on-countries-to-reduce-sugars-intake-among-adults-and-children

Wilczyńska, A., & Żak, N. (2024). Polyphenols as the Main Compounds Influencing the Antioxidant Effect of Honey—A Review. *International journal of molecular sciences, 25(19), 10606*.

Witek, K., Wydra, K., & Filip, M. (2022). A High-Sugar Diet Consumption, Metabolism and Health Impacts with a Focus on the Development of Substance Use Disorder: A Narrative Review. *Nutrients*, *14*(14), 2940.

Wu, H., Chen, M., Guang, C., Zhang, W., & Mu, W. (2020). Characterization of a recombinant D-mannose-producing D-lyxose isomerase from Caldanaerobius polysaccharolyticus. *Download full issue*, 138, 109553.

Xia, Y., Cheng, Q., Mu, W., Hu, X., Sun, Z., Qiu, Y., Liu, X., & Wang, Z. (2021). Research Advances of D-allulose: An Overview of Physiological Functions, Enzymatic Biotransformation Technologies, and Production Processes. *Foods*, *10*(9), 2186.

Yan, S., Wang, W., Zhao, W., Tian, W., Wang, X., Wu, L., & Xue, X. (2023). Identification of the maturity of acacia honey by an endogenous oligosaccharide: A preliminary study. *Food chemistry*, *399*, 134005.

Yang, J., Fan, D., Zhao, F., Lin, Y., Zheng, S., & Han, S. (2022). Characterization of D-Allulose-3-Epimerase From Ruminiclostridium papyrosolvens and Immobilization Within Metal-Organic Frameworks. *Frontiers in Bioengineering and Biotechnology*, *10*, 869536.

Yang, W., Zhang, C., Li, C., Huang, Z. Y., & Miao, X. (2019). Pathway of 5-hydroxymethyl-2-furaldehyde formation in honey. *Journal of food science and technology*, 2417–2425.

Yang, Y., Battesti, M.-J., Djabou, N., Muselli, A., Paolini, J., Tomi, P., & Costa, J. (2012). Melissopalynological origin determination and volatile composition analysis of Corsican ‘‘chestnut grove’’ honeys. *Food Chemistry*, 2144–2154.

Yin, K.-J., Xie, D.-Y., Zhao, L., Fan, G., Ren, J.-N., Zhang, L.-L., & Pan, S.-Y. (2020). Effects of different sweeteners on behavior and neurotransmitters release in mice. *Journal of food science and technology*, 113–121.

Yoshihara, A., Kozakai, T., Shintani, T., Matsutani, R., Ohtani, K., Iida, T., . . . Gullapalli, P. K. (2017). Purification and characterization of d-allulose 3-epimerase derived from Arthrobacter globiformis M30, a GRAS microorganism. *Journal of Bioscience and Bioengineering*, 170-176.

Yuan, Y., Yan, S., Wu, L., Xu, H., Mu, G., & Xue, X. (2024). Exploring formation of turanose in honey via stable isotope labelling and high-resolution mass spectrometry analysis. *Food Chemistry*, 460(Pt 1), 140471.

Zerrouk, S., & Bahloul, R. (2020). Palynological and physicochemical properties of multifloral honey produced in some regions of Algeria. *Journal of Apicultural Research*, *62*(2), 345–354.

Zhang, G.-Z., Tian, J., Zhang, Y.-Z., Li, S.-S., Zheng, H.-Q., & Hu, F.-L. (2021). Investigation of the Maturity Evaluation Indicator of Honey in Natural Ripening Process: The Case of Rape Honey. *Foods*, *10*(11), 2882

Zhang, L., Jiang, B., Mu, W., & Zhang, T. (2009). Bioproduction of D-psicose using permeabilized cells of newly isolated Rhodobacter sphaeroides SK011. *Frontiers of Chemical Engineering in China*, 393-398.

Zhang, W., Fang, D., Zhang, T., Zhou, L., Jiang, B., & Mu, W. (2013). Characterization of a Metal-Dependent d-Psicose 3-Epimerase from a Novel Strain, Desmospora sp. 8437. *Journal of Agricultural and Food Chemistry*, *61*(47), 11468–11476.

Zhang, W., Li, H., Zhang, T., Jiang, B., Zhou, L., & Mu, W. (2015). Characterization of a d-psicose 3-epimerase from Dorea sp. CAG317 with an acidic pH optimum and a high specific activity. *Journal of Molecular Catalysis B: Enzymatic*, 68-74.

Zhang, W., Zhang, T., Jiang, B., & Mu, W. (2015). Biochemical characterization of a d-psicose 3-epimerase from Treponema primitia ZAS-1 and its application on enzymatic production of d-psicose. *Journal of the Science of Food and Agriculture*, *96*(1), 49–56

Zhu, Y., Men, Y., Bai, W., Li, X., Zhang, L., Sun, Y., & Ma, Y. (2012). Overexpression of d-psicose 3-epimerase from Ruminococcus sp. in Escherichia coli and its potential application in d-psicose production. *Biotechnology Letters* , 1901–1906.

Zhu, Z., Gao, D., Li, C., Chen, Y., Zhu, M., Liu, X., Tanokura, M., Qin, H. M., & Lu, F. (2019). Redesign of a novel D-allulose 3-epimerase from Staphylococcus aureus for thermostability and efficient biocatalytic production of D-allulose. *Microbial cell factories*, *18*(1), 59.

Zulkifli, M. F., Radzi, M. N., Saludes, J. P., Dalisay, D. S., & Ismail, W. I. (2022). Potential of Natural Honey in Controlling Obesity and its Related Complications. *Journal of evidence-based integrative medicine*, 27:2515690X221103304.

Zulkifli, M. F., Sivakumar, M., Maulidiani, M., & Ismail, W. I. (2023). Bibliometric approach to trehalulose research trends for its potential health benefits. *Food Bioscience*, *53*, 102677.

CHAPTER III. COMPOSITIONAL PROFILING FOR THE QUALITY ASSESSMENT OF CANADIAN HONEYS

Connecting Statement I

In chapter II, a thorough literature review was conducted on the current knowledge of enzymatic activities and carbohydrate profiling for monofloral honeys worldwide. Chapter III performed the analysis for five enzymatic activities and sugar profiling on monofloral honeys, notably four of key honey types relevant to the Canadian market (buckwheat, clover, goldenrod, and blueberry).

The results from this study were presented at the BÉNÉFIQ2023 conference, held in Québec City, Canada, and at the 11th International Symposium on Recent Advances in Food Analysis, RAFA 2024, held in Prague, Czech Republic.

3.1 Abstract

The value of honey, a natural sweetener with long human consumption history, has gained renewed attention in the last decades. This has driven a growing consumer demand for high quality, authentic honeys, particularly monofloral ones. In the present study, the compositional profiles of 163 honey samples from four botanical origins relevant to the Canadian market (e.g. buckwheat, clover, goldenrod, and blueberry) were characterized in terms of enzymatic activity and sugar profiling using spectrophotometry and LC/MC-QToF. The results showed that clover and goldenrod honeys exhibited higher diastase and invertase activities, with average values of 11.90 DN/4.24 IN and 12.52 DN/5.33 IN, respectively. The highest average acid phosphatase activity was detected in buckwheat honey activity (683.202 mg P/100g), while blueberry honey exhibited the highest average glucose oxidase activity (11.843 µg H_2O_2/g/ h) and average catalase activity (27.629 µg H_2O_2/g/ min), respectively. The concentration of fructose and glucose ranged from 37.0% to 38.1% (w/w), and from 32.0% to 34.1% (w/w), respectively, across the four floral types. Maltose was identified as the most abundant disaccharide, with an average concentration ranging from 0.94% to 1.12% (w/w), followed by isomaltose at 0.593% to 0.657% (w/w). Sucrose, trehalose, gentiobiose, nigerose, and erlose were also successfully detected and quantified in most of the samples. A multi-targeted analysis of potential predictor variables (e.g. enzyme activities, sugar compounds) was conducted with the goal to identify the markers for discriminating the botanical origins. Negative correlations were observed between diastase activity and HMF peak area as well as between glucose and F/G ratio, nigerose, isomaltose, gentiobiose, or tetrasaccharide content. Mathematical models were trained and used for botanical origin prediction with the average accuracy detected between 80% - 90%. Acid phosphatase and catalase were identified as the most impactful features to differentiate clover, blueberry, and buckwheat honey across various models.

3.2 Introduction

Beekeeping not only aids in pollination for various crops but also produces honey and other hive products. Recovering from the overwinter colony losses in 2022, the honey production in Canada during the 2023 season has reached a new height of 91.8 million pounds with a total value of 277.22 million dollars (AAFC, 2024; Shahbandeh, 2024). With a long history of human consumption, honey continues to be a preferred natural sweetener among consumers. Honeys are categorized into honeydew honey and nectar honey, also referred to as floral honey (FAO, 2022). While honeydew honey is produced from either the secretion of living plants or the excretion of plant-sucking insects on the living parts of the plant, the latter comes from the nectar of blossoms (Pita-Calvo & Vázquez, 2017). From a market perspective, nectar honey can be categorized into two types: monofloral and multifloral honey. Monofloral honey is characterized by a predominant nectar source, generally comprising more than 45% of the total pollen count from a single floral source. In contrast, multifloral honey is composed of nectar from multiple floral sources, with none of these sources contributing more than 45% to the total pollen count (Nedić, et al., 2022). Most commercially available honey is produced from a blend of botanical sources without a dominant type In the recent decades, more attentions were drawn towards monofloral honeys attributing to their unique and favorable sensory properties. The growing awareness of the nutraceutical benefits linked to certain monofloral honeys, like Manuka honey, has further increased their popularity and economic value (Seraglio, et al., 2021; Edo, et al., 2023; Wang, Qiu, & Zhu, 2024).

Honey is widely identified as one of the food commodities that are most prone to fraud on a global scale. This has led to high interest in developing and implementing novel authentication methods to identify the botanical origin of honeys (Jandric, et al., 2015; Moore, Spink, & Lipp, 2012; CFIA, 2024). The traditional method utilized by the industry for honey authentication is pollen analysis; however, its efficiency is greatly affected by the analyst's experience and is susceptible to issues such as pollen addition or improper honey filtration (Jaafar, et al., 2020). Moreover, a high variability in the pollen content in honey was reported due to the differences in plants' pollen-producing capacity and geographic origins, leading to diverse regulatory requirements across the different countries (Bodor, Kovacs, Benedek, Hitka, & Behling, 2021). Therefore, there is an urgent need for rapid and accurate routine authentication methods. In this field, chromatographic

and spectroscopic techniques combined with chemometric tools can provide promising ground application (Bose & Padmavati, 2024; Brar, et al., 2023).

Enzymes play a crucial role in the ripening of honey. For example, diastase and invertase, excreted by workers bees, are accumulated during the transportation of nectars and catalyse the hydrolysis of amylose and sucrose into glucose, maltose and fructose. In addition, invertase facilitates the formation of oligosaccharides via its transglycosylation activity (Schievano, Sbrizza, Zuccato, Piana, & Tessari, 2020; Silva, et al., 2019; Alaerjani, et al., 2022). Both diastase and 5-hydroxy-methylfurfural (HMF) content are indicators of freshness and temperature discrimination during processing and storage. Another important enzyme, glucose oxidase (GOX), secreted from the hypopharyngeal glands of honeybees, catalyses the conversion of glucose into gluconic acid and hydrogen peroxides, which are associated with the antimicrobial properties of honey (Bouzo, et al., 2020; Sahin, Kolayli, & Beykaya, 2020). GOX deactivates with increased glucose concentration, long-term storage and exposure to light (Alshareef, Al-Farhan, & Mohammed, 2022). Conversely, catalase counteracts with the oxidation reactions of GOX by reducing hydrogen peroxide into oxygen and water, and its level in honey varies with the botanical origin (Brudzynski, 2020). Similarly, acid phosphatase, another plant-derived enzyme sourced from pollen and nectar, can serve as a potential indicator of the floral origin of honeys (Miłek, Bocian, Kleczyńska, Sowa, & Dżugan, 2021).

Sugars are the main components of honey, comprising up to 70-85% of its composition. The sugar profile of honey is dominated by monosaccharides (about 75-95%), but also contains 10-15% of disaccharides, and about 2% of trisaccharides along with oligosaccharides/oligomers (Crăciun, Pârvulescu, Donise, Dobre, & Stanciu, 2020). Honey also contains bioactive microcomponents such as protein, free amino acids, mineral, vitamins, volatile compounds, and phenolic compounds, which contribute to its sensory properties and nutritional and health-promoting benefits. Many studies were focused on the profiling of phenolic compounds for honey authentication (Gašić, Milojković-Opsenica, & Tešić, 2017; Ntakoulas, et al., 2024). Carbohydrate profiling was also reported as an effective tool for identifying fraudulent in honey, such as added syrup (Megherbi, Herbreteau, Faure, & Salvador, 2009; Qu, et al., 2019; Nyarko, Mensah, & Greenlief, 2024). However, only few studies have been conducted to investigate correlations between the complex carbohydrate profiles and the botanical origins of honey through the application of multivariate

analysis in European countries (de la Fuente, Ruiz-Matute, Valencia-Barrera, Sanz, & Martínez Castro, 2011). There is a need to investigate North America honeys to gain a comprehensive understanding of the relationships between the botanical origin of honeys and the sugar and enzyme profiles and. In the present study, the profiling of carbohydrates and enzymatic activities of 163 Canadian honeys were investigated. The correlation between the two components was analyzed, and a model aiming to differentiate botanical origins was established.

3.3 Materials and Methods

3.3.1 Honey samples

Honey samples were collected from local markets and retail stores in Montréal region (Canada) and on-line stores between 2021 and 2022 and were stored in amber vials at -20 °C before further processing for the analysis. A total of 163 honey samples were selected for the study based on their floral type, as indicated on the self-reported product labels. The selection included four key types of honey relevant to the Canadian market: 59 of buckwheat (*Fagopyrum spp.*) honey, 52 of clover (*Trifolium*) honey, 11 of goldenrod (*Solidago*) honey, and 41 of blueberry honey. These samples were primarily sourced from British Columbia, Ontario, and Quebec.

3.3.2 Materials

Deionized water used were from a Millipore Milli-Q Academic water purification system. Acetonitrile, hydrogen peroxide, methanol, LC/MS graded water, o-dianisidine, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl phosphate, potassium iodide, sodium chloride, sodium citrate, tris (hydroxymethyl) aminomethane, cellobiose, gentiobiose, isomaltose, isomaltotriose, maltose, maltotriose, melezitose, palatinose hydrate, panose, raffinose, sucrose, trehalose, and turanose were purchased from Fisher Scientific (Ontario, Canada). Ammonium acetate was purchased from Honeywell Research Chemical (Morris Plains, US). Ammonia, α-glucosidase, amylase, citric acid, glucose oxidase, glacial acetic acid hydrochloric acid, iodine, myo-inositol, peroxidase from horseradish, phosphatase, p-nitrophenol, sodium acetate, sodium hydroxide, sodium phosphate dibasic dihydrate, sodium phosphate monobasic, soluble starch, fructose, glucose, erlose, kojibiose, and nigerose were purchased from Millipore Sigma (Oakville, Canada).

3.3.3 Enzymatic activity profiling of selected Canadian Honeys

The activity of five enzymes (diastase, invertase, glucose oxidase, catalase, and acid phosphatase) were determined in 163 Canadian honey samples. Enzyme (honey sample) blanks and substrate blanks were incubated simultaneously with all enzymatic reaction triplicates to eliminate the matrix effect and serve as procedural blanks.

3.3.3.1 Diastase activity assay

The diastase activity was determined by measuring the consumption rate of starch in a honey/starch mixture incubated at 40°C. The method described by the International Honey Commission (IHC) was used (Bogdanov, 2009). A standard curve was constructed using starch solution (60-400 mg/L) and iodine assay which forms a complex between the amylose helix and iodine atoms, with a maximum absorbance between 600-680 nm (Brust, Orzechowski, & Fettke, 2020; Pesek, Lehene, Brânzanic, & Silaghi-Dumitrescu, 2022). To perform the iodine assay, a diluted iodine solution was prepared by mixing 4 g of potassium iodide and 0.4 mL of an iodine stock solution (2.2% w/v sublimated iodine and 4.4% w/v potassium iodide) in 100 mL deionized water. The 20% (w/v) honey reaction solution was prepared by mixing 0.2 g honey with 100 µL acetate buffer (1.3 M, pH 5.3, pH adjusted with glacial acetic acid), 60 µL of 2.9% (w/v) sodium chloride solution and deionized water was used to make up the final volume to 1 mL. The reaction mixture consisted of 1 mL of honey reaction solution and 1 ml of 2% (w/v) soluble starch solution and was incubated in an amber tube at 40°C for 1 hr. Every 20 min, 25 µL of the aliquot was sampled and mixed with 1 mL of deionized water and 250 µL of diluted iodine solution. The absorbance was then recorded at 660 nm (DU 800 UV/Visible Spectrophotometer, Beckman). The diastase activity was expressed in diastase number (DN), which represents the quantity of starch (in 10 mg) hydrolyzed by the diastase in 1 gram of honey in one hr at 40ºC (Rodríguez-Flores, Escuredo, & Seijo, 2016).

3.3.3.2 Invertase activity assay

The invertase activity was determined using the official IHC method validated by substituting the honey portion with commercial α-glycosidase (Bogdanov, 2009). This assay is based on the quantification of p-nitrophenol (p-NP) released upon the hydrolysis of p-nitrophenyl-α-D-glucopyranoside (pNPG) substrate by invertase. A standard curve of p-NP (0-100 µM) was established at 400 nm absorbance under basic conditions. Honey samples were dissolved in 0.1 M

phosphate buffer (pH 6) to yield a final concentration of 20% (w/v). 0.1 mL of the honey solution was added to 1 mL of 20 mM pNPG substrate solution. For each honey sample, reaction mixtures were prepared in triplicates and placed in a 40 °C shaking incubator. Starting from time 0 until one hr, one triplicate was taken from the incubator every 15 min, and 0.1 mL of Tris-HCl buffer (3 M, pH 9.5) was added. Adjusting the pH to 9.5 allowed the termination of the honey invertase (α-glycosidase) reaction and enabled the transformation of the p-NP into nitrophenolate anion which had an optimum absorption at 400 nm. The common expression of invertase activity is in invertase number (IN), which indicates the amount sucrose (in 10 mg) hydrolyzed by 1 g of honey per hr. IN was calculated by dividing the invertase activity in international unit (µmol of pNPG/min/kg of honey) with a conversion factor of 7.34 (Julika W. N., et al., 2020).

3.3.3.3 Acid phosphatase activity assay

The acid phosphatase activity in honey was measured using a method described by Miłek (2021). For the method validation, commercial acid phosphatase enzyme was used as a positive control. The substrate solution consisted of 5 mM p-nitrophenyl phosphate (p-NPP) in citrate buffer (0.1 M, pH 4.8). To initiate the enzymatic assay, 1 mL of the substrate solution was mixed with 1 mL of a 20% (w/v) honey solution (citrate buffer, 0.1 M, pH 4.8), and the mixture was incubated at 37 °C for 80 min. Every 20 min, 200 µL aliquots were collected from the reaction mixture and added to 1 mL of 0.25 M NaOH to terminate the reaction and enable the formation of nitrophenolate under basic condition. Absorbance measurements were recorded at 400 nm. The enzymatic activity was estimated from the released p-NP and expressed in mg P/ 100g honey/ 24 hrs.

3.3.3.4 Glucose oxidase activity assay

The determination of glucose oxidase activity in honey was conducted using the modified method described by Flanjak (2016). The glucose oxidase activity was quantified by measuring the generated hydrogen peroxide from the oxidation of glucose to gluconic acid. This was achieved through a peroxidase-o-dianisidine reaction system, in which the oxygen released from hydrogen peroxide reduction oxidizes o-dianisidine, and forms a chromophore with a maximum absorption at 420 nm. The reaction mixture contained 600 µL phosphate buffer (0.1 M, pH 6.1), 100 µL o-dianisidine solution (0.1% w/v, 1:49 methanol/phosphate buffer), 100 µL peroxidase solution (500 U/ml, 0.1 M pH 6.1 phosphate buffer), 200 µL honey solution (20% w/v, 0.1 M pH 6.1 phosphate

buffer). The mixture was incubated in an amber vial at 37 °C for 2 hrs. To terminate the reaction, 100 µL of 1 M HCl was added. The absorbance was measured at 420 nm against a blank reaction mixture in which the honey was substituted with 200 µL phosphate buffer (0.1 M, pH 6.1). The baseline (time zero) was established by adding the honey sample after the hydrochloric acid. A hydrogen peroxide calibration curve (0-200 µM) was constructed using peroxidase and o-dianisidine. Glucose oxidase activity was expressed as µg of released hydrogen peroxide per gram of honey per hr.

3.3.3.5 Catalase activity assay

The catalase activity in honey was determined using a modified method adapted from Huidobro (2005) in which the consumption rate of hydrogen peroxide was measured using a peroxidase-o-dianisidine reaction system. The reaction mixture comprised of 15% honey (w/v) and 3 mM hydrogen peroxide in 60 mM, pH 7 phosphate buffer. The reaction mixtures in amber containers were incubated at 37 °C for 20 min. To determine the quantity of the remaining hydrogen peroxide, 50 µL of the reaction mixture was mixed with 750 µL phosphate buffer (0.1 M, pH 6.1), 100 µL o-dianisidine solution (0.1% w/v, 2:98 methanol/phosphate buffer), and 100 µL peroxidase solution (500 U/mL, 0.1 M pH 6.1 phosphate buffer) at 37 °C for 5 min. The reaction was terminated by the addition of 100 µL of 1 M hydrochloric acid. Absorbance was measured at 420 nm (DU 800 UV/Visible Spectrophotometer, Beckman). Catalase activity was expressed as µg of consumed H_2O_2/ gram of honey per min.

3.3.4 Carbohydrate profiling

3.3.4.1 Determination of monosaccharides

The quantification of the fructose and glucose in honey was carried out using high performance anion exchange chromatography (HPAEC) on a Dionex ICS-3000 system. To separate and detect the monosaccharides, the CarboPac PA20 column (3 × 150 nm) and a pulsed amperometric detector (PAD) were used with an isocratic mobile phase made of 10 mM sodium hydroxide at a flow rate of 0.25 mL/min. Honey samples were diluted to 20 mg/L using deionized water and filtered through 0.22 µm syringe filters, and 250 µL sample were injected using an autosampler. Sugar concentrations were estimated using the calibration curve of the standard fructose and glucose solution (1-10 mg/L), which covers the theoretical fructose and glucose concentration in

20 mg/L honey solution. Chromatograms were processed and integrated using the Chromeleon™ Chromatography Management System.

3.3.4.2 Determination of di-, trisaccharides

Disaccharides and trisaccharides were profiled in honey using liquid chromatography (LC) on an Agilent 1290 Infinity II LC system coupled to the 6560 ion mobility quadrupole time of flight-mass spectrometry (Q-TOF-MS) (Agilent Technologies, Santa Clara, USA). The analytes were separated on an Hypercarb™ Porous Graphitic Carbon HPLC Column (1 × 150 mm, 5 µm). The mobile phases consisted of LC/MS grade water with 0.25% ammonia and 5 mM ammonium acetate (Phase A) and acetonitrile with the same concentrations of ammonia and ammonium acetate (Phase B). The flow rate was set at 0.12 mL/min, the column temperature maintained at 60°C, and the injection volume was 0.5 µL. The gradient profile of mobile phase started with 2% B for the first 0.5 min, increased to 10% B over 14.5 min, held at 10% B for 3 min, then ramped up to 99% B over 8 min and held it for 3 min, before dropping back to 2% B over 3 min and held at 2% B for the final 3 min for stabilisation. The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. The MS conditions were set as follows: the drying gas temperature at 150 °C, drying gas flow at 11 L/ min, sheath gas temperature at 200 °C, sheath gas flow at 11 L/ min, pressure on the nebulizer at 35 psi, capillary voltage at 4000 V, nozzle voltage at 500 V, and the fragmentor voltage at 175 V. Full scan MS data were recorded between mass-to-charge ratios (*m/z*) 50-1700 with a scan rate of 0.63 spectra/s, and were collected at both centroid and profile mode. Data treatment was conducted using Quantitative Analysis 10.0 from Agilent MassHunter Workstation Software. Sugar standards (disaccharide: sucrose, maltose, cellobiose, gentiobiose, isomaltose, melibiose, kojibiose, turanose, trehalose, palatinose, nigerose; trisaccharide: erlose, maltotriose, isomaltotriose, raffinose, melezitose, panose, kestose) were dissolved in LC/MS graded water and diluted to 60 mg/L for retention time identification purposes. Sugar mixtures of all standard solutions were prepared at 0-100 mg/L to construct the calibration curve, which would cover the anticipated concentration ranges of these sugars in honey. The honey samples were prepared in LC/MS graded water, diluted and filtered to a final concentration of 0.5% (w/v). A final concentration of 5 ppm of myo-inositol was used as the internal standard for all the sugar standards, calibration mixtures, and honey samples.

3.3.5 Characterization of other physicochemical properties

3.3.5.1 Color

In order to assess the color of the honey, liquid honey, with no residues or bubbles, was filled in the sample cup and compared to the Jack's scale honey color grader under natural light.

3.3.5.2 HMF

For the quantification of HMF honey samples were analyzed by LC-MS as described by Tian et al. (2024) using an Agilent 1290 Infinity II LC system coupled to the 6545 ion mobility Q-TOF-MS (Agilent Technologies, Santa Clara, USA). The analytes were separated on an InfinityLab Poroshell 120 EC-C18 Column (3 x 100 mm, 2.7 µm) and an InfinityLab Poroshell 120EC-C18 guard column was used (Agilent Technologies, Santa Clara, USA). The mobile phases consisted of LC/MS grade water containing 0.1% formic acid (Phase A) and methanol containing the same concentrationof formic acid (Phase B). The flow rate was set at 0.4 ml/min, the column temperature maintained at 30°C, and the injection volume was 20 µl. The gradient profile of mobile phase started with 30% B increased to 100% for the first 3 min, held at 100% B for 3 min, before dropping back to 30% B over 2 min with 1 min post-column run. The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. The MS conditions were set as follows: the drying gas temperature at 110 °C, drying gas flow at 12 L/ min, sheath gas temperature at 150 °C, sheath gas flow at 10 L/ min, pressure on the nebulizer at 40 psi, capillary voltage at 4000 V, nozzle voltage at 500 V, and the fragmentor voltage at 150 V. Full scan MS data were recorded between mass-to-charge ratios (*m/z*) 100-1700 . The peak area was obtained using Quantitative Analysis 10.0 from Agilent MassHunter Workstation Software. The honey samples were prepared in 50:50 ACN/water (v/v), diluted and filtered to a final concentration of 10% (w/v), and the first 1.4 min elution was discarded to prevent the entries of high concentration sugars into the mass spectrometer.

3.3.6 Multivariate statistical analyses

A distribution-based cluster analysis was performed using the enzymatic activity profiling data as well as the fructose and glucose content and the fructose/glucose ratio to visually observe sample grouping and differentiation. A heatmap was generated after submitting all quantitative results to a correlation analysis to assess the correlations among the enzymatic and carbohydrate profiles

and other honey properties (HMF, color). A principal component analysis (PCA) was performed with all the quantitative data for the clover, buckwheat and blueberry honey samples to investigate grouping according to floral types, with goldenrod sample removed due to smaller sample size. Data normalization was carried out for the unidentified sugars and HMF content. The python and various open-source libraries, including Pandas, Sklearn, Scipy, Matplotlib, and Seaborn were used for all data analyses and visualizations.

3.3.7 Honey classification and prediction

Using the python library Pandas, various mathematical classification models were investigated for honey botanical origin prediction using the data of enzymatic and carbohydrate profiling. All models were examined for their classification accuracy by generating the confusion matrix based on the actual label (self-claimed botanical origin obtained form the label) and the predicted botanical origins. Due to the limited number of goldenrod samples, only buckwheat (59), blueberry (41), and clover (52) honey samples were subjected to this analysis. This set of models employed 5-fold cross-validation using 55 samples, where the results of their pollen analysis corresponded with their claimed botanical origins. Samples were deemed a match if they contained over 45% of the targeted pollen or had less than 45% of the targeted pollen but exhibited sensory attributes (eg. color and taste) consistent with the targeted monofloral honey. Among the models examined, the one model with the highest accuracy score was chosen for further cross-validation and confusion matrix generation.

3.4 Results and Discussion

3.4.1 Enzymatic activity profiling of selected Canadian Honeys

The enzymatic activity profiles (diastase, invertase, acid phosphatase, glucose oxidase, and catalase) were established for 169 Canadian honeys of varying botanical sources. The experimental findings of the enzyme activities are presented in the supplementary materials (**Fig 3.1**, complete data set in **Supplementary Table 3.1**). All results were obtained in parallel with standards each day to ensure accuracy.

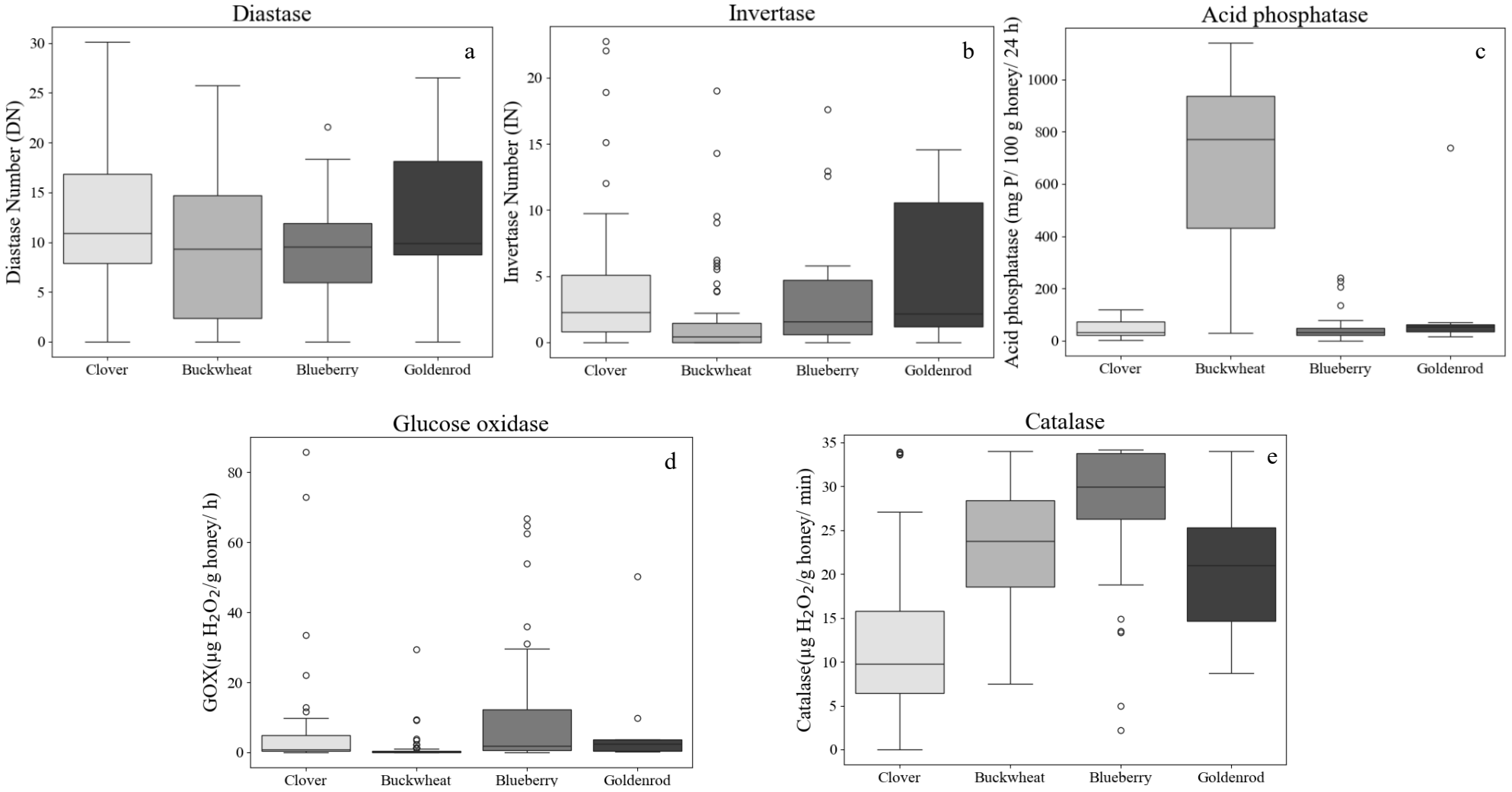


Figure 3.1. Levels of enzymatic activities in monofloral honey samples (clover: 52, buckwheat: 59, blueberry: 41, goldenrod: 11). (a): diastase (DN); (b): invertase (IN); (c) acid phosphatase (mg P/ 100 g honey/ 24 h); (d) glucose oxidase (µg H_2O_2/g honey/ h); (e): catalase (µg H_2O_2/g honey/ min). The boxes display the median and interquartile range (IQR) and whiskers extend up to 1.5 IQR.

The diastase activity in honeys is responsible for breaking down the starches into simple sugars (e.g maltose and glucose) and is a key indicator of honey quality and freshness. Low activities can reflect indirect assessment of excessive heating or prolonged storage or even adulteration through the addition of syrups (Takahashi, Yoshida, Yokozeki, Igarashi, & Fujita, 2023). Large variations in the diastase activity (**Fig 1. (a)**) were observed across four types of monofloral honeys. While the minimum value determined in each floral type is 0 DN, the maximum value differed among the four types. One sample among the clover honeys exhibited the highest maximum value of diastase activity at 30.127 DN, while the highest maximum value for the other floral types was 25.716 DN for buckwheat honey, 21.583 DN for blueberry honey, and 26.572 DN for goldenrod honey. Outliers beyond or below the range of the whiskers were not found in all floral types except for one blueberry sample at 21.58 DN.

Indicated in **Fig 3.1 (a)**, the median value and IQR for the four types of honey are as follows: clover honey has a median of 10.88 DN with an IQR of 7.31-16.7 DN; buckwheat honey has a median of 9.36 DN with an IQR of 2.34-15.11 DN; blueberry honey exhibits a median of 9.52 DN with and IQR of 5.43-12.11 DN; goldenrod honey shows a median of 9.89 DN with an IQR of 8.03-19.23 DN (**Supplementary Table 3.1**). The median values were found to be similar across the four floral types, however, blueberry honey, exhibits a narrower range of diastase activity comparing to the other three. The diastase activity for clover honey in this study was generally higher than that reported by Can et al. (2015), ranging from 6.6-9.2 DN, but aligned with the results obtained by Bell and Grainger (2023) and Ghoniemy et al. (2022), with the average activity ranging between 10.16-11.80 DN. The buckwheat honey exhibited a lower diastase activity compared to the values reported in previous studies for Polish and Italian buckwheat honeys, ranging from 21.1 to 66.43 DN; however, it aligns with the values (8.1-17.7 DN) reported for a blend of imported and EU buckwheat honey (Kowalski & Lukasiewicz, 2017; Miłek, Bocian, Kleczyńska, Sowa, & Dżugan, 2021; Tomczyk, et al., 2022; Pasini, Gardini, Marcazzan, & Caboni, 2013). The average diastase activity in goldenrod honeys from this study is also lower than the values reported for fresh goldenrod honeys in 2017 from Podkarpackie (Poland), which had an average of 33.08± 3.34 DN, but aligns with the values reported in 2022 from the same region, ranging from 9.8-26.38 DN (Wesołowska & Dżugan, 2017; Dżugan, et al., 2022). These differences revealed the variability in the enzymatic activities in honey over different years and underline the complexity of factors that impact honey's enzymatic activity.

The quality of honey, as a key factor tied to its economic value, is regulated for international trade by the CODEX with specific guidelines varying across different countries (Thrasyvoulou, et al., 2018; Mădaş, et al., 2020). According to the regulation by Canadian Food Inspection Agency (CFIA) graded Canadian No.1 honey must have a diastase content higher than 8 if the HMF content is less than or equal to 40 mg/mL, or higher than 3 if the HMF content is no more than 15 mg/mL (CFIA, 2021). Most of the honey samples in this study comply with Canadian regulations, with 14 clover, 25 buckwheat, 15 blueberry, and 2 goldenrod honey samples showing diastase activities below 8. Of these, 15 were not claimed to be Canadian No. 1 graded, and 5 were labeled as Ontario No. 1, thus not required to meet the diastase activity standards set for Canada No 1. grade honey (Government of Ontario, 2021). The lower diastase activity in some samples can be attributed to the storage conditions before purchasing them, as several studies have shown that diastase in honey is sensitive to heat and light (Can et al., 2015; Ghoniemy et al., 2022; Pashayanm, 2024).

Similar to diastase, the level of **invertase activity** can be an indicator for assessing the freshness, purity, processing history and compliance with quality standards. The results of invertase activity are presented in **Fig 3.1 (b)**. Outliers were detected in all floral types except for goldenrod honeys. The median value and IQR for the four types of honey are as follows: clover honey has a median of 2.21 IN with an IQR of 0.83-4.92 IN; buckwheat honey has a median of 0.42 IN with an IQR of 0-1.48 IN; blueberry honey exhibits a median of 1.58 IN with and IQR of 0.6-4.74 IN; goldenrod honey shows a median of 2.15 IN with an IQR of 1.04-11.88 IN. Goldenrod honey had the lowest maximum value of 14.58 IN, followed by blueberry honey with a maximum of 17.59 IN. Clover honey had the highest maximum of 22.76 IN (**Supplementary Table 3.1**). Despite a high maximum of 19.00 IN, 20 out of 59 buckwheat honey samples exhibited the invertase activity below detection limit of the method in use. Upon the unit conversion to IU via multiplying the IN value by the conversion factor 7.34, the majority of the invertase activities for buckwheat honeys in this study are significantly lower than that from the study conducted by Kowalski and Lukasiewicz (2017), which were ranged between 39.1-82.8 IU. The wide range of differences, low median value and presence of multiple outliers in invertase activities within each floral type can be related to the weaker stability of the enzyme when exposed to heat. In comparison to diastase, as a study suggested, invertase activity decreased significantly after 24 hrs, even at temperatures as low as 35°C (Kamboj, Sandhu, & Nanda, 2024).

The levels of **glucose oxidase** (GOX) in honey samples are also presented in **Fig. 3.1 (c)**. GOX in honey serves not only as a natural preservative upon the generation of gluconic acid but also an indicator of quality, authenticity and processing. Outliers were found in all floral types, spreading in a wide range beyond the upper extremes and showing large variations between the GOX activities measured for the same floral type. The buckwheat honey had a significantly lower GOX activity compared to the other three floral types, with a median value of 0.24 μg H_2O_2/g/h and an IQR of 0.06-0.47 μg H_2O_2/g/h. Although clover and goldenrod honey display comparable IQRs of 0.37-4.63 μg H_2O_2/g/h and 0.36-3.72 μg H_2O_2/g/h, respectively, goldenrod honey has a larger median value of 2.38 μg H_2O_2/g/h compared to clover honey, which has a median value of 0.78 μg H_2O_2/g/h. With a median of 1.87 μg H_2O_2/g/h, the blueberry honey exhibits a wider IQR of 0.63-12.38 μg H_2O_2/g/h (**Supplementary Table 3.1**). The levels of GOX detected in the clover honeys in this study are lower than the values reported by Ghoniemy (2022) from Giza Governorate of Egypt, ranging between 22.37 and 26.00 μg H_2O_2/g/h. Ghoniemy (2022) also investigated the effects of storage conditions on the enzymatic activities, reporting that the GOX activity decreased to 11.70 μg H_2O_2/g/h and 14.13 μg H_2O_2/g/h for samples stored at room temperature for 12 months in transparent and amber vessels, respectively. This suggested that GOX is highly sensitive to light and heat, and may loss its activity faster than diastase, which is commonly recognized as a freshness indicator in honey regulations (Ghoniemy, Esmail, Mahmoud, & Mohamed, 2022; Guttentag, Krishnakumar, Cokcetin, Harry, & Carter, 2021; Alshareef, Al-Farhan, & Mohammed, 2022; Sahin, Kolayli, & Beykaya, 2020). The lower GOX activity observed in the buckwheat honey samples in this study, along with previously noted reductions in diastase and invertase activities for the same floral type, suggests that regional differences may influence enzymatic activities between Canadian buckwheat honeys and those from Polish studies (Kowalski & Lukasiewicz, 2017; Miłek, Bocian, Kleczyńska, Sowa, & Dżugan, 2021; Tomczyk, et al., 2022). In addition to geographic locations, factors related to bees, such as age, diet, the seasonal changes in their physiological status, the density of the population, and the colony strength may also account for the observed differences (Babacan & Rand, 2005; Al-Sherif, et al., 2017; Lichtenberg-Kraag, 2015). Moreover, the nectar flow, nectar viscosity and sucrose concentration are also suggested to be negatively correlated with the abundance of salivary enzymes in honey (Lichtenberg-Kraag, 2015; Flanjak, Strelec, Kenjerić, & Primorac, 2016).

Acid phosphatase in honeys is another enzyme that plays multiple roles from helping in honey production to serving as a biomarker for freshness, quality, and origin. While no outliers were detected in clover and buckwheat sample, 4 blueberry samples and 1 goldenrod sample were identified as outliers (**Fig 3.1 (d)**). Buckwheat honey exhibited the highest acid phosphatase activity levels ranging from 30.50-1139.88 U, with a median of 770.62 U and an IQR of 427.21-939.14 U. Clover honey has a median activity of 33.58 U and an IQR of 20.2-72.03 U. Similarly, blueberry honey exhibits a similar median activity of 32.49 U and an IQR of 20.25-51.67 U. For goldenrod honey, the sole outlier is the maximum value of 738.34 U, while the median activity is the median value is 53.32 U with an IQR of 27.86-62.44 U (**Supplementary Table 3.1**). The high levels of acid phosphatase in buckwheat honey sample align with the findings reported by Miłek et al. (2021), where buckwheat honeys exhibited the highest levels of acid phosphatase activity when compared to honeys from other botanical origins

The **catalase** activity in honeys contributes to their chemical stability by regulating the level of hydrogen peroxide and can be an indicator of raw or processed honeys. Outliers were identified in clover and blueberry honeys, with all 2 outliers for clover honey found above the upper extreme and all 5 outliers for blueberry honey found beneath the lower extreme (**Fig 3.1 (e)**). The highest catalase activity was obtained in blueberry honey with a median value of 29.96 μg H_2O_2/g/min and an IQR of 26.21-33.74 μg H_2O_2/g/min, followed by buckwheat honey with a median value of 23.77 μg H_2O_2/g/min and an IQR of 18.35-28.42 μg H_2O_2/g/min, and goldenrod honey with a median value of 21.02 μg H_2O_2/g/min and an IQR of 12.25-26.94 μg H_2O_2/g/min. The lowest activities were observed for clover honey with a median value of 9.80 μg H_2O_2/g/min and an IQR of 26.21-33.74 μg H_2O_2/g/min (**Supplementary Table 3.1**). To date, very few studies were conducted on the quantification of catalase in honey samples. Large variations of catalase activities were found in honey with different botanical origin sourced from the same locality, which further suggests the impact of plant source on the presence of the enzyme (Alshareef, Al-Farhan, & Mohammed, 2022; de Abreu Franchini, Costa Matos, & Matos, 2011). Low activity of catalase in clover honey compared to other floral types in this study agreed with the observations previous reported (Borutinskaite, Treigyte, Čeksteryte, Kurtinaitiene, & Navakauskiene, 2018). Positive correlations were suggested between the antimicrobial properties and the glucose oxidase or catalase in honey (Osés, Rodríguezk, Valencia, Fernández-Muiño, & Sancho, 2024; Lu, et al., 2013).

3.4.2 Carbohydrate profiling of selected Canadian Honeys

Carbohydrate profiling of 169 Canadian honeys from four different self-claimed botanical sources was conducted, and the results are summarized in **Fig 3.3**, with details presented in **Supplementary Table 3.2** and **3.3.**.

Some targeted carbohydrate compounds, including turanose, kojibiose, melibiose, and maltotriose, had weak signals and overlapped with large peaks, making them unquantifiable (**Fig 3.2**). While fructose and glucose were detected in larger quantities as expected across the four floral types, sucrose, maltose, isomaltose, nigerose and erlose were minor compounds. Gentiobiose was not detected in 4 clover, and 3 buckwheat honeys, while trehalose was missing in one blueberry, one goldenrod sample and four different clover samples. Palatinose, cellobiose, melezitose, isomaltose, kestose, panose, and raffinose were either not present in the honey samples or present but below the level of detection, thus these carbohydrates will not be further discussed.

Fructose (**Fig 3.3 a**) was the dominant sugar in all honeys with an average concentration of 37.8% in blueberry honeys, 37.0% in buckwheat honeys, 38.1% in clover honeys and 37.9% is goldenrod honeys. Similarly, glucose (**Fig 3.3 b**), the second most abundant sugar in honey, showed minor variations, with an average concentration varying between 32.0 - 34.1% across the four floral types. The differences in the fructose/glucose (F/G) ratio (**Fig 3.3 c**) were not statistically significant ($p>0.05$) among the blueberry, buckwheat, and clover samples, with the average values ranging between 1.12-1.135. However, the goldenrod honey had an average F/G ratio of 1.87±0.063. The F/G ratio in buckwheat honey aligned with the results reported by Pasini (2013), in which 9 out of 10 samples showed an F/G ratio between 1.1-1.2.

The third most abundant sugar in honey is maltose (**Fig 3.3 d**) with an average value in each floral type ranging between 0.94% - 1.12%, and this is in consistent with results obtained for honeys from other geographic regions (eg. Spain, Italy) (de la Fuente, Ruiz-Matute, Valencia-Barrera, Sanz, & Martínez Castro, 2011; Pasini, Gardini, Marcazzan, & Caboni, 2013; Pascual-Maté et al., 2018;). However, some studies had shown a higher sucrose (0.6%, 1.26% and 1.79%) than maltose (0%, 0.26% and 0.74%) content in buckwheat honey from China, USA, and Poland, respectively (Corey, et al., 2022; Nešović, et al., 2020; Deng, et al., 2018).

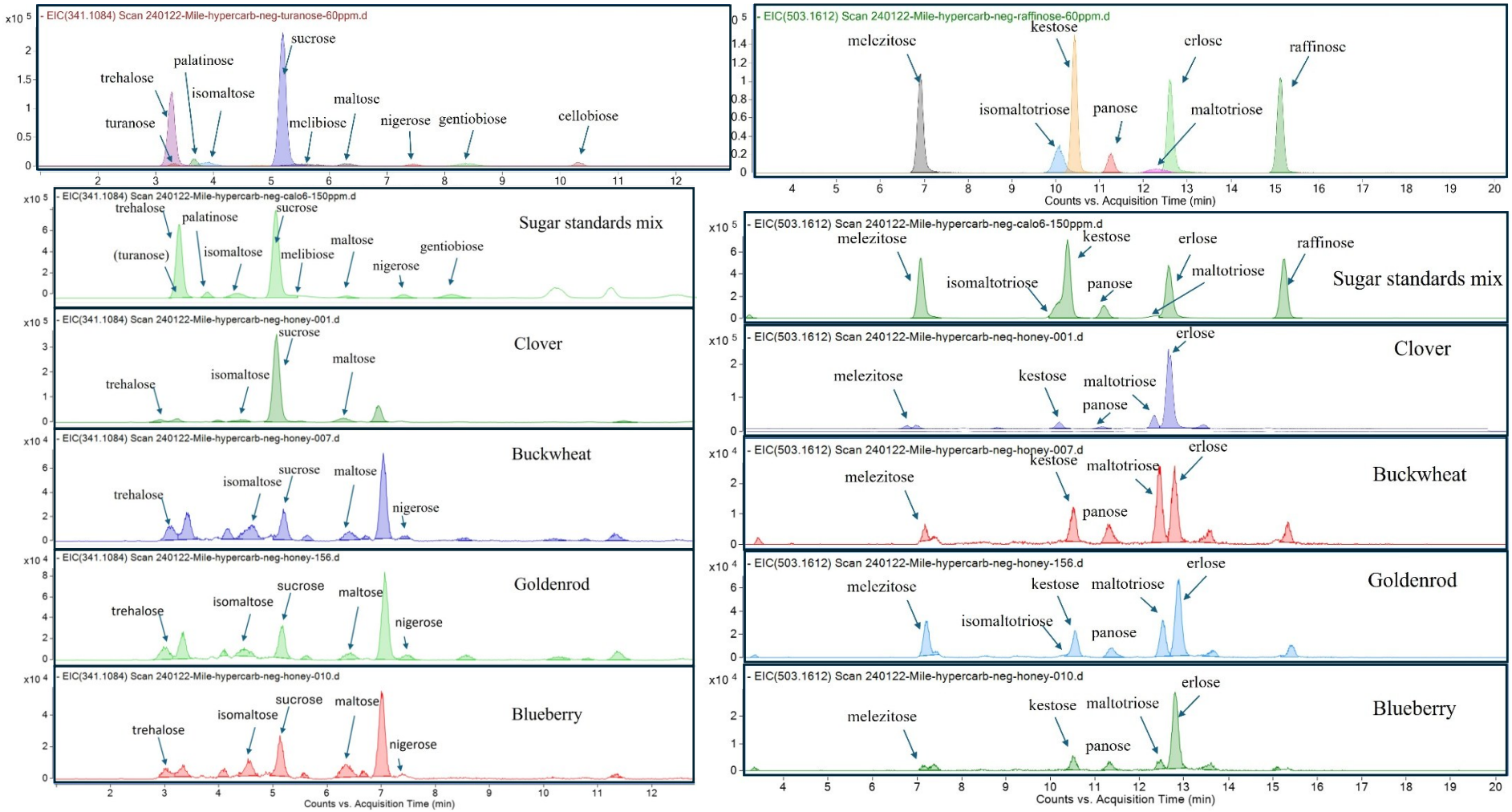
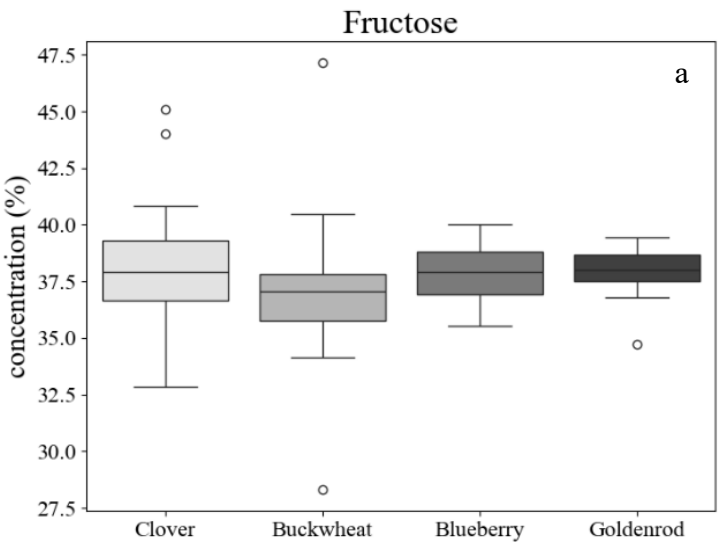
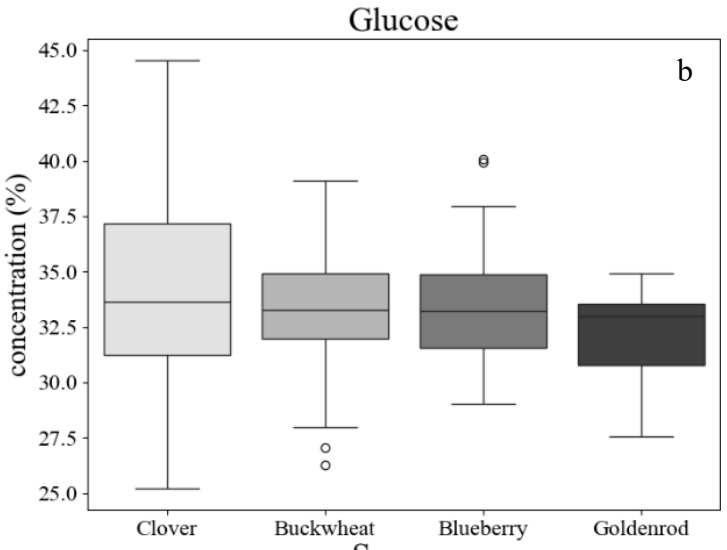
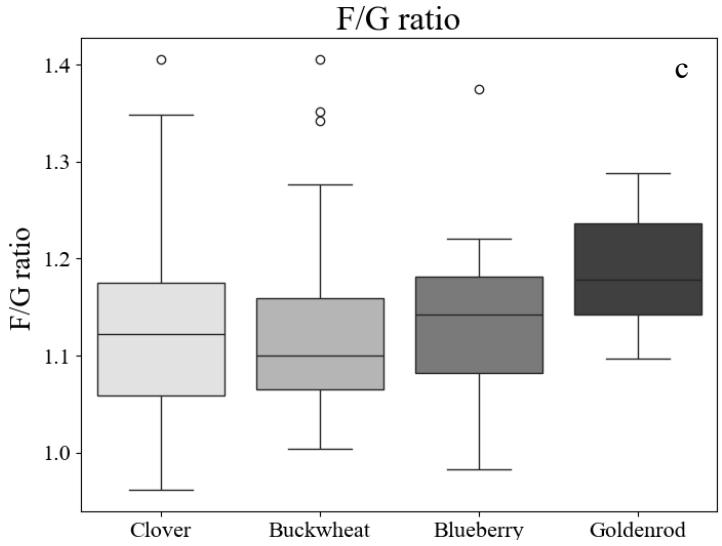
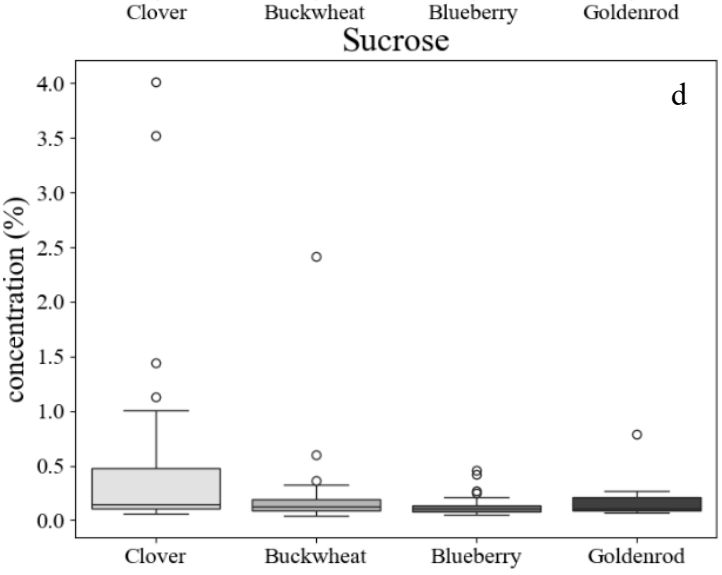


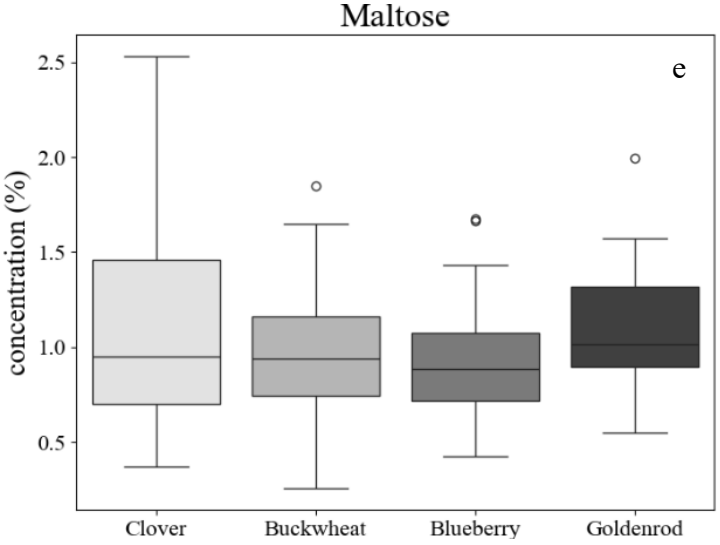
Figure 3.2. LCMS-QToF chromatogram of 11 disaccharides and 7 trisaccharides in a standard mixture and honey samples (a: disaccharides in standard mixture; b: trisaccharides in standard mixture; c: honey disaccharide profile obtained from LCMS-QToF; d: honey trisaccharide and other polysaccharide profile obtained from LCMS-QToF)

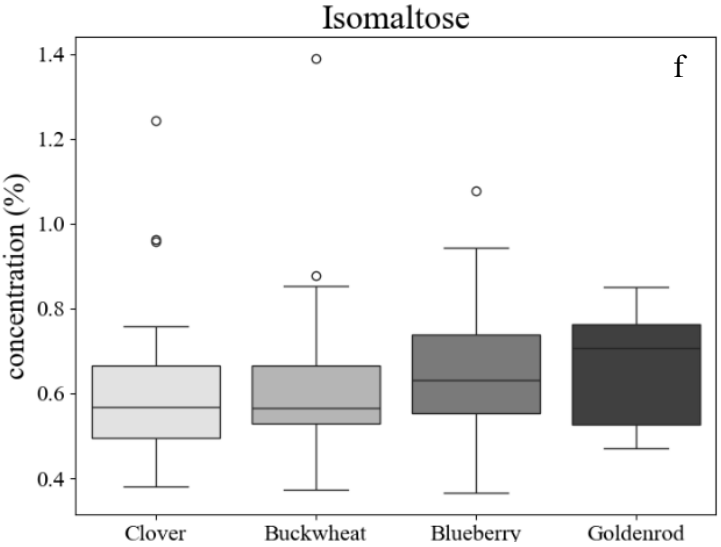

Fructose
a
concentration (%)
47.5
45.0
42.5
40.0
37.5
35.0
32.5
30.0
27.5
Clover
Buckwheat
Blueberry
Goldenrod


Glucose
b
concentration (%)
45.0
42.5
40.0
37.5
35.0
32.5
30.0
27.5
25.0
Clover
Buckwheat
Blueberry
Goldenrod


F/G ratio
c
F/G ratio
1.4
1.3
1.2
1.1
1.0
Clover
Buckwheat
Blueberry
Goldenrod


Sucrose
d
concentration (%)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Clover
Buckwheat
Blueberry
Goldenrod


Maltose
e
concentration (%)
2.5
2.0
1.5
1.0
0.5
Clover
Buckwheat
Blueberry
Goldenrod


Isomaltose
f
concentration (%)
1.4
1.2
1.0
0.8
0.6
0.4
Clover
Buckwheat
Blueberry
Goldenrod

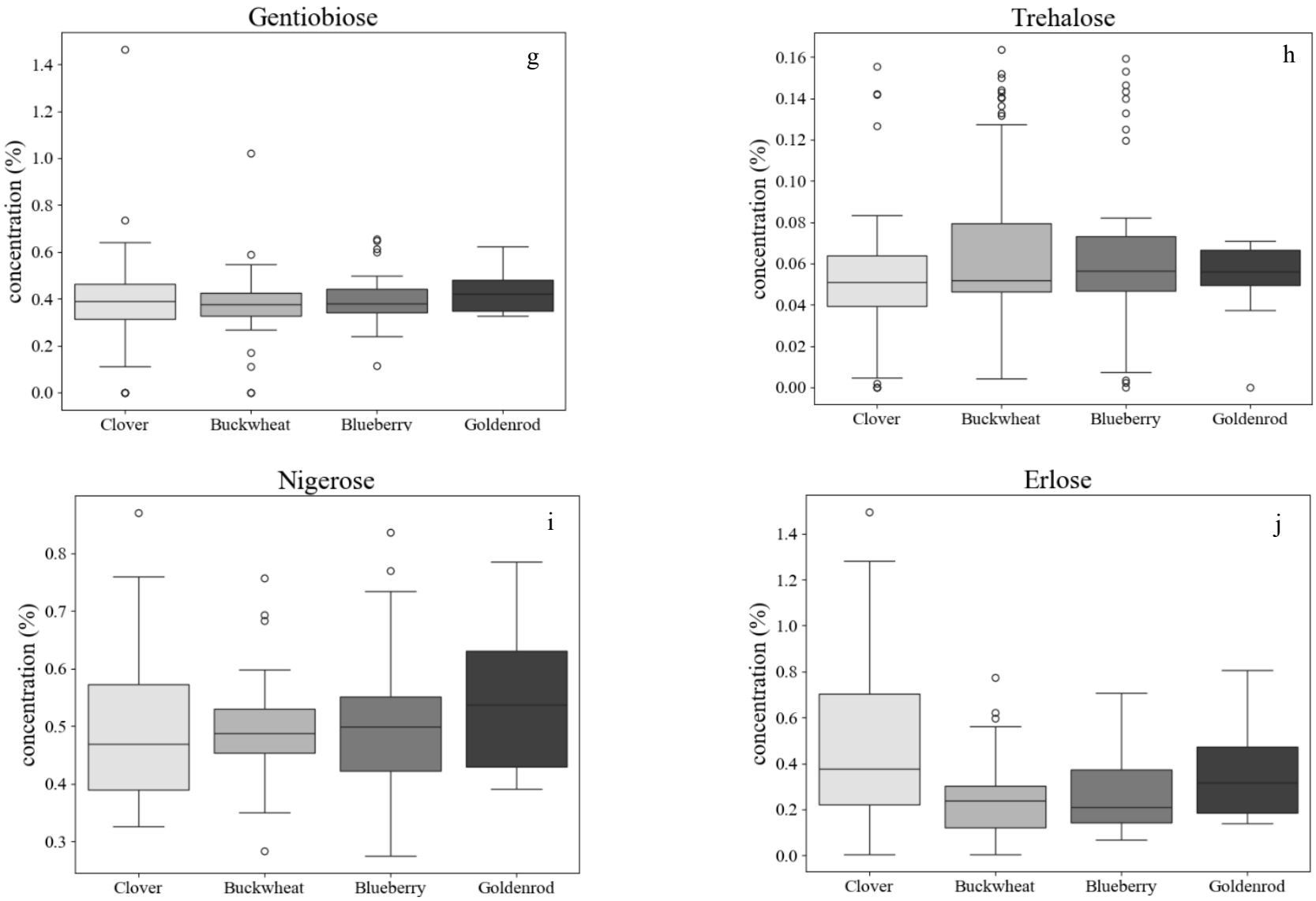


Figure 3.3. Sugar content of detectable monosaccharides (a, b: fructose, glucose), c: F/G ratio, disaccharides (d-i: sucrose, maltose, isomaltose, gentiobiose, trehalose, nigerose), and trisaccharide (j: erlose) in honey samples samples (clover: 52, buckwheat:59, blueberry: 41, goldenrod: 11).

All samples in this study, including the outliers (**Fig 3.3 (d)**) comply with the sucrose requirement (less than 5%) for Canadian blossom honey, with a median of 0.15%, 0.13%, 0.10% and 0.11%, and an IQR of 0.10-0.47%, 0.09-0.20%, 0.08-0.14% and 0.09-0.24% for clover, buckwheat, blueberry, and goldenrod honeys, respectively (CFIA, 2021). These values align with the sucrose content reported in previous studies of 0.18-0.27% for clover honey, and 0.1-0.9% for buckwheat honey (Pasini, Gardini, Marcazzan, & Caboni, 2013; Pascual-Maté, et al., 2018). Lower sucrose content in honey is an indicator of a higher level of honey maturation, as sucrose is converted to fructose and glucose via invertase during the ripening process (Yan, et al., 2023; Pascual-Maté, et al., 2018; Pasini, Gardini, Marcazzan, & Caboni, 2013).

Isomaltose was also quantified across all samples, and the median values and the IQRs for the four floral types (**Fig. 3.3 (e)**) were estimated as follows: 0.57% and 0.49-0.66% for clover honey, 0.57% and 0.53-0.67% for buckwheat honey, 0.63% and 0.54-0.74% for blueberry honey, and 0.71% and 0.51-0.77% for goldenrod honey. Compared to previous studies of Pascual-Maté (2018) and Pasini (2013), the isomaltose contents were either slightly lower or closer to the lower range. Trehalose was detected in most honey samples, except for 4 clover, 1 blueberry and 1 goldenrod honey sample, and the average concentration varied between 0.051% - 0.066%. Gentiobiose was detected in all honeys, except for 4 clover and 3 buckwheat samples. The highest gentiobiose content of 1.463% (w/w) was observed in one clover honey sample, although goldenrod honeys generally had a higher average concentration of 0.428% (w/w). Nigerose was detected in all samples with an average concentration ranging from 0.489 to 0.547% (w/w) across the four floral types. Erlose, the only trisaccharide quantified in this study, was present in all honey samples, with an average concentration ranging from 0.245 to 0.461% (w/w). The highest erlose content was detected in clover honey at 1.495% (w/w).

The sum of the minor sugars, quantified against each individual standard curves constructed based on their LC/MS results are shown in **Fig 3.4**, and a higher total concentration of these sugars is observed in clover and goldenrod honeys. In total, 5 unknown disaccharides, 6 unknown trisaccharides, 3 unknown tetrasaccharides, and 3 unknown pentasaccharides were detected in most of the samples; the rarest oligosaccharide, although unidentified, was detected in at least 7 samples. Relatively lower percentage of di-, tri-, and oligosaccharide were recorded for buckwheat and blueberry honey when compared to clover and goldenrod honeys (**Fig 3.4**).

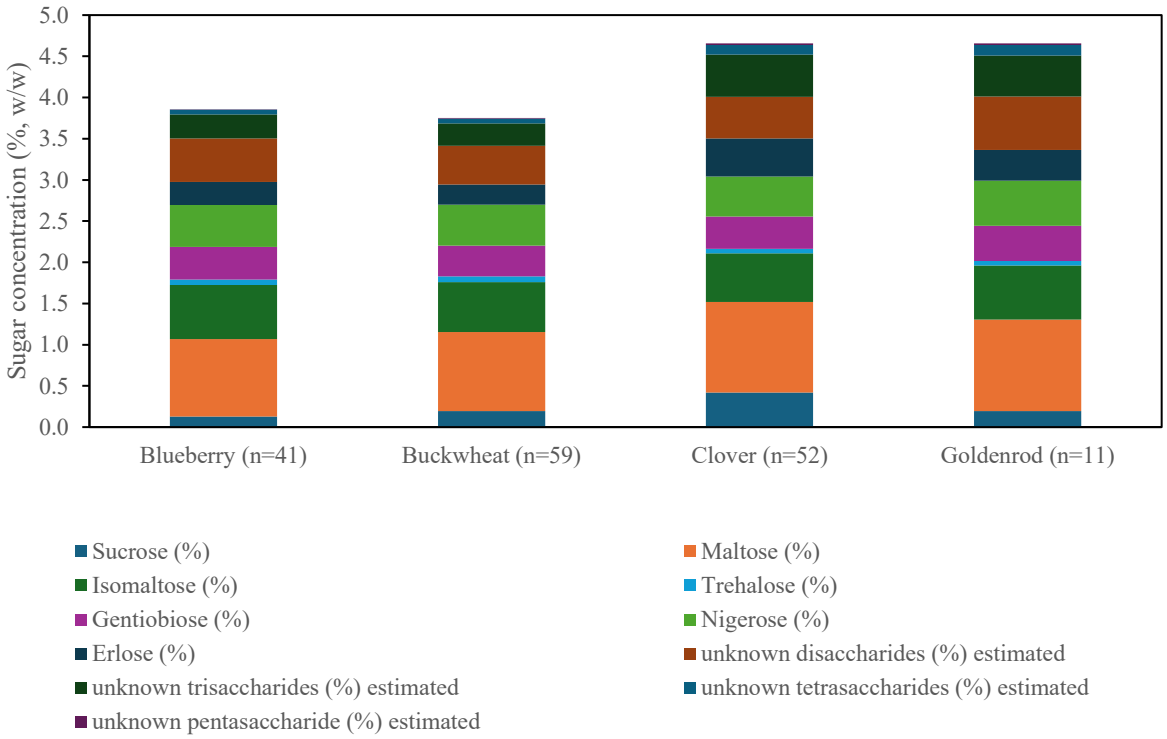


Figure 3.4. Profile distribution of disaccharides (sucrose, maltose, isomaltose, trehalose, gentiobiose, nigerose and unknown), trisaccharides (erlose and unknown), tetra-, and pentasaccharides (unknown)

3.4.3 Correlation statistical analyses

Fig 3.5 shows a biplot distribution-based cluster analysis of enzymatic activities, fructose concentration, glucose concentration, F/G ratio date. The distribution cluster with acid phosphate activity distinguished the buckwheat honey samples from the other types. This is in agreement with **Fig. 3.1**, where buckwheat honeys exhibited a significantly higher acid phosphatase activity compared to clover, blueberry and goldenrod honey samples. Additionally, the cluster plot of catalase activity, reveals the separation of clover, buckwheat and blueberry honey samples based on the level of this activity. Both acid phosphatase and catalase, significantly contributed to clustering of honeys, which can be explained by being plant originated enzymes (Alonso-Torre, et al., 2006). The results (**Fig. 3.1**) also shows that other enzymes - diastase, invertase, and glucose oxidase - which are secreted from the hypopharyngeal or salivary glands of bees during nectar and pollen collection, didn't correlate directly with the botanical origin of the honey.

A heatmap was generated to visualize the correlation among all quantitative data of honeys including enzymatic activities, carbohydrate profiling, HMF content and the Jack's scale pfund (**Fig 3.6**). The strongest negative correlations observed were between glucose content and the F/G ratio ($r = -0.8$) and glucose content and nigerose content ($r = -0.67$), both highly significant ($p < 0.0001$). In addition, correlations between HMF content and diastase activity ($r = -0.45$) or invertase activity ($r = -0.39$) were found to be moderately negative and statistically significant ($p < 0.0001$). Glucose concentration with diastase activity ($r = -0.36$), isomaltose concentration ($r = -0.5$), gentiobiose concentration ($r = -0.57$), or with the unknown trisaccharide ($r = -0.35$), tetrasaccharide ($r = -0.46$), and pentasaccharide ($r = -0.37$) concentrations also exhibited mildly negative correlation with a high level of significance ($p< 0.0001$). The slight negative correlation between glucose and invertase activity can be attributed to the inhibitory effect of high glucose concentrations on invertase activity, as previously documented (Zhou, et al., 2020). It has been reported that the formation of di and oligosaccharides in honey with α-, and β-glycosidic linkages, such as maltose, isomaltose, gentiobiose, kestose, and panose is determined by both enzymatic, via invertase, and non-enzymatic transglycosylation (Silva, et al., 2019). Hence, the mildly negative correlations observed in this study, between the glucose content and other di-, tri-, and oligosaccharide concentrations may be due to the transglycosylation process that uses glucose as a starting substrate to generate the oligomers.

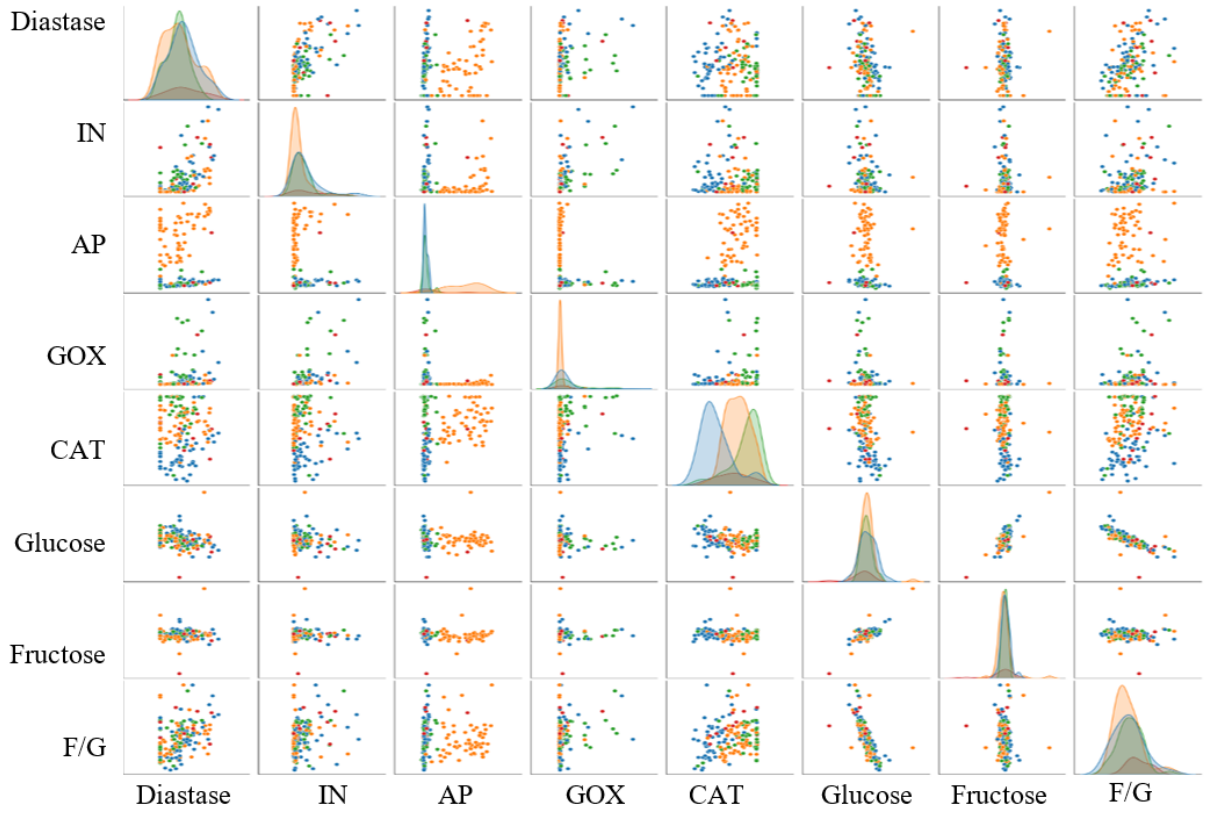


Figure 3.5 Biplot distribution-based cluster analysis of enzymatic activities and monosaccharide contents (top to bottom and left to right: diastase, **IN**: invertase, **AP**: acid phosphatase, **GOX**: glucose oxidase, **CAT**: catalase, glucose, fructose, F/G ratio) expressed in different monofloral honeys (clover (■); buckwheat (■); blueberry (■); goldenrod (■)

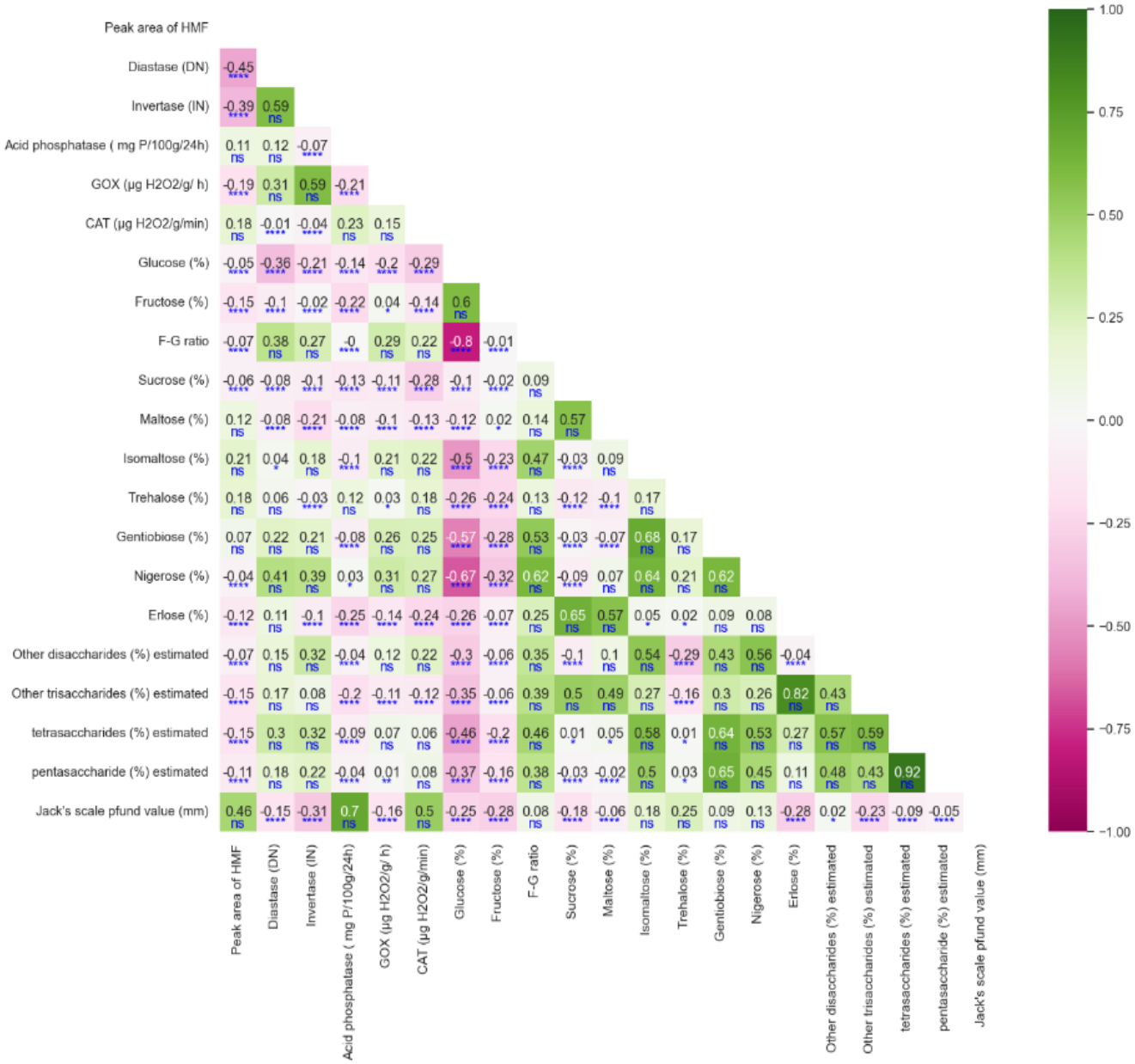


Figure 3.6 Correlative analysis of enzymatic activities, sugar profile, HMF content and color scales of all honey samples (****: p value ≤ 0.0001, ***: p value ≤ 0.001, **: p value ≤ 0.01, *: p value ≤ 0. 0.05, ns: not significant)

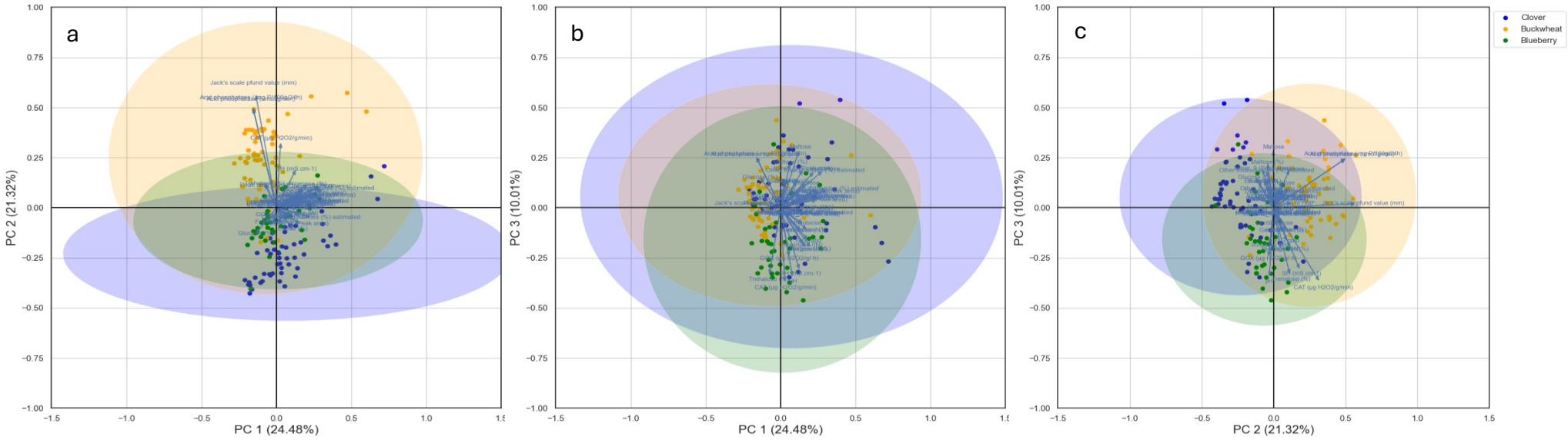


Figure 3.7 Principal component analysis for the enzymatic activity and carbohydrate profiling data of clover, buckwheat and goldenrod honey samples with samples colored by floral type (clover ( ); buckwheat ( ); blueberry ( ))

In order to determine the significant variables in identifying the botanical origins of honeys, a Principal Component Analysis (PCA) was conducted using the data obtained for clover, buckwheat and blueberry samples, and the results are presented in **Fig 3.7**. With over 30 attributes taken into consideration, PCA helped to reduce the dimensionality of the data set and to visualise the variations between samples in reduced data space (Saha & Manickavasagan, 2021). Three principal components contributed to 55.90% of the total variance, with principal component 1 (PC 1) explained 24.48% of the variance, followed by principal component 2 (PC 2) and principal component 3 (PC 3), expressing 21.32% and 10.01% respectively (3D plot see **Supplementary Figure 3.2**). The vectors representing the loadings of the inputs are presented, where the vector length indicates the magnitude of the contribution towards the PCs, and the angle between the vectors indicates correlations. Analysis of **Fig. 3.7 (a)** revealed grouping patterns of blueberry, clover and buckwheat honey samples projected along PC2, with color, acid phosphatase and catalase activity showing the most significant contribution. However, variables parallel to PC1 have shorter vector length, thus did not contribute significantly to grouping of samples. The blueberry honey samples clustered negatively along PC3 and are perpendicular to PC1 and PC2 (**Fig 3.7 (b) (c)**). Buckwheat and clover honey were grouped positively along PC3, with clover positioned on the left and buckwheat on the right along PC2 (**Fig 3.7 (c)**). Moreover, the vector representing acid phosphatase activity projects in the direction of buckwheat honey groupings, indicating a stronger influence on sample classification and aligning with the previous findings shown in **Fig 3.5**.

3.4.4 Predicted classification of botanical origins of honey

Different mathematical models were constructed using the carbohydrate and enzyme activity profiling data and were assessed for the prediction of botanical origins of honey. The cross-validated accuracy scores of all models configured with different training and predicting sample sets are shown in the appendix (**Supplementary Figure 3.1 a-d**). In total, four sets of models were generated and analyzed. Model set 2 exploited (**Supplementary Figure 3.1 (b)**) based solely on a small sample set was eliminated from further discussion.

To develop a classification model considering all 152 samples of interest (buckwheat: 59, blueberry: 41, clover: 52), XGB was selected due to its highest accuracy score of 0.9 (90%) upon 10-fold cross-validation (**Supplementary Figure 3.1 (a)**), indicating robust overall performance.

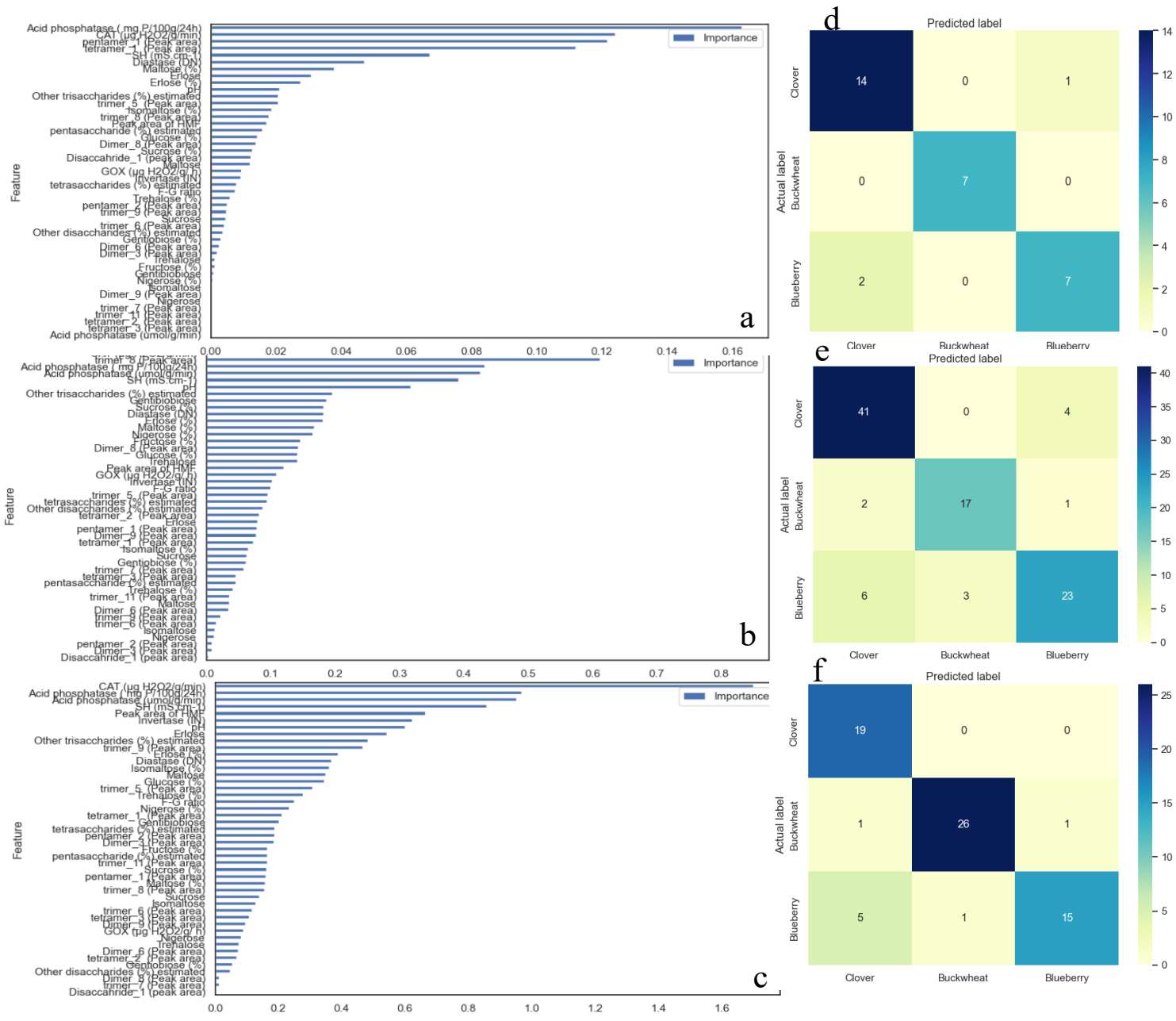


Figure 3.8 Importance score of features in different models (a: Model 1 XGB Classifier; b: Model 2 Ridge Regrssion; c: Model 3 Logistic Regression) and the corresponding confusion matrix (d-f) generated for the botanical origin identification of blueberry, buckwheat, and clover honeys based on their self-claimed floral origin.

According to the confusion matrix (**Fig 3.8 (d)**) the model successfully identified all 7 buckwheat honey samples. However, it encountered challenges in differentiating between blueberry and clover honey, misidentifying 1 out of 15 clover honey samples as a blueberry one and 2 out of 9 blueberry honey samples as a clover one. This model captured a high number of features due to a high inclusiveness of the samples used for both training and prediction, although the impact scores of the dominant features were relatively modest with the most significant predictor being acid phosphatase activity, at 0.16, followed by catalase activity at 0.12.

The second model, a Ridge Regression, was trained with 55 samples with confirmed floral types via pollen analysis and was tested on 97 unmatched samples (**Supplementary Figure 3.1 (b)**). This Ridge Regression model achieved an average accuracy of 97% in cross-validation with a standard deviation of 0.067. The confusion matrix revealed strong predictive capabilities, with correct identification of over 90% of clover samples, 89% of buckwheat samples, and 79% of blueberry honey (**Fig 3.8 (e)**). Due to the possible overlapping of features, the model shows difficulties in distinguishing between clover and blueberry honeys. All misidentified clover samples were confused for blueberry and 6 out of 9 misidentified blueberry samples were confused for clover. The importance score results (**Fig 3.8 (b)**) identify catalase activity as the most influential feature with a score of 0.8, followed by an unknown trisaccharide (RT= 12.088 min) at 0.6, and acid phosphatase activity at 0.45. Although high accuracy was found with this model, the small training sample size with the unmatched larger prediction set may lead to overfitting and compromising the generalization and validity of the predictions.

Lastly, the Logistic Regression model was employed among the models trained on a set of 84 non-outlier samples and predicted on a 68-sample set containing at least one outlier (enzymatic activity or carbohydrate profiling) (**Supplementary Figure 3.1 (c)**). With cross validation, this model yields 88% accuracy with a standard deviation of 0.121, and the confusion matrix (**Fig 3.8 (e)**) suggested that 100% true clover honey was properly identified, 93% of the buckwheat samples were categorized correctly. However, 6 out of 21 blueberry samples were misclassified, among which 5 were confused for clover honey and 1 for buckwheat. Although this model successfully identified all clover honeys, a high number of false positive (FP) occurred when 6 non-clover honey were identified as clover honey. Importance score shown in **Fig 3.8 (c)** shows that catalase

activity (1.7) and acid phosphatase activity (1) are two of the most influential features to the model, followed by electro conductivity, peak area of HMF, invertase activity, pH and erlose content.

Catalase and acid phosphatase activities have been identified as the key predictors in all three models discussed, regardless of the training set composition. Such findings were also confirmed by the distribution analysis and align with the potentiality of these two plant-originated enzymes in honey botanical origin identification suggested (Flanjak, Strelec, Kenjerić, & Primorac, 2016). The XGB model shows the most promising results in predicting buckwheat honey from non-buckwheat ones in this study, as all true buckwheat samples were identified with no FP detected.

3.5 Conclusion

The diastase activity results from this study confirmed the quality of 163 monofloral honeys purchased from the market complying with the CFIA standards. All the targeted enzymes, namely diastase, invertase, glucose oxidase, catalase and acid phosphatase, were detected in Canadian honeys and they were quantified. When performing biplot cluster analysis, the buckwheat honey distinguished from the rest with a significantly higher acid phosphatase activity at 683.202 mg P/ 100g honey. Clover, blueberry and buckwheat honey clusters were formed when considering catalase activities. This further confirmed the significance of the plant originated enzymes, catalase and acid phosphatase, on the authentication of the botanical origins of monofloral honeys. Negative correlations ($p<0.0001$) were observed between glucose and nigerose, isomaltose, or gentiobiose content, as well as between the HMF content and diastase or invertase. The PCA analysis further confirms the significance of acid phosphatase and catalase activities alongside other quantifiable features in establishing a more robust honey authentication model for buckwheat, clover and blueberry honeys. Promising identification of buckwheat honey was achieved by using the XGB model. Catalase and acid phosphatase have been identified as the most significant parameters in all models examined. However, this XGB model requires further strengthening to better differentiate clover and blueberry honey. By identifying and incorporating multiple biomarkers pertinent to assessing the profile of honey quality, our study significantly contributes to enhancing the prediction of honey authenticity and quality. However, additional research is necessary to identify and quantify the unknown sugars present in honey samples. Moreover, increasing the diversity and number of honey varieties analyzed will further enhance the reliability of these indicators in honey authentication.

Reference

AAFC. (2024). *Statistical Overview of the Canadian Honey and Bee Industry 2023.* Agriculture and Agri-Food Canada.

Alaerjani, W. M. A., Abu-Melha, S., Alshareef, R. M. H., Al-Farhan, B. S., Ghramh, H. A., Al-Shehri, B. M. A., Bajaber, M. A., Khan, K. A., Alrooqi, M. M., Modawe, G. A., & Mohammed, M. E. A. (2022). Biochemical Reactions and Their Biological Contributions in Honey. *Molecules*, *27*(15), 4719.

Alonso-Torre, S. R., Cavia, M. M., Fernández-Muiño, M. A., Moreno, G., Huidobro, J. F., & Sancho, M. T. (2006). Evolution of acid phosphatase activity of honeys from different climates. *Food Chemistry*, 750-755.

Alshareef, R., Al-Farhan, B., & Mohammed, M. (2022). Glucose Oxidase and Catalase Activities in Honey Samples from the Southwestern Region of Saudi Arabia. *Applied Sciences*, *12*(15), 7584.

Al-Sherif, A., Mazeed, A., Ewis, M., Nafea, E., Hagag, E.-S., & Kamel, A. (2017). Activity of salivary glands in secreting honey-elaborating enzymes in two subspecies of honeybee (Apis mellifera L). *Physiological Entomology*, 397-403.

Babacan, S., & Rand, A. (2005). Purification of Amylase from Honey. *Food Chemistry and Toxicology*, 413-418.

Barreiros, J., Cepeda, A., Franco, C., Nebot, C., & Vázquez, B. (2024). Analysis of minerals in honey and their nutritional implications. *Journal of Food Composition and Analysis*, *136*, 106733.

Bell, A. R., & Grainger, M. N. (2023). Accelerated loss of diastase in manuka honey: Investigation of manuka specific compounds. *Food Chemistry*, *426*, 136614.

Bodor, Z., Kovacs, Z., Benedek, C., Hitka, G., & Behling, H. (2021). Origin Identification of Hungarian Honey Using Melissopalynology, Physicochemical Analysis, and Near Infrared Spectroscopy. *Molecules*, *26*(23), 7274.

Bogdanov, S. (2009). *Harmonised Mehtods of the International Honey Commission.* International Honey Commission.

Borutinskaite, V., Treigyte, G., Ceksteryte, V., Kurtinaitiene, B., & Navakauskiene, R. (2018). Proteomic identification and enzymatic activity of buckwheat (Fagopyrum esculentum) honey based on different assays. *Journal of Food and Nutrition Research*, 57-69.

Bose, D., & Padmavati, M. (2024). Honey Authentication: A review of the issues and challenges associated with honey adulteration. *Food Bioscience*, *61*, 105004.

Bouzo, D., Cokcetin, N. N., Li, L., Ballerin, G., Bottomley, A. L., Lazenby, J., Whitchurch, C. B., Paulsen, I. T., Hassan, K. A., & Harry, E. J. (2020). Characterizing the Mechanism of Action of an Ancient Antimicrobial, Manuka Honey, against Pseudomonas aeruginosa Using Modern Transcriptomics. *mSystems*, *5*(3), e00106-20.

Brar, D. S., Pant, K., Krishnan, R., Kaur, S., Rasane, P., Nanda, V., Saxena, S., & Gautam, S. (2023). A comprehensive review on unethical honey: Validation by emerging techniques. *Food Control*, *145*, 109482.

Brudzynski, K. (2020). A current perspective on hydrogen peroxide production in honey. A review. *Food Chemistry*, *332*, 127229.

Brust, H., Orzechowski, S., & Fettke, J. (2020). Starch and Glycogen Analyses: Methods and Techniques. *Biomolecules*, *10*(7), 1020.

Can, Z., Yildiz, O., Sahin, H., Turumtay, E. A., Silici, S., & Kolayli, S. (2015). An investigation of Turkish honeys: Their physico-chemical properties, antioxidant capacities and phenolic profiles. *Food Chemistry*, 133-141.

CFIA. (2021, April 28). *Canadian Grade Compendium: Volume 6 - Honey*. Retrieved from Govenment of Canada: https://inspection.canada.ca/en/about-cfia/acts-and-regulations/list-acts-and-regulations/documents-incorporated-reference/canadian-grade-compendium-volume-6

CFIA. (2024, May 24). *Report: Enhanced honey authenticity surveillance (2018 to 2019)*. Retrieved from Government of Canada: https://inspection.canada.ca/en/science-and-research/our-research-and-publications/report-0

- Corey, K., Hamama, A. A., Li, H., Siddiqui, R. A., Kim, C., & Bhardwaj, H. L. (2022). Composition of Buckwheat Honey. *Journal of Agricultural Science*, *14*(9), 59.

Crăciun, M. E., Pârvulescu, O. C., Donise, A. C., Dobre, T., & Stanciu, D. R. (2020). Characterization and classification of Romanian acacia honey based on its physicochemical parameters and chemometrics. *Scientific reports*, *10*(1), 20690.

de Abreu Franchini, R. A., Costa Matos, M. A., & Matos, R. C. (2011). Amperometric Determination of Catalase in Brazilian Commercial Honeys. *Analytical Letters* , 232-240.

de la Fuente, E., Ruiz-Matute, A., Valencia-Barrera, R. M., Sanz, J., & Martínez Castro, I. (2011). Carbohydrate composition of Spanish unifloral honeys. *Food Chemistry*, 1483–1489.

Deng, J., Liu, R., Lu, Q., Hao, P., Xu, A., Zhang, J., & Tan, J. (2018). Biochemical properties, antibacterial and cellular antioxidant activities of buckwheat honey in comparison to manuka honey. *Food Chemistry*, 243-249.

Dżugan, M., Miłek, M., Kielar, P., Stępień, K., Sidor, E., & Bocian, A. (2022). SDS-PAGE Protein and HPTLC Polyphenols Profiling as a Promising Tool for Authentication of Goldenrod Honey. *Foods*, 2390.

Edo, G. I., Akpoghelie, P. O., Jikah, A. N., Onoharigho, F. O., Oghenwogaga, O. J., & Okoronkwo, K. A. (2023). Quality, composition and health effects of natural effects of natural honey: a review. *Natural Resrouces for Human Health*, 449-461.

FAO. (2022). *STANDARD FOR HONEY.* Codex Alimentarius.

Flanjak, I., Strelec, I., Kenjerić, D., & Primorac, L. (2016). Croatian produced unifloral honey characterized according to the protein and proline content and enzyme activities. *Journal of Apicultural Science*, 39-48.

Gašić, U. M., Milojković-Opsenica, D. M., & Tešić, Ž. L. (2017). Polyphenols as Possible Markers of Botanical Origin of Honey. *Journal of AOAC International*, 852-861.

Ghoniemy, H. A., Esmail, A. H., Mahmoud, A. A.-T., & Mohamed, A. M. (2022). Evaluation of vitamin C, proline, enzymes and hydroxymethylfurfural levels in clover honey at different storage conditions. *Egyptian Journal of Chemistry*, 427-434.

Government of Ontario. (2021, January 1). *O. Reg. 119/11: Produce, Honey and Maple Products*. Retrieved from Government of Ontario: https://www.ontario.ca/laws/ regulation/110119

Guttentag, A., Krishnakumar, K., Cokcetin, N., Harry, E., & Carter, D. (2021). Factors affecting the production and measurement of hydrogen peroxide in honey samples. *Microbiology* .

Huidobro, J. F., & Sánchez, P. (2005). Precise Method for the Measurement of Catalase Activity in Honey. *Journal of AOAC International*, 800-804.

Jaafar, M. B., Othman, M. B., Yaacob, M., Talip, B. A., Ilyas, M. A., Ngajikin, N. H., & Fauzi, N. A. (2020). A Review on Honey Adulteration and the Available Detection Approaches. *The International Journal of Intergrated Engineering*, 125-131.

Jandric, Z., Haughey, S. A., Frew, R. D., McComb, K., Galvin-King, P., Elliott, C. T., & Cannavan, A. (2015). Discrimination of honey of different floral origins by a combination of various chemical parameters. *Food Chemistry*, 52-59.

Julika, W. N., Ajit, A., Ismail, N., Aqilah, N., Naila, A., & Sulaiman, A. Z. (2020). Sugar profile and enzymatic analysis of stingless bee honey collected from lcoal market in Malaysia. *IOP Conference Series: Materials Science and Engineering*, (p. 736).

Kamboj, R., Sandhu, R. S., & Nanda, V. (2024). Effect of heating and pH on hydroxymethylfurfural content, diastase and invertase activity of Dalbergia honey using response surface methodology. *Food Chemistry Advances*, *5*, 100766.

Kowalski, S., & Lukasiewicz, M. (2017). Diastase and Invertase Activity Changes and 5-Hydroxymethyl-2-Furfural Formation in Honeys Under Influence of Microwave Irradiation. *Journal of Food Process Engineering*, e12410.

Lichtenberg-Kraag, B. (2015). Evidence for correlation between invertase activity and sucrose content during the ripening process of honey. *Journal of Apicultural Research*, 365-373.

Lu, J., Carter, D. A., Turnbull, L., Rosendale, D., Hedderley, D., Stephens, J., Gannabathula, S., Steinhorn, G., Schlothauer, R. C., Whitchurch, C. B., & Harry, E. J. (2013). The Effect of New Zealand Kanuka, Manuka and Clover Honeys on Bacterial Growth Dynamics and Cellular Morphology Varies According to the Species. *PloS one*, *8*(2), e55898.

Mădaş, M. N., Mărghitaş, L. A., Dezmirean, D. S., Bobiş, O., Abbas, O., Danthine, S., Francis, F., Haubruge, E., & Nguyen, B. K. (2020). Labeling Regulations and Quality Control of Honey Origin: A Review. *Food Reviews International*, 215-240.

Megherbi, M., Herbreteau, B., Faure, R., & Salvador, A. (2009). Polysaccharides as a Marker for Detection of Corn Sugar Syrup Addition in Honey. *Journal of Agricultural and Food Chemistry*, 2105-2111.

Miłek, M., Bocian, A., Kleczyńska, E., Sowa, P., & Dżugan, M. (2021). The Comparison of Physicochemical Parameters, Antioxidant Activity and Proteins for the Raw Local Polish Honeys and Imported Honey Blends. *Molecules*, *26*(9), 2423.

Moore, J. C., Spink, J., & Lipp, M. (2012). Development and Application of a Databaseof Food Ingredient Fraud and EconomicallyMotivated Adulteration from 1980 to 2010. *Joural of Food Science*, R118-R126.

Nedić, N., Nešović, M., Radišić, P., Gašić, U., Baošić, R., Joksimović, K., Pezo, L., Tešić, Ž., & Vovk, I. (2022). Polyphenolic and Chemical Profiles of Honey From the Tara Mountain in Serbia. *Frontiers in nutrition*, *9*, 941463.

Nešović, M., Gašić, U., Tosti, T., Horvacki, N., Šikoparija, B., Nedić, N., Blagojević, S., Ignjatović, L., & Tešić, Ž. (2020). Polyphenol profile of buckwheat honey, nectar and pollen. *Royal Society open science*, *7*(12), 201576.

Ntakoulas, D. D., Tsagkaris, A. S., Raptis, S., Pasias, I. N., Raptopoulou, K. G., Kharoshka, A., Schulzova, V., & Proestos, C. (2024). Study of authenticity, quality characteristics and bioactivity in honey samples from different botanical origins and countries. *Journal of Food Composition and Analysis*, *136*, 106716.

Nyarko, K., Mensah, S., & Greenlief, C. M. (2024). Examining the Use of Polyphenols and Sugars for Authenticating Honey on the U.S. Market: A Comprehensive Review. *molecules*, *29*(20), 4940.

Osés, S. M., Rodríguezk, C., Valencia, O., Fernández-Muiño, M. A., & Sancho, T. (2024). Relationships among Hydrogen Peroxide Concentration, Catalase, Glucose Oxidase, and Antimicrobial Activities of Honeys. *Foods*, *13*(9), 1344.

Pascual-Maté, A., Osés, S., Marcazzan, G., Gardini, S., Fernández Muiño, M., & Sancho, M. (2018). Sugar composition and sugar-related parameters of honeys from the northern Iberian Plateau. *Journal of Food Composition and Analysis*, 34-43.

Pashayanm, S. A. (2024). Functional value of biologically active honey components. *BIO Web of Conferences*, (p. 108).

Pasini, F., Gardini, S., Marcazzan, G. L., & Caboni, M. F. (2013). Buckwheat honeys: Screening of composition and properties. *Food Chemistry*, 2802-2811.

Pesek, S., Lehene, M., Brânzanic, A. M., & Silaghi-Dumitrescu, R. (2022). On the Origin of the Blue Color in The Iodine/Iodide/Starch Supramolecular Complex. *Molecules*, *27*(24), 8974.

Pita-Calvo, C., & Vázquez, M. (2017). Differences between honeydew and blossom honeys: A review. *Trends in Food Science & Technology*, 79-87.

Qu, L., Jiang, Y., Huang, X., Cui, M., Ning, F., Liu, T., Gao, Y., Wu, D., Nie, Z., & Luo, L.(2019). High-Throughput Monitoring of Multiclass Syrup Adulterants inHoney Based on the Oligosaccharide and Polysaccharide Profiles by MALDI Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, 11256-11261.

Rodríguez-Flores, S., Escuredo, O., & Seijo, M. C. (2016). Characterization and antioxidant capacity of sweet chestnut honey produced in North-West Spain. *Journal of Apicultural Science*, 19-30.

Saha, D., & Manickavasagan, A. (2021). Machine learning techniques for analysis of hyperspectral images to determine quality of food products: A review. *Current Research in Food Science*, 28-44.

Sahin, H., Kolayli, S., & Beykaya, M. (2020). Investigation of Variations of Invertase and Glucose Oxidase Degrees against Heating and Timing Options in Raw Honeys. *Journal of Chemistry*, *2020*, 5398062,

Schievano, E., Sbrizza, M., Zuccato, V., Piana, L., & Tessari, M. (2020). NMR carbohydrate profile in tracing acacia honey authenticity. *Food Chemistry*, *309*, 125788.

Seraglio, S. K., Schulz, M., Brugnerotto, P., Silva, B., Gonzaga, L. V., Fett, R., & Costa, A. C. (2021). Quality, composition and health-protective properties of citrus honey: A review. *Food Research International*, *143*, 110268.

Shahbandeh, M. (2024, May 22). *Value of honey produced in Canada 2012-2023*. Retrieved from Statista: https://www.statista.com/statistics/540278/production-value-honey-canada/

Silva, S., Moreira, A., Domingues, M., Evtuguin, D., Coelho, E., & Coimbra, M. (2019). Contribution of non-enzymatic transglycosylation reactions to the honey oligosaccharides origin and diversity. *Pure and Applied Chemistry*, 1231-1242.

Stryjewski, L. (2010). *Semantic Scholar*. Retrieved from 40 years of boxplots: https://www.semanticscholar.org/paper/40-years-of-boxplots-Stryjewski/d31505d5b6d61ad75a5ae6ded8fa5b7202e66372

Takahashi, Y., Yoshida, I., Yokozeki, T., Igarashi, T., & Fujita, K. (2023). Investigation of Foreign Amylase Adulteration in Honey Distributed in Japan by Rapid and Improved Native PAGE Activity Staining Method. *Journal of Applied Glycoscience*, 67-73.

Thrasyvoulou, A., Tananaki, C., Goras, G., Karazafiris, E., Dimou, M., Liolios, V., Kanelis, D., & Gounari, S. (2018). Legislation of honey criteria and standards. *Journal of Apicultural Research*, 88–96.

Tian, L., Bilamjian, S., Liu, L., Akiki, C., Cuthbertson, D. J., Anumol, T., & Bayen, S. (2024). Development of a LC-QTOF-MS based dilute-and-shoot approach for the botanical discrimination of honeys. *Analytica chimica acta*, *1304*, 342536.

Tomczyk, M., Bocian, A., Sidor, E., Miłek, M., Zaguła, G., & Dżugan, M. (2022). The Use of HPTLC and SDS-PAGE Methods for Coniferous Honeydew Honey Fingerprinting Compiled with Mineral Content and Antioxidant Activity. *Molecules*, 720.

Wang, S., Qiu, Y., & Zhu, F. (2024). An updated review of functional ingredients of Manuka honey and their value-added innovations. *Food chemistry*, *440*, 138060.

Wesołowska, M., & Dżugan, M. (2017). Activity and thermal stability of diastase present in honey from Podkarpacie region. *Food. Science Technology. Quality*, 103-112.

Yan, S., Wang, W., Zhao, W., Tian, W., Wang, X., Wu, L., & Xue, X. (2023). Identification of the maturity of acacia honey by an endogenous oligosaccharide: A preliminary study. *Food chemistry*, *399*, 134005.

Zhou, G., Peng, C., Liu, X., Chang, F., Xiao, Y., Liu, J., & Fang, Z. (2020). Identification and Immobilization of an Invertase With High Specific Activity and Sucrose Tolerance Ability of Gongronella sp. w5 for High Fructose Syrup Preparation. *Frontiers in microbiology*, *11*, 633.

Appendix

Supplementary Table 3.1 Enzymatic activity of blueberry, buckwheat, clover and goldenrod honey samples

	Diastase (DN)	Invertase (IN)	Acid phosphatase (mg P/100g/24h)	GOX (µg H2O2/g/ h)	CAT (µg H2O2/ g/ min)
Blueberry (n=41)					
Max	21.583	17.587	240.867	66.672	34.168
Min	ND	ND	ND	0.046	2.212
Average	8.832	3.164	48.851	11.843	27.629
STD	5.512	3.735	55.897	19.136	8.102
Buckwheat (n=59)					
Max	25.716	18.999	1139.882	29.376	34.037
Min	ND	ND	30.496	ND	7.497
Average	9.919	1.857	683.202	1.276	23.604
STD	8.205	3.594	295.382	4.130	6.231
Clover (n=52)					
Max	30.127	22.758	119.730	85.725	33.893
Min	ND	ND	3.913	ND	ND
Average	11.892	4.236	45.387	6.248	12.029
STD	7.751	5.396	32.621	15.947	8.470
Goldenrod (n=11)					
Max	26.572	14.575	738.336	50.115	33.976
Min	ND	ND	15.759	0.151	8.690
Average	12.521	5.333	109.348	6.779	20.917
STDV	8.925	5.563	209.420	14.646	8.796

Supplementary Table 3.2. Molecular structure, m/z, retention time for all sugar compounds, excluding fructose and glucose, detected in honey samples

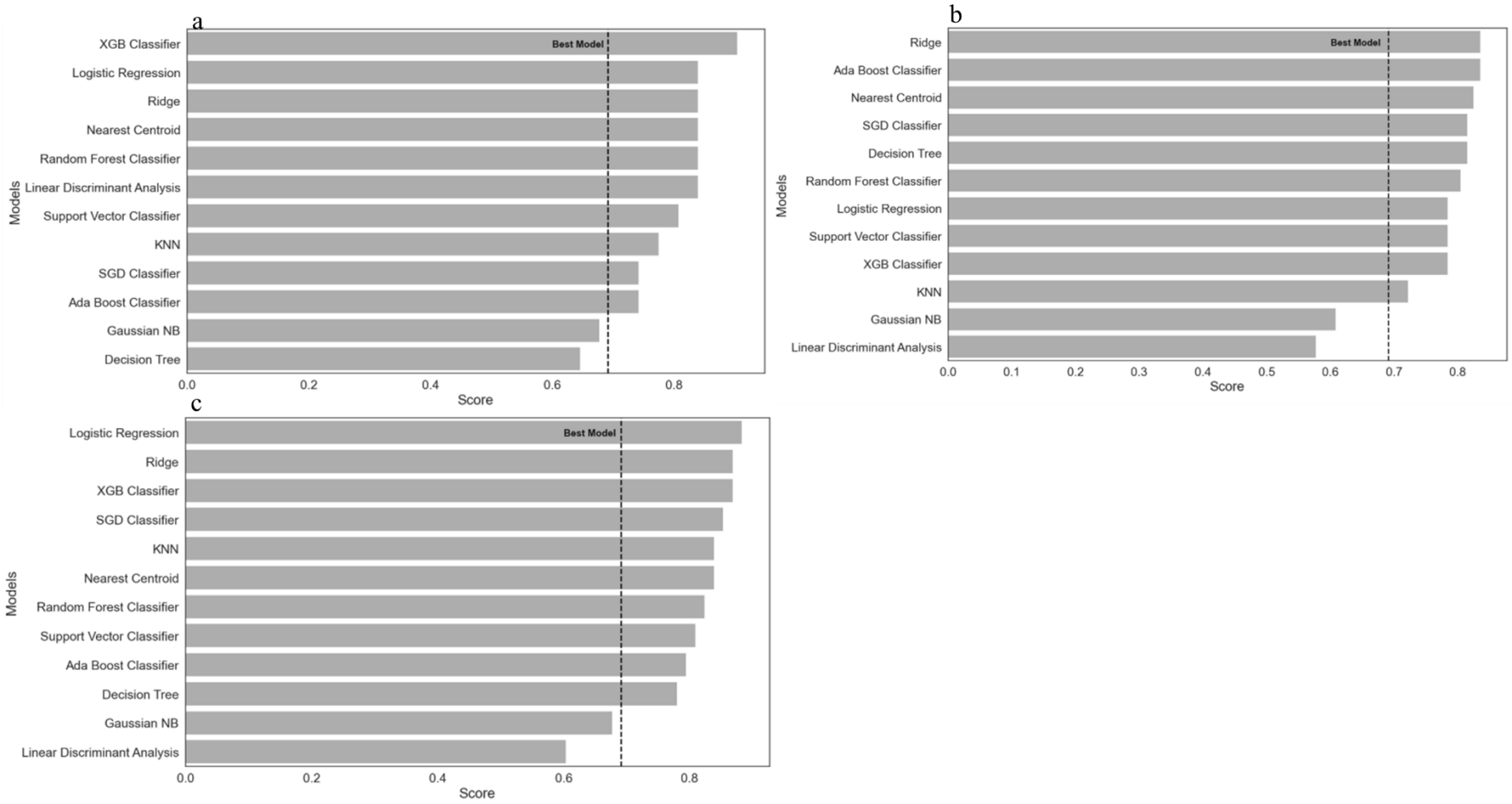
Sugar	Formular	m/z	RT
Unknown_1	C12 H22 O11	341.1084	2.928
Trehalose	C12 H22 O12	341.1084	3.208
Unknown_2	C12 H22 O13	341.1084	3.979
Isomaltose	C12 H22 O14	341.1084	4.402
Sucrose	C12 H22 O15	341.1084	5.072
Unknown_3	C12 H22 O16	341.1084	5.529
Maltose	C12 H22 O17	341.1084	6.309
Unknown_4	C12 H22 O18	341.1084	6.611
Unknown_5	C12 H22 O19	341.1084	6.945
Nigerose	C12 H22 O20	341.1084	7.394
Gentiobiose	C12 H22 O21	341.1084	8.504
Unknown_6	C18 H32 O16	503.1612	10.529
Unknown_7	C18 H32 O16	503.1612	11.47
Unknown_8	C18 H32 O16	503.1612	12.088
Unknown_9	C18 H32 O16	503.1612	12.665
Unknown_10	C18 H32 O16	503.1612	12.978
Erlose	C18 H32 O16	503.1612	13.766
Unknown_11	C18 H32 O16	503.1612	15.215
Unknown_12	C24 H42 O21	665.214	16.588
Unknown_13	C24 H42 O21	665.214	17.283
Unknown_14	C24 H42 O21	665.214	18.376
Unknown_15	C30H52O26	827.2669	20.463
Unknown_16	C30H52O26	827.2669	20.811

Supplementary Table 3.3 Identified sugar profiling and corresponding F/G ratio of blueberry, buckwheat, clover and goldenrod honeys

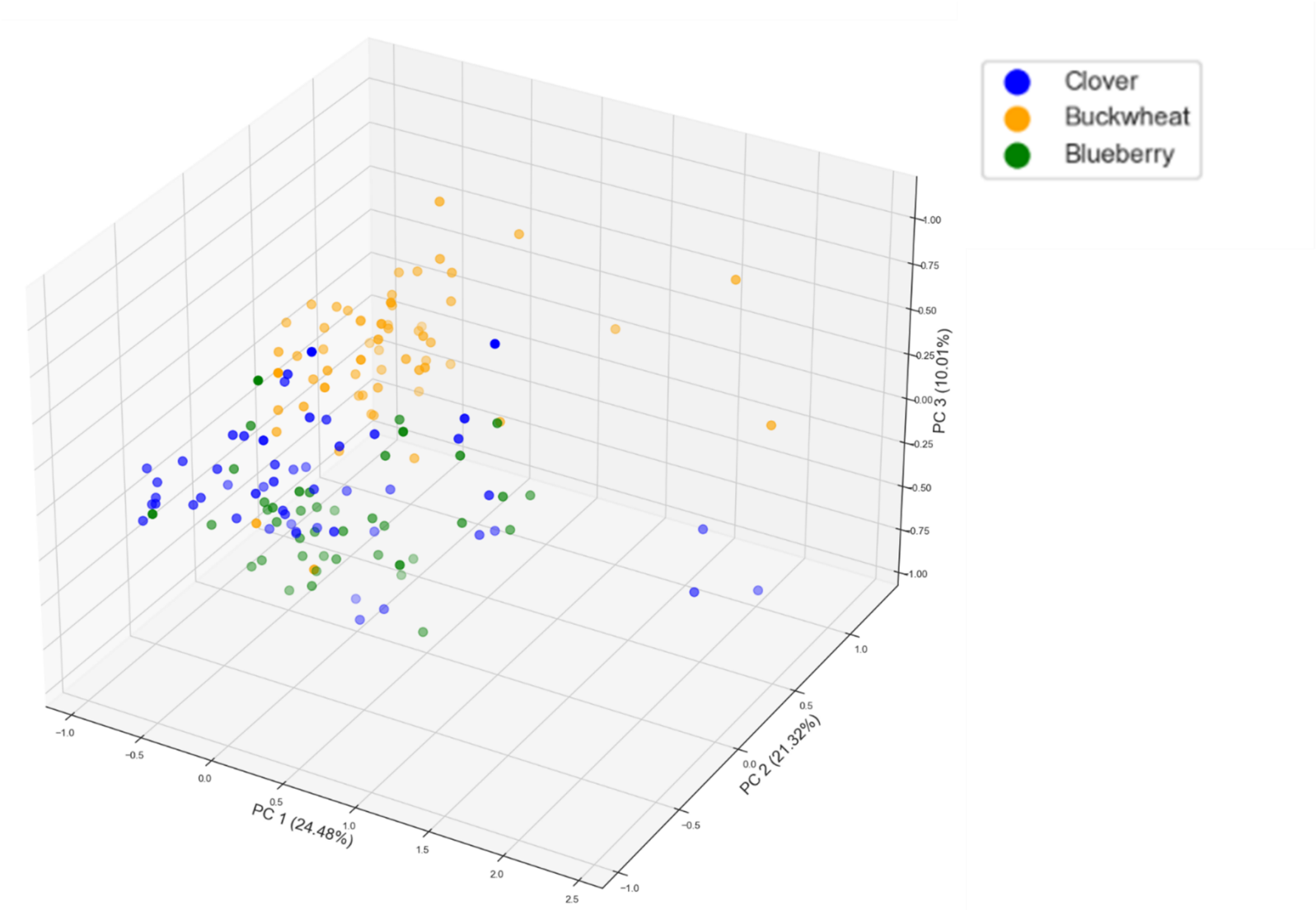
	Glucose (%)[a]	Fructose (%)[a]	F/G ratio[b]	Sucrose (%)[a]	Maltose (%)[a]	Isomaltose (%)[a]	Trehalose (%)[a]	Gentiobiose (%)[a]	Nigerose (%)[a]	Erlose (%)[a]
Blueberry (n=41)										
Max	40.057	40.024	1.374	0.455	1.677	1.078	0.160	0.656	0.836	0.708
Min	29.014	35.513	0.983	0.049	0.422	0.366	0.000	0.114	0.275	0.069
Average	33.463	37.800	1.135	0.127	0.943	0.655	0.066	0.396	0.508	0.281
STDV	2.567	1.296	0.075	0.086	0.306	0.150	0.042	0.111	0.118	0.176
Buckwheat (n=59)										
Max	39.094	47.154	1.405	2.419	1.849	1.389	0.164	1.021	0.757	0.775
Min	26.250	28.311	1.004	0.041	0.256	0.375	0.004	0.000	0.284	0.006
Average	33.159	37.009	1.120	0.193	0.961	0.607	0.068	0.374	0.495	0.245
STDV	2.473	2.241	0.082	0.310	0.346	0.157	0.041	0.146	0.081	0.158
Clover (n=52)										
Max	44.538	45.104	1.405	4.013	2.532	1.243	0.155	1.463	0.870	1.495
Min	25.211	32.833	0.962	0.058	0.370	0.380	0.000	0.000	0.326	0.005
Average	34.110	38.098	1.127	0.421	1.097	0.593	0.051	0.392	0.489	0.461
STDV	3.977	2.166	0.095	0.738	0.511	0.157	0.035	0.224	0.122	0.324
Goldenrod (n=11)										
Max	34.898	39.436	1.288	0.786	1.995	0.851	0.071	0.623	0.785	0.807
Min	27.549	34.726	1.097	0.066	0.551	0.472	0.000	0.327	0.392	0.140
Average	32.001	37.881	1.187	0.194	1.112	0.657	0.053	0.428	0.547	0.371
STDV	2.305	1.321	0.063	0.207	0.425	0.144	0.020	0.094	0.130	0.235

a. Sugar concentrations presented in weight-by-weight percentage, calculated as $\frac{mass\ of\ the\ sugar\ quantified\ (g)}{total\ mass\ of\ the\ honey\ (g)}$

b. The ratio of fructose content over glucose content



Supplementary Figure 3.1 Accuracy score of different models generated for different training and prediction sets with cross validations. (a) 10-fold cross validation for all 152 samples; (b) 152 samples, trained with the set with pollen analysis directly confirming the botanical origin (n=55); (c) 152 samples, trained with the set that had no variables identified as an outlier (n= 84)



Supplementary Figure 3.2 Principal component analysis (PCA) plot representing clover, buckwheat and blueberry honeys.

CHAPTER IV. BIOSYNTHESIS OF D-ALLULOSE FOR THE BIOTRANSFORMATION OF FUNCTIONAL HONEY

Connecting Statement II

Chapter III covered the profiling of enzymatic activities and sugars for Canadian honeys with different botanical origins and the development of models for their authentication. Selected targeted honey samples were biotransformed into functional ones via the enzymatic conversion of D-fructose into D-allulose. Chapter IV shows the results of this biotransformation and its optimization using D-allulose-3-epimerase. Additionally, the effect of the biotransformation on the physiochemical properties of honey from different botanical origins was also investigated.

4.1 Abstract

This study focused on the application and the optimization of the endogenous biotransformation of D-fructose in honey into D-allulose by D-allulose-3-epimerase (DAEase), aiming at producing a reduced-caloric functional honey. The enzymatic activity of DAEase was higher at 20% and 70% (w/v) clover honey solutions compared to fructose solutions. A three-variable central composite rotatable design was used for the optimization of the following biotransformation parameters: reaction time, initial honey concentration, and DAEase enzymatic units. The biotransformation was assessed by quantifying the amount of produced D-allulose and the bioconversion yield (%, w/w) of fructose. Coupled with the analysis of variance, the optimum reaction conditions were determined with the critical parameters identified. Both honey concentration and reaction time affect the final D-allulose concentration and bioconversion yield significantly with a quadratic model. The optimized parameters were applied for the biotransformation of 12 honey samples from three different monofloral sources, namely clover, buckwheat, and blueberry. Results indicated a significantly lower bioconversion yield of D-allulose in buckwheat sample when compared to clover and blueberry; however, a maximum bioconversion rate of 29.46% could be obtained in clover honeys. While the biotransformation had no significant impact on the viscosity and pH changes of all honey solutions, it reduced the post-reaction total color differences compared to the standard of blueberry honeys.

4.2 Introduction

The demand for low-calorie sugar alternatives has been increasing over the past few decades due their contribution to limiting chronic diseases such as diabetes and obesity (Xie, Li, Ban, Yang, & Li, 2024). Alternative sugars are widely used by the agri-food industry to substitute the sucrose and are characterized by a higher sweetness intensity and/or a lower caloric content, that include synthetic sweeteners (e.g. acesulfame-K and aspartame), sugar alcohol (e.g. erythritol, and sorbitol), sweet glycoside (e.g. stevia, monk fruit extract), rare sugar (eg. D-allulose), and protein sweeteners (e.g. thaumatins, and miraculin) (Mooradian, 2024).

Among alternatives, D-allulose, known as D-psicose, has attracted a high interest due to its low-calorie content (0.4 kcal/g) with low glycemic response, good sweetness ability (70% of sucrose sweetness) and physicochemical properties, such as low hygroscopicity and high solubility (Zhang, et al., 2023; CAS, 2021; FDA, 2020; Drabo, et al., 2023; Jiang, et al., 2020).. D-allulose is structurally the C3 epimer of D-fructose and is generally presented in viscous liquid form or in white crystals with a melting point of 58°C or 109°C (CAS, 2021). As a type of rare sugar, D-allulose can be found naturally in trace amounts in *Itea* plants, wheat, processed cane, steam treated coffee, beet molasses and macroalgae (Bayu, Warsito, Putra, Karnjanakom, & Guan, 2021; Tsukamoto, et al., 2014; Ayers, et al., 2014; Kim, et al., 2020). It was designated as “general regarded as safe” (GRAS) by the FDA in 2014 and is one of the most promising bulk rare sugars to be widely utilized in the food industry and pharmaceuticals (Li, et al., 2024). D-allulose demonstrated prebiotic, anti-diabetic, anti-obesity, anti-hyperlipidemic, anti-atherosclerotic, anti-inflammatory, and neuroprotective effects; in addition, it also showed promising enhancement in food applications such as improved gelling properties and flavor profiles and reduced oxidation during the Maillard reaction, highlighting its potential uses in the food industry (Tsukamoto, et al., 2014; Choi, Kwon, Kim, & Choi, 2018; Xia, et al., 2021; Chen, Gao, & Li, 2022; O'Charoen, Hayakawa, & Ogawa, 2015).

D-allulose production was mainly achieved through chemical synthetic routes; however, expensive substrate, sophisticated processing procedures, hazardous by-product, and long reaction time have hindered the mass production of D-allulose (Drabo, et al., 2023). As a result, D-allulose production was switched from chemical synthesis to biosynthesis via either enzymatic bioconversion or microbiological fermentation (Guo, et al., 2024). For instance, Izumori (2006)

has investigated the use of D-tagatose-3-epimerase (DTEase) from *Pseudomonas sp.* ST-24 to produce D-allulose from D-fructose. Other epimerase enzymes with higher affinity towards D-allulose were discovered and named as D-allulose-3-epimerase (DAEase). In previous studies, DAEase were sourced from various microbial origins such as *Agrobacterium tumefaciens* (Kim, Hyun, Kim, Lee, & Oh, 2006)*, Dorea sp*. (Zhang, et al., 2015), *Treponema primitia* (Zhang, Zhang, Jiang, & Mu, 2015), *Flavonifractor plautii* (Park, et al., 2016)*, Paenibacillus senegalensis* (Yang, et al., 2019), *Novibacillus thermophilus* (Jia, et al., 2021)*, Arthrobacter psychrolactophilus* (Laksmi, Nirwantono, Nuryana, & Agustriana, 2022)*, Blautia produca* (Tang, et al., 2022)*, Ruminiclostridium papyrosolvens* (Yang, et al., 2022).

Honey is an important natural sweetener valued by health-oriented populations. Compared to refined sugar, honey has a lower glycemic index and contains various beneficial bioactive compounds, including vitamins, minerals, and phenolic compounds (Bobiş, Dezmirean, & Moise, 2018). Its therapeutical potentials, such as supporting the immune system through antimicrobial, anti-inflammatory, and antioxidant activities, have been well-studied (Samarghandian, Farkhondeh, & Samini, 2017). However, despite the numerous health benefits honey offers, it is still classified as a source of free sugar, and its daily intake should be moderated (Ahmed, et al., 2023). As one of the major sugar components, fructose comprised approximately 38.4% of the total content of honey (Khan, et al., 2018). Hence lowering its concentration by endogenous bioconversion into D-allulose would help lowering its caloric content and further improve its nutritional profile and health benefits. The aim of the present study is to investigate and optimize the catalytic efficiency of DAEase from *Dorea sp.* in a naturally fructose-rich sweetener, honey, for the production of a fortified functional product that contains allulose to reduce caloric content. Additionally, the physicochemical properties of biotransformed honeys were investigated.

4.3 Material and Methods

4.3.1 Materials

Escherichia coli BL21 (DE3) competent cell, yeast extract, tryptone, sodium phosphate monobasic, sodium phosphate dibasic, carbenicillin disodium, imidazole and lysozyme DNAse were purchased from Millipore Sigma (Oakville, Canada). Sodium chloride and isopropyl-β-d-1-thiogalactopyranoside (IPTG) were purchased from Fisher Scientific (Ontario, Canada). His-Trap FF column was purchased from Cytiva. Luria-Bertani (LB) agar was purchased from BioBasic

(Markham, Canada). Clover, buckwheat and blueberry honeys were purchased from local (Montreal) retail stores and stored in amber vials at -20 °C before the experiment.

4.3.2 Expression and purification of selected D-allulose-3-epimerase

The targeted gene sequence of DAEase from *Dorea sp.* (accession no CDD07088.1) was obtained from Genebank database according to the study conducted by Zhang et al (2018). The gene was synthesized by GeneScript (USA) and cloned into the expression vector pET-22b (+) with an added His-tag to the C-terminal, and *E.coli* BL21 (DE3) competent cell was selected as the expression host. The recombinant plasmid transformation was performed by mixing 2 µl of diluted plasmid (25 ng/ µl) with 50 µl competent cell suspension, then incubated on ice for 30 min. The suspension was thereafter placed in 42 °C hot water bath for 45 s, followed by the addition of 450 µl LB broth without antibiotic, and incubated at 200 rpm under 37 °C for 1 hr. The suspension was concentrated by centrifuging at 10,000 G for 3 min then resuspended in 50 µl LB broth and was inoculated onto a carbenicillin (100 µg/ml) contained LB agar plate to incubate overnight at 37 °C. Saturated liquid cultures were obtained by incubating one colony from the plate in 2 ml of LB broth containing carbenicillin (100 µg/ml) at 250 rpm and 37 °C for 20-24 hrs. Upon removing the liquid culture from the incubator, it was mixed with 50% glycerol at 1:1 ratio, then stored in -80 °C freezer until further usage.

The recombinant strain was precultured into LB agar plate containing carbenicillin (100 µg/ml), and the preculture was incubated overnight at 37°C, 250 rpm. The preculture was then added into LB broth supplemented with carbenicillin (100 µg/ml) at 1: 50 ratio (v/v) and incubated for 4-6 hrs at 37°C 250 rpm. When the OD_{600} reaches 0.6, IPTG was added for a final concentration of 1mM to induce enzyme expression. Growth of the culture continued at 28°C and 250 rpm for 6 hrs. The cell harvesting was accomplished by centrifuging the broth at 4,500 rpm, 4 °C for 15 min, and the pellets were stored at -80 °C until further processing. The recovered pellets were suspended in lysis buffer (50 mM phosphate buffer, 300 mM NaCl, pH 6.5; 4 ml/g pellet) with the addition of lysozyme (4 mg/g) and DNase (4 µl/ g; 1 mg/ml concentration). The cell suspension was incubated at 18°C under 50 rpm for 1 hr and disrupted via ultrasonication with a microtip in an ice bath for 6 cycles of 15 kHz, 10 seconds on 60 seconds off. The cell debris were removed by centrifuging at 4 °C, under 10,000 rpm for 1 hr and collecting the enzyme containing supernatant.

Ultrafiltration with 3 kDa membrane filter using an ultrafiltration cell (Amicon, Millipore) was performed to condense the crude enzyme extract (supernatant) before purification. The DAEase enzyme was purified via immobilized metal affinity chromatography using a Nickel column (His-Trap FF). Before loading, the enzyme was passed through a 0.22 µm filter and the column was equilibrated with 5 column volume of lysis buffer (50 mM phosphate buffer, 300 mM NaCl, pH 6.5). Then the loaded column was subsequently washed with 13 column volume of lysis buffer and 13 column volume of wash buffer (50 mM phosphate buffer, 300 mM NaCl, 50 mM imidazole, pH 6.5). Finally, the targeted enzyme was eluted in 4 column volume of elution buffer (50 mM phosphate buffer, 300 mM NaCl, 200 mM imidazole, pH 6.5). The purity of the DAEase enzyme was confirmed with Tris-glycine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis at 120 V using freshly cast 15% SDS polyacrylamide gels. Molecular weights of the protein subunits were determined with all protein segments stained with Coomassie Brilliant Blue R250 (Bio-rad,) and distained with an aqueous solution of 10% (v/v) methanol and 10% (v/v) acetic acid, then compared to a premix protein ladder.

4.3.3 D-allulose-3-epimerase activity assay

The epimerization activity of DAEase was measured by the production of D-allulose from D-fructose under standard conditions. The substrate solution consisted of 5% fructose solution in pH 6.0, 50 mM phosphate buffer containing 1 mM Co^{2+}. The enzyme-substrate mixture was incubated in a hot water bath at 70 °C for 3 and 6 min, then boiled in hot water for 10 min to cease the reaction. The quantification of the fructose and D-allulose was carried out by high performance anion exchange chromatography (HPAEC) using a Dionex ICS-3000 system. To separate and detect the two epimers, CarboPac PA20 column (3 x 150 nm) and a pulsed amperometric detector (PAD) were used with an isocratic mobile phase made of 5 mM sodium hydroxide in MilliQ water at a flow rate of 0.25 ml/min. The chromatograms were obtained and integrated using the Chromeleon™ Chromatography Management System. The unit of the DAEase was expressed as 1 µmol D-allulose produced from D-fructose per min at 70 °C, pH 6.0.

4.3.4 Biotransformation reactions

The biotransformation reactions of pure fructose and clover honey-based fructose by DAEase from *Dorea sp.* were carried out at 40 or 70°C over 90 min time course using a substrate concentration of 5% in 100 mM phosphate buffer (pH 6, 1 mM Co^{2+}). Aliquots were collected at 5, 10, 20, 40,

60, and 90 min. To assess the effect of honey concentration, the biotransformation was carried out in the presence of 20% and 70% (w/v) honey at 40 °C over 12 hrs reaction time period. To ensure a complete cessation of the reaction, the collected samples were boiled for 10 min and subsequently stored at -20°C. The concentration of D-allulose produced and fructose consumed was measured by HPAEC-PAD. Standard curves were constructed using different concentrations of fructose and D-allulose (3-10 ppm).

4.3.5 Optimization of the biotransformation of honey

The effects of reaction parameters on the honey biotransformation was investigated using response surface methodology (RSM). A five-level, three variable, central composite rotatable design was created using Design Expert software. RSM is a robust statistical technique that is widely implemented in process development, and optimization (Lamidi, et al., 2023). The reaction parameters investigated include the DAEase units (3-9 U/ml reaction), concentration of the honey solution (50% - 90%, w/v), and the reaction time (3-20 min). Other parameters such as the reaction temperature (70 °C), buffer (100M phosphate buffer, pH 6), and co-factor (1 mM Co2+) were kept constant. The amount of D-allulose (mg/ml) and the bioconversion yield (%, w/w) were quantified as responses. The bioconversion yield is defined as the ratio of the final D-allulose concentration to the initial D-fructose concentration in the reaction mixture.

4.3.6 Effect of floral type on the bioconversion of honey

Three types of monofloral honeys - clover, buckwheat, and blueberry - were selected for investigating the effect of floral type. This selection was based on carbohydrate profile and enzymatic activity levels (e.g. diastase, invertase, acid phosphates, glucose oxidase and catalase). Four samples of each type of honey were selected, considering both fructose concentration and invertase activity. The samples included pairs with high and low levels of both invertase activity and fructose content to ensure a comprehensive analysis.

4.3.7 Characterization of biotransformed honeys

4.3.7.1 End-product profiles

The end-product profiles of bioconversion reactions were analyzed using Agilent 1290 Infinity II LC system coupled to the 6560 ion mobility Q-TOF-MS (Agilent Technologies, Santa Clara, USA) . The analytes were separated on an InfinityLab Poroshell 120 HILIC-Z column (2.1 x 100

mm, 2.7µm). Mobile phase A was LC/MS grade water with 0.25% ammonium hydroxide, and mobile phase B was acetonitrile with 0.25% ammonium hydroxide. The flow rate was set at 0.4 ml/min with a column temperature at 35°C and an injection volume of 1 µl. The mobile phase profile used for the run in negative ion mode started with 85% B (0-0.5 min), decreased to 79% B (0.5-3 min), then to 60% B (3-5 min), then to 40% B (5-5.5 min), held at 40% B (5.5-7.5 min), increased to 85% B (7.5-8 min), held at 85% B (8-9 min). The mass spectrometer was equipped with a Dual AJS ESI ion source operating in negative ionization mode. The MS conditions were set as follows: the drying gas temperature at 250 °C, drying gas flow at 11 L/ min, sheath gas temperature at 325 °C, sheath gas flow at 12 L/ min, pressure on the nebulizer at 30 psi, capillary voltage at 4000 V, nozzle voltage at 2000 V, and the fragmentor voltage at 200V. Full scan MS data were recorded between mass-to-charge ratios (*m/z*) 100-1700 with a scan rate of 2spectra/s, and were collected at both centroid and profile mode. Data treatment was conducted using Quantitative Analysis 10.0 from Agilent MassHunter Workstation Software. The samples were prepared by dilution in 50:50 ACN/water (v/v).

4.3.7.2 Rheology related properties

The biotransformed honeys were subjected to flow test in order to determine the flow behavior index (n), consistency coefficient (m), and apparent viscosity (ŋ) at increasing shear rates ($\hat{y}$, 1-100 s^{-1}) using a stress-controlled rheometer (AR 2000 Rheometer, TA Instrument, New Castle, DE, US) fitted with a stainless steel 60 mm cone of 2° and solvent trap. The rheology properties were determined at 22°C using a circulating bath and a controlled Peltier system. The equipment control, parameter modification and data treatment were conducted using TA Rheology Advantage. The Power-law model was applied for the calculations of the apparent viscosity (Berker, 2002):

$$ŋ = m\,\hat{y}^{(n-1)} \qquad \text{Equation 1}$$

4.3.7.3 Physicochemical properties

The physicochemical properties of biotransformed honeys were investigated and compared to the corresponding original honeys. The pH was ascertained with Mettler Toledo pH meter. The color was characterized using a colorimeter (CR-400 Chroma Meter) equipped with a quartz cell (CM-A97, 2 mm) and the corresponding cell holder for low opacity liquid measurement (Konica Minolta). Data treatment was conducted using the color data software SpectraMagic NX2 version

1.2 (Konica Minolta). Measurements were made in duplicates, and the composite color difference (ΔE^*_{ab}) were calculated using the following equation. ΔE^*_{ab}value greater than 1 would indicate a color difference between two samples that are detectable with naked eyes (Ly, Dyer, Feig, Chien, & Bino, 2020).

$$\Delta E^*_{ab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \qquad \text{Equation 2}$$

To investigate the effect of biosynthesis of endogenous D-allulose on the color of the honeys, ΔE^*_{ab} was measured using the 78% (w/v) honey before (78%-B) and after (78%-A) the enzymatic reaction and using 78%-A and the 100% (w/v) honey solution. Additionally, ΔE^*_{ab} was calculated for 78%-B and the 100% (w/v) honey solution to assess the color changes after diluting honey.

4.4 Results and Discussion

4.4.1 DAEase expression and catalytic efficiency

D-allulose-3-epimerase (DAEase) gene from *Dorea sp.* was expressed in *E. coli* BL21 (DE3). The purified recombinant DAEase obtained through a one-step of nickel affinity chromatography showed one band at 32 kDa on SDS-polyacrylamide gel (**Fig 4.1**). This result is in agreement with the molecular weight reported by Zhang et al. (2015) for DAEase from *Dorea sp.*

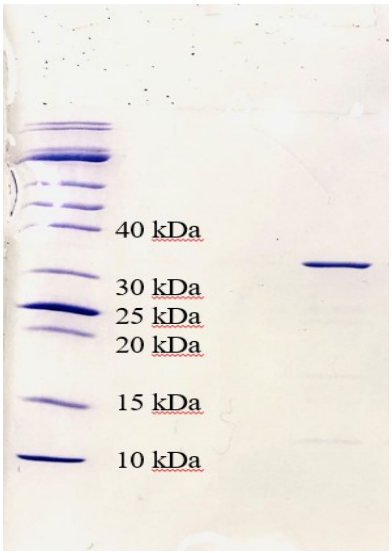


Figure 4.1 SDS-PAGE of the purified enzyme (Left: protein ladder, right: purified DAEase).

The DAEase activity of the crude enzyme extract and its catalytic efficiency to produce D-allulose were assessed using 5% (w/v) pure fructose or honey-based fructose solution at 40°C and 70°C and pH 6.0. These temperatures were chosen based on the enzyme's catalytic efficiency, with the highest activity observed at 70°C and a longer half-life at 40°C (Zhang W. , et al., 2018). It has been reported that the reaction temperature, pH, and the presence of the Co^{2+} co-factor are critical factors for maximizing the enzymatic activity and catalytic efficiency of DAEase enzyme (Zhang, et al., 2018). Under these specific conditions, the enzymatic epimerization of fructose into D-allulose is the predominant conversion reaction.

In order to confirm the bioconversion of D-fructose present honey into D-allulose, LC/MS-QToF was used to characterize the end-products of blank honey and biotransformed honey. The chromatogram of blank honey solution without enzymatic treatment showed only fructose and glucose peak, whereas a monosaccharide (RT=1.536-1.597 min) with the same retention time as the allulose standard was obtained in the biotransformed honey sample after enzymatic treatment. To further confirm the identity of the newly emerged monosaccharide, its MS/MS fragmentation spectra was obtained (**Fig 4.2**) showing a similar pattern to that of the allulose standards. This result confirmed the production of allulose in honey via enzymatic treatment.

Fig. 4.3 shows the relative activity of DAEase in catalyzing D-allulose production and the D-allulose concentration in the reaction mixtures of 5% (w/v) pure fructose solution and a diluted honey solution with a final fructose concentration of 5% (w/v). The results show no further increase in D-allulose concentration beyond 20-min incubation at 70°C. A maximum D-allulose concentration of 9.57 mg/ml was obtained in the presence of pure fructose, while 12.93 mg/ml concentration was achieved in the honey solution used as a fructose source.

DAEase demonstrated elevated activity in reactions using diluted honey as the fructose source compared to the activity calculated from 5% fructose reaction, with an increase of 10% and 17% for 5-min and 10-min sample detected, respectively. Stable epimerization activity was observed during the course of the reaction incubated at 40°C, during which relative activity stabilized between 12% to 18%, and 11% to 19% for 5% fructose and diluted honey reactions, respectively.

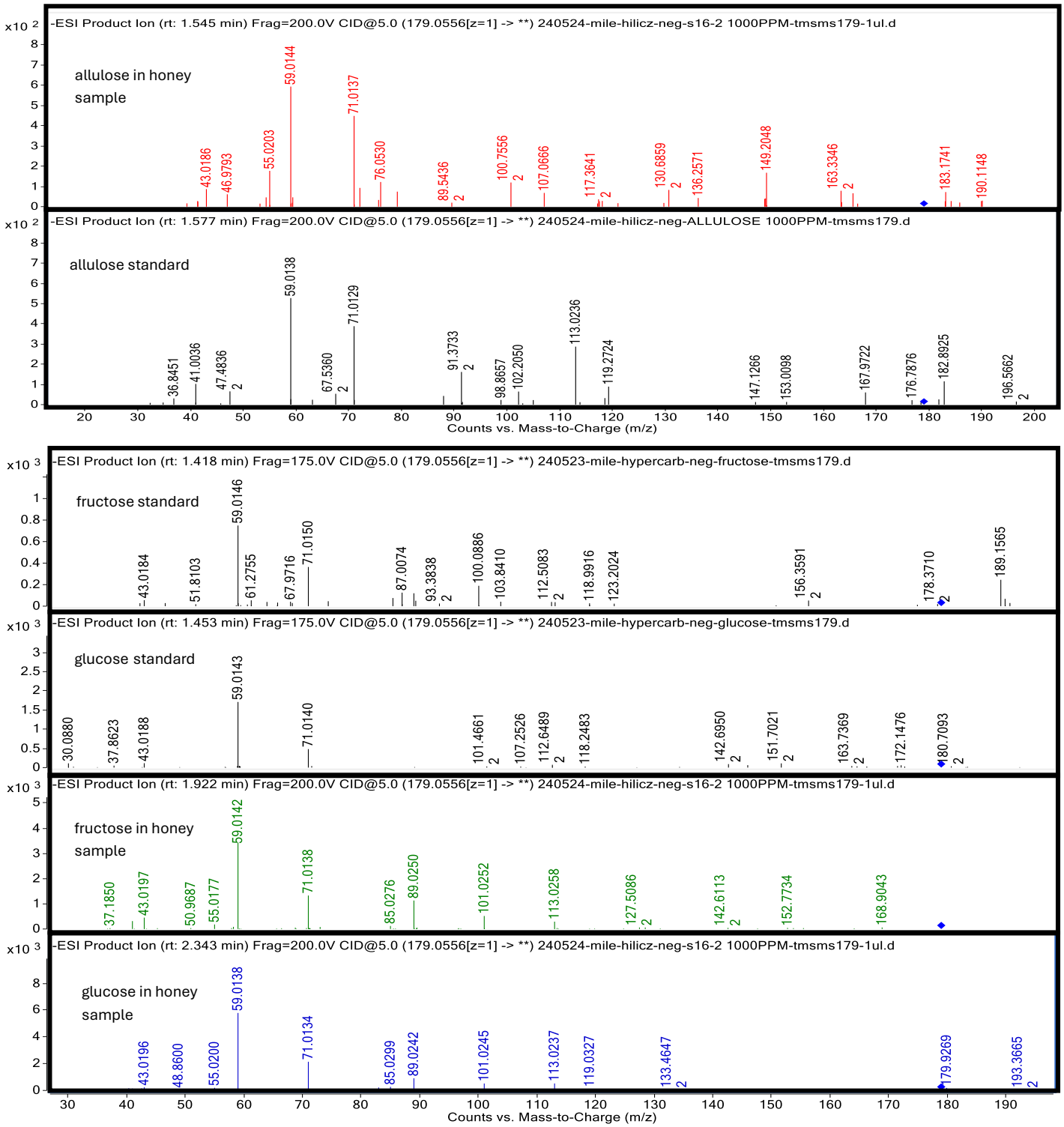


Figure 4.2. MS-MS fragmentation spectra of allulose, fructose, and glucose standards and in the bioconversion end-product (from top to bottom: allulose in honey and allulose standard (RT:1.53), fructose standards (RT: 1.88), glucose standards (RT: 2.32), fructose in honey, glucose in honey)

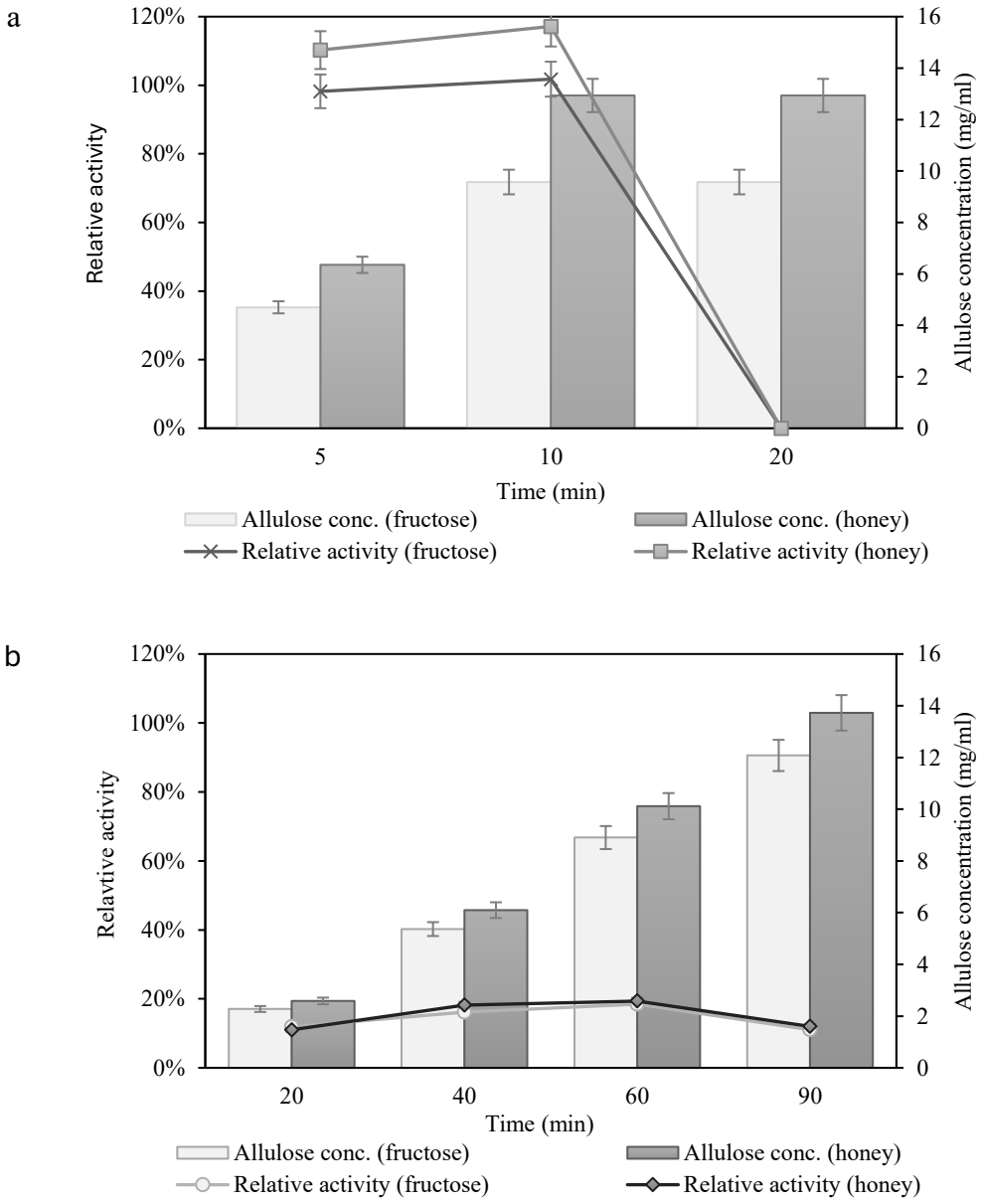


Figure 4.3. D-allulose concentration (mg/ml) and relative activity (%) of DAEase in the reaction mixture containing 5% (w/v) initial fructose from pure fructose and honey at 70°C (a) and 40°C (b).

Compared to previous study, which reported a relative activity of 40% for DAEase from *Dorea* sp. at 40°C relative to 70°C, the enzyme in this study requires higher activation energy, as evidenced by the significant increase in the enzyme activity when the reaction temperature was raised from 40°C to 70°C (Zhang, et al., 2015).

The results indicate that the bioconversion yield reached 24.16% (corresponding to 12.08 mg/ml D-allulose final concentration) and 27.46% (corresponding to 13.73 mg/ml D-allulose final concentration) after 90 min incubation at 40°C, in pure fructose solution and in honey mixture, respectively. Extending the reaction time to 17 hrs, ultimately resulted in a final bioconversion yield of 28.17% in the honey solution (data not shown). This result aligned with the findings of Zhang's team (2015) on the epimerization efficiency of the DAEase obtained from the wild-type *Dorea sp.*, in which the enzyme was found to be able to achieve up to 30% bioconversion yield at equilibrium state.

4.4.2 Biotransformation of honeys catalyzed by DAEase

The time course for the bioconversion of clover honey catalysed by DAEase was investigated using 20 and 70% honey concentrations. The epimerisation of fructose present in honey into D-allulose catalysed by DAEase was carried out at 40 or 70°C. **Fig 4.4** shows the time course for the production of D-allulose over 240 min at 70°C and 12 hr at 40°C. At 70°C, a linear increase in the allulose content was observed within the first 20 min in the reaction containing 70% (w/v) honey to reach 102.07 mg/ml concentration (corresponding to 36.45% bioconversion yield) and remained more or less constant at 60 min; thereafter, a slow decrease in the allulose content to 87.65 mg/ml was obtained after 240 min bioconversion. This decrease could be the result of the reverse epimerization, during which allulose presented in the reaction mixture exceeded the equilibrium state, hence triggers the conversion of D-allulose back to D-fructose (Tan, et al., 2023). A linear increase in the allulose content during the first 4 hrs of incubation at 40°C (**Fig 4.4b**), indicated that the enzyme retained its activity and remained stable, which matches the thermal stability results of the DAEase from wild-type *Dorea sp* strain of having over 70% residual activity after 4 hrs at 40°C incubation (Zhang, et al., 2015).

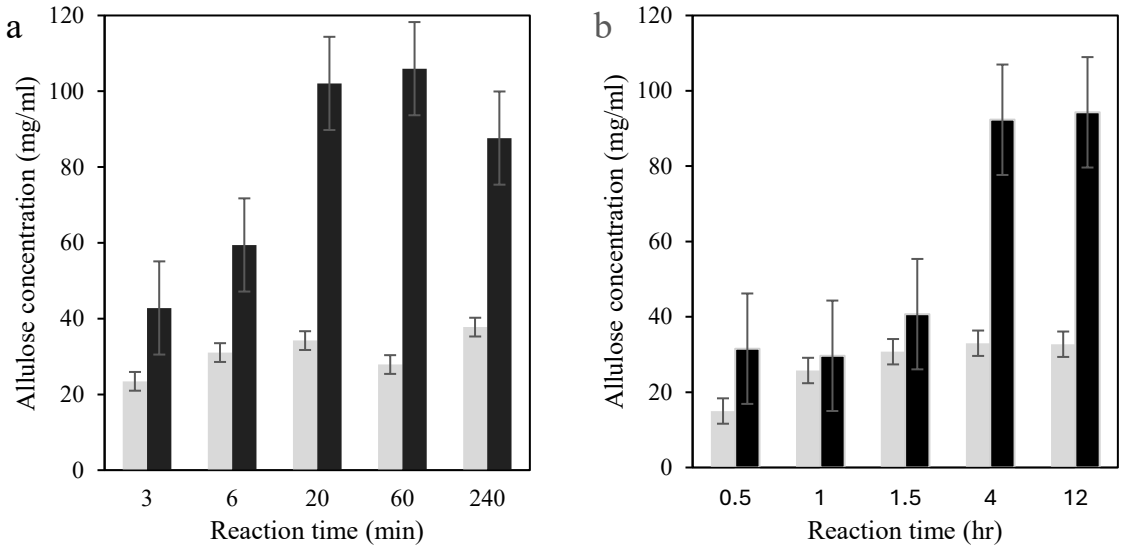


Figure 4.4 Allulose concentrations (mg/ml) produced in honey samples upon biotransformation catalyzed by D-allulose-3-epimerase (DAEase) from *Dorea sp* at (a) 70°C and (b) 40°C.(20% ( ), 70% ( ) honey solution)

At 40°C, the maximum D-allulose content of 94.29 mg/ml (corresponding to 33.68% bioconversion yield) was achieved after 12 hrs of bioconversion in the reaction containing 70% (w/v) honey. For the reaction performed in 20% (w/v) honey solution, the D-allulose production reached the highest value of 37.75 mg/ml (corresponding to 47.18% bioconversion yield) after 4 hrs incubation at 70°C, with a slight decrease in its concentration from 20 min to 1 hr. The reaction, containing 20% honey performed at 40°C, resulted in a maximum concentration of allulose of 32.99 mg/ml (corresponding to 40.91 bioconversion yield) after 4 hrs of reactions. At both honey concentrations, a higher maximum bioconversion was achieved when incubating at 70°C within a shorter period, while the enzyme showed a higher stability and relatively comparable final yield when incubating at 40°C. According to literature, the yield of the allulose production from fructose via free-form enzymatic epimerization varied from 20.0 to 38.9% using various fructose concentrations and enzyme sequence sourced from various bacteria (Li C. , et al., 2021). High bioconversion yield was achieved when introducing the enzyme into the food matrix, honey.

Several studies exploring the possibility of utilizing DAEase to produce functional beverages employed cascades of enzymes, which contains invertase for the generation of more fructose (Li, et al., 2021; Li, et al., 2022). Honey, on the other hand, naturally contains invertase which would aid in the production of D-allulose, hence, making honey as a promising starting material for the production of D-allulose enriched functional food.

4.4.3 Optimization of D-allulose production in honey

The effects of reaction parameters, including honey concentration (50-90%, w/v), reaction time (3-20 min), enzyme unit (3-9U/ ml reaction), on the bioconversion of honey by DAEase were studied. Clover honey was selected as the substrate honey type for the optimization processes, due to its renowned popularity worldwide as a monofloral honey. The D-allulose concentration as well as the bioconversion yield (% w/w) were used as responses, and the results are displayed in **Table 4.1**. The lowest D-allulose concentration of 17.87 mg/ml was recorded with formulation #10, while the highest concentration of 46.87 mg/ml was recorded with formulation #3. However, taking into consideration the differences in honey concentrations, hence the initial fructose content within the reaction, the highest and the lowest bioconversion yield of 7.6% and 17.5% were obtained with formulation #15 and #3, respectively.

Table 4.1 Experimental design factors and responses for the bioconversion of fructose into allulose in honey samples

Reactions	Factor 1	Factor 2	Factor 3	Response 1	Response 2
	Honey (%, w/v) [a]	Reaction time (min)	Enzyme (U)	Allulose concentration (mg/ml)	Bioconversion yield (%) [b]
1	58.1	6.4	7.8	33.10	15.0
2	81.9	6.4	7.8	30.50	9.8
3	70.0	11.5	9.0	46.87	17.6
4	81.9	16.6	4.2	40.64	13.0
5	58.1	16.6	7.8	25.43	11.5
6	70.0	20.0	6.0	26.16	9.8
7	70.0	11.5	6.0	41.58	15.6
8	70.0	11.5	6.0	40.29	15.1
9	58.1	16.6	4.2	32.73	14.8
10	58.1	6.4	4.2	17.87	8.1
11	50.0	11.5	6.0	21.16	11.1
12	90.0	11.5	6.0	41.01	12.0
13	70.0	11.5	6.0	40.03	15.0
14	70.0	11.5	3.0	26.56	10.0
15	81.9	6.4	4.2	23.77	7.6
16	70.0	11.5	6.0	46.03	17.3
17	81.9	16.6	7.8	32.25	10.3
18	70.0	3.0	6.0	26.65	10.0
19	70.0	11.5	6.0	41.33	15.5
20	70.0	11.5	6.0	45.51	17.1

a. The honey concentration expressed in percentage weight by volume corresponds to the quantity of the selected honey sample in the reaction mixture before the addition of the enzyme

b. The bioconversion yield was calculated as the amount of allulose produced in the end-product over the initial fructose content in the honey sample in % (w/w)

Table 4.2 Analysis of variance (ANOVA) for allulose production using DAEase in honey solution

	Allulose concentration (mg/ml)[a]			Bioconversion yield (%, w/w)[b]		
	F-value	**p-value**	**Equation**	**F-value**	**p-value**	**Equation**
Model[c]	8.79	0.0006	-234.40590	10.04	0.0003	-292.49835
A	5.81	0.0329	+16.27601	9.07	0.0108	+22.43916
B	9.39	0.0098	+4.54748	1.06	0.3242	+5.83284
C	2.22	0.1622	+8.83165	2.99	0.1092	+13.10522
AC	8.60	0.0125	-0.522004	10.00	0.0082	-0.726602
A^2	3.65	0.0802	-0.717840	3.84	0.0737	-0.950497
B^2	12.78	0.0038	-0.030221	15.26	0.0021	-0.042642
C^2	24.59	0.0003	-0.232077	34.84	< 0.0001	-0.356645
LOF	4.37	0.0618		3.44	0.0963	

a. R^2= 0.8368, adjusted R^2=0.7416, predicted R^2=0.3086, Adequate precision=8.9694

b. R^2= 0.8541, adjusted R^2=0.7691, predicted R^2=0.4376, Adequate precision=9.1381

c. A: enzyme unit, B: honey concentration, C: reaction time, LOF: lack of fit

Table 4.2 shows the analysis of variance (ANOVA) of the models used for each response. F-value helps with the assessment of the significance of different models, and the p-value indicates if the variation of the response variables observed with the model are due to the existence of outliers. Lack of fit (LOF) for the model analysis determines if the model sufficiently captures the true relationship between variables. Higher F-value and lower p-value (< 0.05) are desired for the models as they suggest significance in the variable affecting the response. However, high F value and low p value for the LOF, on the other hand, would suggest potential improvement in the model due to extremes and bias (Weisberg, 2013; Sarabia & Ortiz, 2009). The slight decrease in the adjusted R^2 (D-allulose concentration: 0.74, bioconversion yield: 0.76) from R^2 (D-allulose concentration: 0.83, bioconversion yield: 0.85) for models generated for the two responses indicates the potential for model improvements. However, as all values are above or only slightly below 0.75, the current model remains valid for implementation. The LOF for the linear and quadratic models generated for both D-allulose concentration and bioconversion yield has an F-value of 4.37 (p-value of 0.0618) and 3.44 (p-value of 0.0963) respectively. Overall, the current model does not exhibit a significant LOF and is effective enough to capture the relationship among variables without the need to implement more complex models.

Due to difference in the initial honey concentration for different reactions, the results of allulose concentration are not proportional to those of bioconversion yield. In the linear model of allulose concentration response, the honey concentration (F-value of 9.39, p-value of 0.0098) is the most significant parameter, whereas the same parameter has no significant effect on the bioconversion yield according to its predictive linear model. In contrast, the linear effect of enzyme unit is statistically significant in both predictive linear model of allulose concentration (F-value of 5.81, p-value of 0.0329) and bioconversion yield (F-value of 9.07, p-value of 0.0108). The interactive effect of enzyme unit and time was significant in the predictive linear model of allulose concentration (F-value of 8.6, p-value of 0.0125) and quadratic model of bioconversion yield (F-value of 10, p-value of 0.0082). The quadratic model on the two responses suggests that honey concentration and reaction time have greater impact on both allulose concentration (F-value of 12.78, p-value of 0.0038 and F-value of 24.59, p-value of 0.0003, respectively) and bioconversion yield (F-value of 15.26, p-value of 0.0021 and F-value of 34.84, p-value < 0.0001, respectively). Among these, reaction time has the most significant effects due to its highest F value and lowest p-value. (**Table 4.2**).

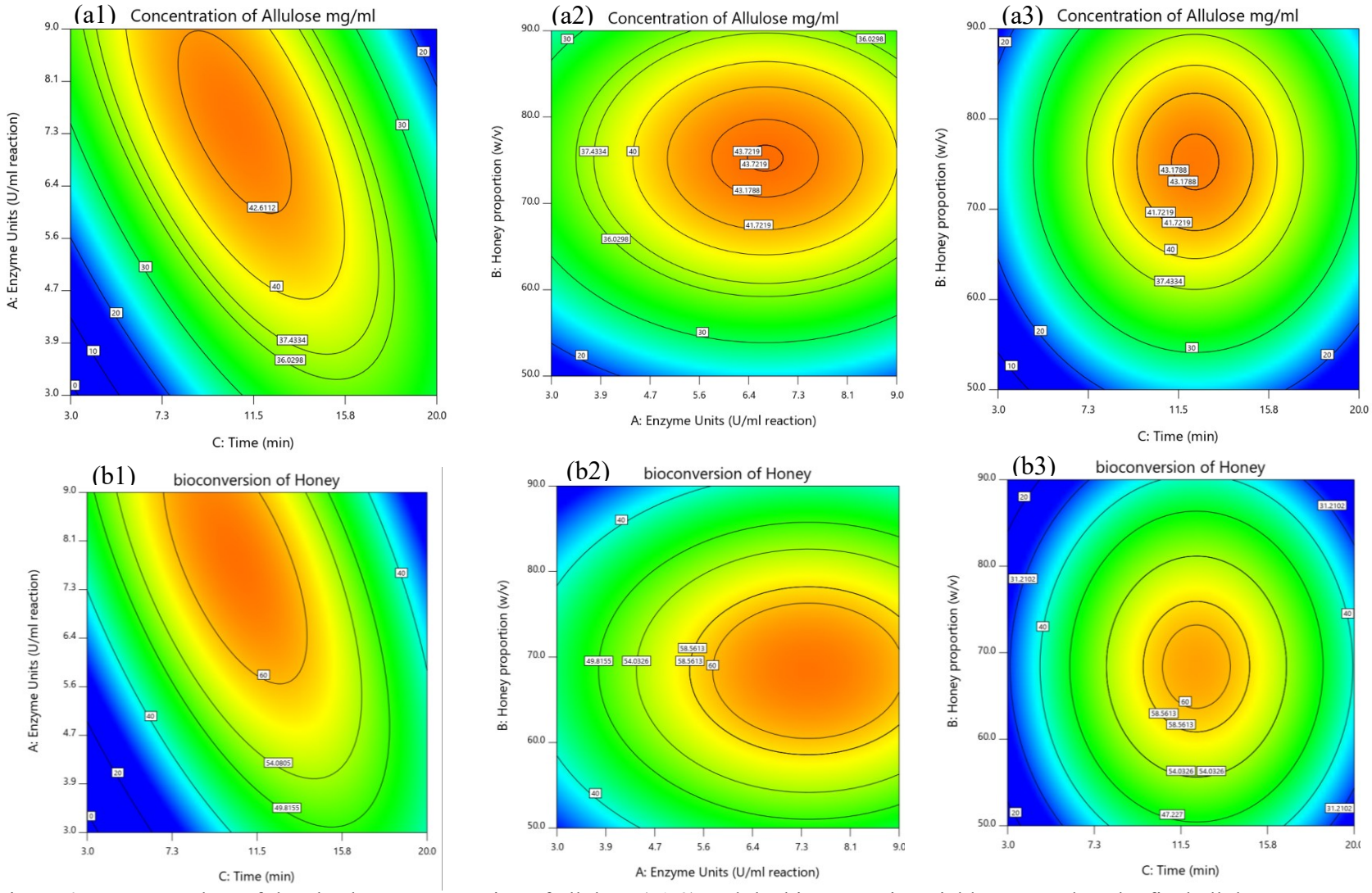


Figure 4.5 Contour plots of the absolute concentration of allulose (a1-3) and the bioconversion yield expressed as the final allulose concentration over the initial fructose concentration (b1-3). All reactions of biotransformation of honey by D-allulose-3-epimerase (DAEase) from Dorea sp were carried out at 70°C at pH 6.0.

Table 4.3 Selected monofloral honeys (blueberry, buckwheat, clover) used for the bioconversion reaction and their corresponding enzymatic activities (diastase, invertase, acid phosphatase, GOX and CAT), sugar content (glucose, fructose, and sucrose), and F/G ratio.

Honey sample[a]	Diastase (DN)[ab]	Invertase (IN)[ab]	Acid phosphatase (U)[b]	GOX (U)[ab]	CAT (U)[ab]	Glucose (%)[c]	Fructose (%)[c]	F/G ratio[ac]	Sucrose (%)[c]
B1	8.37	3.89	15.38	1.58	33.80	36.40	40.02	1.10	0.06
B2	<1.00	5.07	69.97	0.63	32.46	31.83	38.86	1.22	0.07
B3	10.02	0.58	25.85	0.63	33.84	33.32	38.07	1.14	0.09
B4	9.00	1.58	14.05	9.13	34.09	35.40	39.19	1.11	0.10
Bw1	23.22	14.32	1125.86	9.13	23.96	32.27	38.50	1.19	0.08
Bw2	1.54	<0.10	760.77	0.08	28.50	30.52	34.15	1.12	0.13
Bw3	16.15	9.55	993.25	0.50	14.15	35.51	36.57	1.03	0.08
Bw4	<1.00	<0.10	646.92	0.02	23.22	34.89	47.15	1.35	0.11
C1	25.36	12.05	74.03	11.62	20.44	25.21	32.83	1.30	0.10
C2	15.05	1.09	16.09	0.60	6.45	37.31	40.81	1.09	0.13
C3	16.74	7.72	98.02	1.59	7.04	25.25	35.48	1.41	0.20
C4	9.96	4.54	12.49	3.18	8.84	44.54	45.10	1.01	0.10

a: B: blueberry honey, Bw: buckwheat honey, C: clover honey, DN: diastase number, IN: invertase number, GOX: glucose oxidase, CAT: catalase, F/G ratio: fructose to glucose ratio

b: DN: diastase number, 10 mg starch hydrolyzed by 1 g of honey per hr; IN: invertase number, 10 mg sucrose hydrolyzed by 1 g of honey per hr; acid phosphatase: mg P/100g honey/24hr, COX: µg H_2O_2/g honey/ hr, CAT: µg H_2O_2/g/min

c: Glucose, fructose, and sucrose content expressed in % w/w in pure honey; F/G ratio: calculated as the mass of fructose over glucose in pure honey.

Fig 4.5 displays the contour plots of the predictive models. According to the ANOVA analysis, **Fig. 4.5 a1 and b1** reveal similar patterns, where medium to high enzyme units (5.6–9.0 U/mL) positively influence both D-allulose concentration and bioconversion yield in the early stages of the reaction. The level of responses significantly increased within the reaction time range of 7.3–15.8 minutes while maintaining the enzyme unit at above 5.6 U/ml. Reaction time, with its narrower effective range, was identified as a more critical parameter. Nevertheless, longer reaction time (15.8- 20 min) decreased both allulose concentration and bioconversion yield. These observations can be attributed to the occurrence of the reverse reaction of epimerization (Tan, et al., 2023). **Fig 4.5 a2**, **a3** and **b2**, **b3** indicate that the use of the medium levels of enzyme unit (5.6-8.1 U/ml), honey concentration (65-80%, w/v) as well as reaction time (9.5-15 min) can result in a higher allulose concentration and bioconversion yield. The inclusion of honey concentration further narrows down the range of enzyme unit and reaction time in the predictive models. Aligned with the results suggested by the ANOVA, both honey concentration and reaction time shows a stronger quadratic effect on the two responses, allulose concentration and bioconversion yield.

4.4.4 Effect of honey floral type on the bioconversion of fructose into D-allulose

The bioconversion of 12 honey samples from three different monofloral type by DAEase was carried out using the identified optimal conditions. The honey samples showed different enzymatic profiles and sugar content (**Table 4.3**). The fructose contents of the blueberry, buckwheat and clover honey samples were ranged between 38.07- 40.02%, 34.15- 47.15% and 32.83- 45.10% (w/w), respectively. The invertase activity for the blueberry, buckwheat and clover samples varied between 0.58- 5.07 IN, less than 0.1 to 14.32 IN, and 1.09- 12.05 IN, respectively. Diastase, acid phosphatase, glucose oxidase and catalase activities, as well as the glucose and sucrose content are also displayed in **Table 4.3**.

Based on the models, the optimal bioconversion conditions were identified to be reaction time of 10 min, enzyme unit at 7.6 U/ ml and honey concentration at 71-78 % (w/v). Because the RSM suggested a higher hypothetical D-allulose final concentration of 44 mg/ml (corresponding to 14.10% bioconversion yield) with 78% (w/v) honey solution compared to 41 mg/ml (corresponding to 14.44% bioconversion yield) with 71% (w/v) honey solution, the former honey concentration was prepared for the test trails with 100 mM phosphate buffer (pH 6.0) to validate the model.

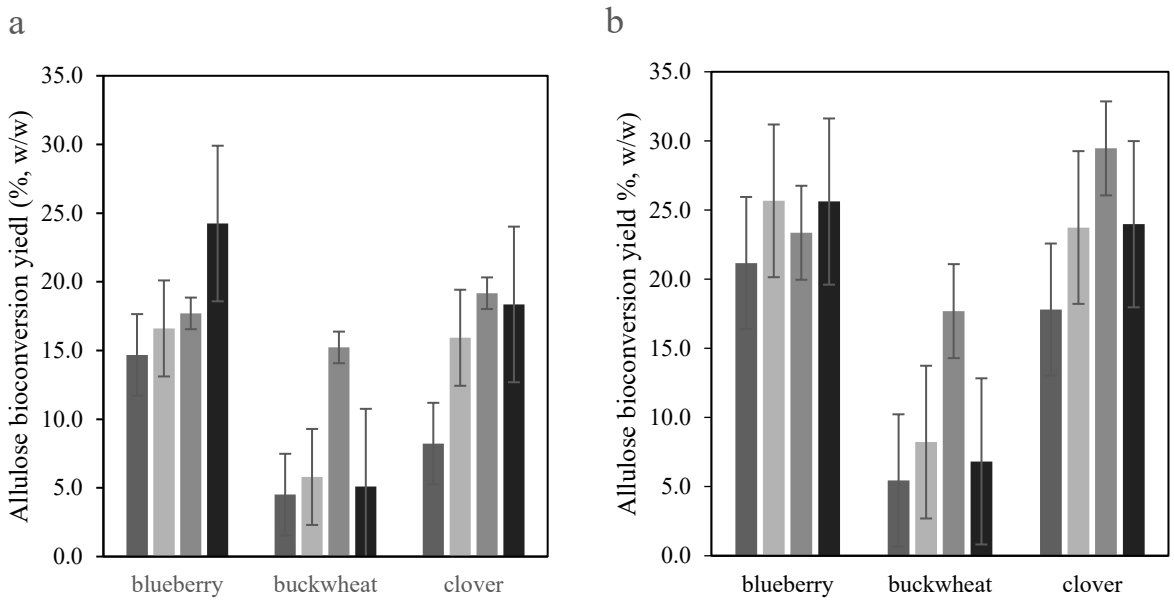


Figure 4.6. Bioconversion yield of allulose in honey sample reactions under the optimum temperature (from left to right: B1-B4, Bw1-Bw4, C1-C4). (a) bioconversion yield after 10 min reaction; (b) bioconversion yield after 30 min reactions. Conversion yield was calculated as the final allulose concentration/ initial fructose concentration (%, w/w).

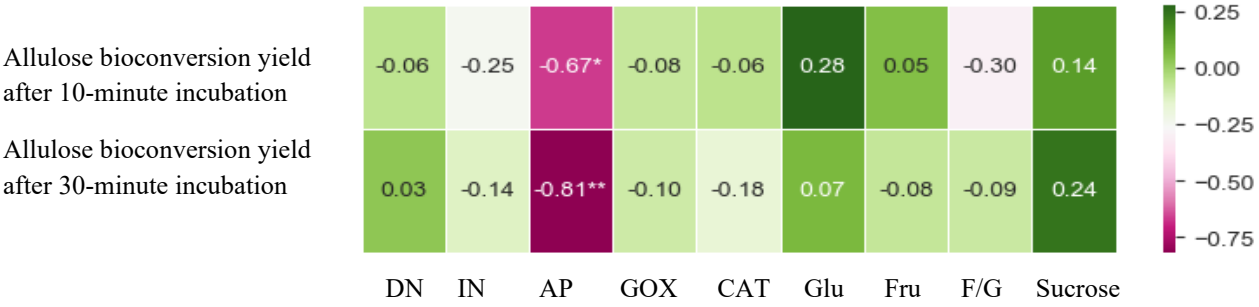


Figure 4.7 Correlation analysis between the bioconversion yield of allulose in honey samples after10 min or 30 min with the enzymatic activities (DN: diastase, IN: invertase, AP: acid phosphatase, GOX: glucose oxidase, CAT: catalase) and sugar content (Glu: glucose, Fru: fructose, F/G: fructose to glucose ratio, sucrose). (*: p-value <0.1; **: p-value <0.01)

With the biotransformation parameters to maximize D-allulose production confirmed, an average of 46.63±18.57 mg/ml final concentration of D-allulose and a bioconversion yield of 15.42±4.99% were obtained in clover honey samples (**Supplementary Table 4.1**). The experimental responses align with the predicted value; hence the predicted optimum bioconversion conditions were valid to apply to honey from other botanical origins. The bioconversion yield of each sample reaction after 10 min and 30 min incubation are displayed in **Figure 4.6**. The highest bioconversion yield at 10 min were found in sample B4 (24.25%, w/w), Bw3 (15.23%, w/w) and C3(19.17%, w/w), and the highest bioconversion yield at 30 min were found in sample B2 (25.67%, w/w), B4 (25.62%, w/w), Bw3 (17.69%, w/w), and C3 (29.46%, w/w).

The correlation analysis between the bioconversion yield of D-allulose and enzymatic activity or major sugar components of the original honey samples are exhibited in **Figure 4.7**. Significant negative correlations were found between the acid phosphatase activity and the bioconversion yield at both 10 min (-0.67, p-value<0.1) and 30 min (-0.81, p-value<0.01). These results reveal enzymatic inhibitions between acid phosphatase, biomarkers for buckwheat honey, and DAEase. However, it is worth mentioning that the observed negative correlation might be due the presence of other microcomponents, such as phenolic compounds, present in buckwheat honey. Additionally, after 30 minutes of incubation, sample C3 achieved the bioconversion yield of 29.46%, aligning with the previously reported theoretical equilibrium state of allulose and fructose in DAEase-aided bioconversions (Zhang, et al., 2015).

4.4.5 Effect of bioconversion of honeys on their physicochemical properties

Table 4.4 shows the pH and color measurements at time 0 and the end of the biotransformation reaction (30 min). The study of Zhang (2015) reported maximum activity at pH 6 while retaining over 90% of the relative activity between pH 5.5-7.5. The recorded value prior to the biotransformation fell within the range of 5.4-5.72 with buffer addition, hence, significant reduction in enzymatic activity due to the acidic nature of honeys were not expected. The three samples with the highest bioconversion yield during the first 10 minutes of reaction were revealed to have the closest initial pH to that of the optimum condition for DAEase. Among the four clover samples, sample C1 showed both the lowest bioconversion yield after 10 minutes of reaction and the lowest initial pH. However, as pH continued to decrease throughout the incubation period, this trend became less apparent. No correlation was observed between the pH value after 30 minutes

Table 4.4 pH and color measurement for 12 honey solutions (78% w/v) before and after the biotransformation, and color measurement for 100% (w/v) solutions.

Sample	Mixture before biotransformation reaction				30-min biotransformation reaction				100% (w/v) honey		
	pH	**L* value**	**a* value**	**b* value**	**pH**	**L* value**	**a* value**	**b* value**	**L* value**	**a* value**	**b* value**
B1	5.46±0.02	70.42±0.08	-2.43±0.01	31.56±0.04	5.28±0.04	65.89±0.89	-0.96±0.16	36.27±0.22	67.51	-1.54	26.68
B2	5.48±0.01	75.38±0.17	-3.32±0.04	16.67±0.10	5.33±0.04	70.84±0.21	-2.73±0.01	20.87±0.21	72.54	-3.67	20.52
B3	5.48±0.01	75.21±0.07	-3.84±0.02	18.28±0.01	5.39±0.01	70.44±0.08	-3.03±0.04	23.90±0.05	74.38	-4.43	22.95
B4	5.6±0.03	75.42±0.08	-3.08±0.04	16.79±0.07	5.55±0.06	72.01±1.67	-2.91±0.08	20.88±0.02	72.91	-3.45	19.89
Bw1	5.4±0.06	66.36±0.03	0.11±0.02	39.13±0.02	5.29±0.01	58.86±0.93	2.49±0.04	40.40±0.52	60.22	1.91	42.87
Bw2	5.45±0.01	47.44±0.01	16.93±0.01	45.95±0.04	5.26±0.04	41.86±2.23	16.98±0.03	40.64±1.52	44.37	21.00	43.95
Bw3	5.58±0.03	60.47±0.23	4.78±0.14	44.04±0.11	5.39±0.01	56.78±1.24	5.67±0.35	43.65±0.59	52.05	6.46	41.61
Bw4	5.52±0.04	43.07±0.23	18.52±0.06	40.22±0.28	5.22±0.03	40.29±0.22	18.42±0.36	36.02±0.98	45.31	16.19	25.68
C1	5.58±0.01	75.545±0.02	-3.47±0.02	15.74±0.03	5.55±0.01	68.14±0.51	-2.46±0.20	21.33±0.19	72.58	-3.92	22.47
C2	5.70±0.02	78.52±0.04	-1.38±0.00	2.83±0.04	5.55±0.03	71.49±0.82	-1.13±0.09	6.20±0.02	77.79	-1.68	4.05
C3	5.68±0.01	78.97±0.28	-1.47±0.02	2.34±0.04	5.59±0.02	72.18±0.07	-1.17±0.06	5.49±0.18	79.81	-1.86	3.64
C4	5.72±0.01	78.36±0.08	-1.43±0.03	3.29±0.04	5.60±0.01	73.95±0.01	-1.37±0.01	6.82±0.01	78.67	-1.73	4.36

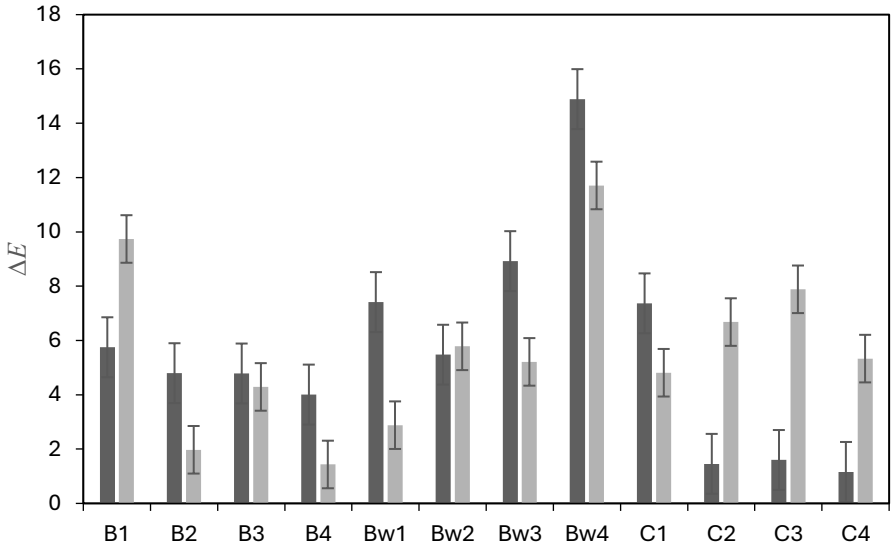


Figure 4.8. The impact of the enzymatic biotransformation in the total color difference between the 100% (w/v) honey with mixture before the biotransformation (■) or with the reaction end-product (■).

of reaction and the bioconversion yield of D-allulose within either the blueberry or clover samples. Therefore, the slight fluctuations in pH during the incubation process do not have a significant impact on the overall bioconversion of fructose into D-allulose in honey. Significant differences were not found between the apparent viscosity of all three types of honey undergone the bioconversion reaction at η_{50}, a shear rate measurement of fluid that correlates with the perception of swallowing (Ross, Tyler, Borgognone, & Eriksen, 2019). All samples before and after the biotransformation exhibits a slight shear thinning property which associated with the rheology behavior of undiluted honeys (Faustino & Pinheiro, 2021). Either increased or decreased shear thinning properties were found with the end-product when compared to 100% (w/v) honey solutions (**Supplementary Figure 4.1**).

Color measurement presented in **Table 4.4** consist of the L, a, b values for each individual samples before and after the reaction. L value indicates the level of pigmentation of samples with values scale from 0 (black) to 100 (white); a* value indicates the level of red/green of samples with the positive and negative value describing red or green, respectively; b* value indicates the level of yellow and blue of samples with the positive and negative value describing yellow or blue,

respectively (Ly, Dyer, Feig, Chien, & Bino, 2020). The total color differences, depicted in **Fig 4.8**, showed values all above 1, hence the dilution to 78% may have a noticeable impact on the appearance of the honey solution. Reduction in total color difference were observed after the biotransformation with three out of four blueberry honeys (B2-4, p value of 0.06) and buckwheat honeys (Bw1, 3, 4, p-value of 0.1). However, the dark-colored buckwheat honey, despite having some decrease in the post-reaction total color difference, still appeared different from the 100% (w/v) honey solution, as the values were still significantly higher than 1. On the other hand, the biotransformation showed negative impact on the color of 78% (w/v) clover honey samples, as three out four samples (C 2-4) had increased color differences (p-value of 0.007) after the reaction. An increase in the b* value can be seen with all blueberry and clover honey samples and buckwheat sample Bw1 after the biotransformation reaction, indicating an increase in the yellowness of the mixture. Decreased L* values are associated with all samples, suggesting increased intensity in pigmentation, possibly attributed to the Maillard reaction induced by heating (Guo, et al., 2022). No significant changes in a* values were observed except for sample Bw1, indicating small changes in the red/blue scale for the honey.

4.5 Conclusion

This study examined the potential of using DAEase, an epimerase from wild-type *Dorea sp.* sequences in the endogenous biogeneration of D-allulose in honey. The ANOVA identified the critical attributes for the D-allulose production as well as the bioconversion yield. Initial honey concentration as well as the reaction time were the most critical factors for maximizing the concentration of D-allulose production and the bioconversion yield in honey. With 78% (w/v) honey solution and 9 units of enzyme used, blueberry honeys were able to achieve a bioconversion yield of 24.25% (w/v) after 10 min of reaction, while clover honeys were able to reach 29.45% of bioconversion of fructose into D-allulose after 30 min of reaction. Negative correlations between the acid phosphatase activity and DAEase activity were drawn with a significantly lower bioconversion yield observed for all buckwheat honey samples tested. For future studies, other parameters such as phenolic compounds can be studied to determine the factor that influences the activity of DAEase in buckwheat samples.

Reference

Ahmed, A., Tul-Noor, Z., Lee, D., Bajwah, S., Ahmed, Z., Zafar, S., Syeda, M., Jamil, F., Qureshi, F., Zia, F., Baig, R., Ahmed, S., Tayyiba, M., Ahmad, S., Ramdath, D., Tsao, R., Cui, S., Kendall, C. W. C., de Souza, R. J., Khan, T. A., … Sievenpiper, J. L. (2023). Effect of honey on cardiometabolic risk factors: a systematic review and meta-analysis. *Nutrition reviews*, 758–774.

Ayers, B. J., Hollinshead, J., Saville, A. W., Nakagawa, S., Adachi, I., Kato, A., Izumori, K., Bartholomew, B., Fleet, G. W., & Nash, R. J. (2014). Iteamine, the first alkaloid isolated from Itea virginica L. inflorescence. *Phytochemistry*, 126-131.

Bayu, A., Warsito, M. F., Putra, M. Y., Karnjanakom, S., & Guan, G. (2021). Macroalgae-derived rare sugars: Applications and catalytic synthesis. *Carbon Resources Conversion*, 150-163.

Berker, A. (2002). Rheology for adhesion science and technology. In D. A. Dillard, A. Pocius, & M. Chaudhury, *Adhesion Science and Engineering* (pp. 443-498). Elsevier Science.

Bobiş, O., Dezmirean, D. S., & Moise, A. R. (2018). Honey and Diabetes: The Importance of Natural Simple Sugars in Diet for Preventing and Treating Different Type of Diabetes. *Oxidative medicine and cellular longevity*, 4757893.

CAS. (2021, August 09). *D-Psicose*. Retrieved from American Chemical Society: https://www.acs.org/molecule-of-the-week/archive/p/d-psicose.html

Chen, Z., Gao, X.-D., & Li, Z. (2022). Recent Advances Regarding the Physiological Functions and Biosynthesis of D-Allulose. *Frontiers in Microbiology*, *13*, 881037.

Choi, B.-R., Kwon, E.-Y., Kim, H.-J., & Choi, M.-S. (2018). Role of Synbiotics Containing d-Allulose in the Alteration of Body Fat and Hepatic Lipids in Diet-Induced Obese Mice. *Nutrients*, *10*(11), 1797.

Drabo, P., Fischer, M., Emondts, M., Hamm, J., Engelke, M., Simonis, M., . . . Delidovich, I. (2023). Solvent effects on catalytic activity and selectivity in amine-catalyzed D-fructose isomerization. *Journal of Catalysis*, 13-21.

Faustino, C., & Pinheiro, L. (2021). Analytical Rheology of Honey: A State-of-the-Art Review. *Foods*, *10*(8), 1709.

FDA. (2020). *The Declaration of Allulose and Calories from Allulose on Nutrition and Supplement Facts Labels: Guidance for Industry.* College Park: Food and Drug Administration.

Guo, Q. X., Liu, H. M., Liu, M. W., Wang, C. X., Qin, Z., & Wang, X. D. (2022). Effects of roasting temperature and duration on color and flavor of a sesame oligosaccharide-protein complex in a Maillard reaction model. *Food chemistry: X*, *16*, 100483.

Guo, Q., Dong, Z.-X., Luo, X., Zheng, L.-J., Fan, L.-H., & Zheng, H.-D. (2024). Engineering Escherichia coli for D-allulose biosynthesis from glycerol. *Journal of Biotechnology*, 103-111.

Izumori, K. (2006). Izumoring: A strategy for bioproduction of all hexoses. *Journal of Biotechnology*, *124*(4), 717-722.

Jia, D. X., Sun, C. Y., Jin, Y. T., Liu, Z. Q., Zheng, Y. G., Li, M., Wang, H. Y., & Chen, D. S. (2021). Properties of d-allulose 3-epimerase mined from Novibacillus thermophilus and its application to synthesis of d-allulose. *Enzyme and microbial technology*, *148*, 109816.

Jiang, S., Xiao, W., Zhu, X., Yang, P., Zheng, Z., Lu, S., Jiang, S., Zhang, G., & Liu, J. (2020). Review on D-Allulose: In vivo Metabolism, Catalytic Mechanism, Engineering Strain Construction, Bio-Production Technology. *Frontiers in bioengineering and biotechnology*, *8*, 26.

Khan, S. U., Anjum, S. I., Rahman, K., Ansari, M. J., Khan, W. U., Kamal, S., Khattak, B., Muhammad, A., & Khan, H. U. (2018). Honey: Single food stuff comprises many drugs. *Saudi journal of biological sciences*, 320-325.

Kim, D.-B., Nam, T. G., Jung, Y. S., Kim, H.-J., Sa, S., & Yoo, M. (2020). Optimization and validation for quantification for allulose of jelly candies using response surface methodology. *Journal of Food Science and Technology* , 2670-2676.

Kim, H.-J., Hyun, E.-K., Kim, Y.-S., Lee, Y.-J., & Oh, D.-K. (2006). Characterization of an Agrobacterium tumefaciensd-Psicose 3-Epimerase That Converts d-Fructose to d-Psicose. *Applied and environmental microbiology*, *72*(2), 981–985.

Laksmi, F. A., Nirwantono, R., Nuryana, I., & Agustriana, E. (2022). Expression and characterization of thermostable D-allulose 3-epimerase from Arthrobacter psychrolactophilus (Ap DAEase) with potential catalytic activity for bioconversion of D-allulose from d-fructose. *International journal of biological macromolecules*, 426–438.

Lamidi, S., Olaleye, N., Bankole, Y., Obalola, A., Aribike, E., & Adigun, I. (2023). Applications of Response Surface Methodology (RSM) in Product Design, Development, and Process Optimization. In P. Kayaroganam, *Response Surface Methodology - Research Advances and Applications.* IntechOpen.

Li, C., Li, L., Feng, Z., Guan, L., Lu, F., & Qin, H.-M. (2021). Two-step biosynthesis of d-allulose via a multienzyme cascade for the bioconversion of fruit juices. *Food Chemistry*, 129746.

Li, J., Dai, Q., Zhu, Y., Xu, W., Zhang, W., Chen, Y., & Mu, W. (2024). Low-calorie bulk sweeteners: Recent advances in physical benefits, applications, and bioproduction. *Critical Reviews in Food Science and Nutrition*, 6581-6595.

Li, L., Zhang, Q., Wang, T., Qi, H., Wei, M., Lu, F., . . . Qin, H.-M. (2022). Engineering of Acid-Resistant d-Allulose 3-Epimerase for Functional Juice Production. *J Agric Food Chem*, 6298-16306.

Ly, B. C., Dyer, E. B., Feig, J. L., Chien, A. L., & Bino, S. D. (2020). Research Techniques Made Simple: Cutaneous Colorimetry: A Reliable Technique for Objective Skin Color Measurement. *Journal of Investigative Dermatology*, 3-12.

Mooradian, A. D. (2024). Sugar or Sweetener? *Southern Medical Journal*, 513-516.

O'Charoen, S., Hayakawa, S., & Ogawa, M. (2015). Food properties of egg white protein modified by rare ketohexoses through Maillard reaction. *International Journal of Food Science and Technology*, 194-202.

Park, C.-S., Kim, T., Hong, S.-H., Shin, K.-C., Kim, K.-R., & Oh, D.-K. (2016). D-Allulose Production from D-Fructose by Permeabilized Recombinant Cells of Corynebacterium glutamicum Cells Expressing D-Allulose 3-Epimerase Flavonifractor plautii. *PloS one*, *11*(7), e0160044.

Ross, A. I., Tyler, P., Borgognone, M. G., & Eriksen, B. M. (2019). Relationships between shear rheology and sensory attributes of hydrocolloid-thickened fluids designed to compensate for impairments in oral manipulation and swallowing. *Journal of Food Engineering*, 123-131.

Samarghandian, S., Farkhondeh, T., & Samini, F. (2017). Honey and Health: A Review of Recent Clinical Research. *Pharmacognosy research*, 121-127.

Sarabia, L., & Ortiz, M. C. (2009). Response Surface Methodology. In S. D. Brown, R. Tauler, & B. Walczak, *Comprehensive Chemometrics: Chemical and Biochemical Data Analysis* (pp. 345-390). Elsevier.

Tan, J. H., Chen, A., Bi, J., Lim, Y. H., Wong, F. T., & Ow, D. S.-W. (2023). The Engineering, Expression, and Immobilization of Epimerases for D-allulose Production. *International journal of molecular sciences*, *24*(16), 12703.

Tang, X., An, Y., Iqbal, M. W., Cong, H., Zhang, G., Zhang, Y., Ravikumar, Y., Zabed, H. M., Zhao, M., Zhou, H., & Qi, X. (2022). The Characterization of a Novel D-allulose 3-Epimerase from Blautia produca and Its Application in D-allulose Production. *Foods*, *11*(20), 3225.

Tsukamoto, I., Hossain, A., Yamaguchi, F., Hirata, Y., Dong, Y., Kamitori, K., . . . Tokuda, M. (2014). Intestinal absorption, organ distribution, and urinary excretion of the rare sugar D-psicose. *Drug Design, Development and Therapy*, 1955–1964.

Weisberg, S. (2013). Testing and Analysis of Variance. In S. Weisberg, *Applied Linear Regression* (pp. 143-163). John Wiley & Sons, Incorporated.

Xia, Y., Cheng, Q., Mu, W., Hu, X., Sun, Z., Qiu, Y., Liu, X., & Wang, Z. (2021). Research Advances of D-allulose: An Overview of Physiological Functions, Enzymatic Biotransformation Technologies, and Production Processes. *Foods*, *10*(9), 2186.

Xie, X., Li, C., Ban, X., Yang, H., & Li, Z. (2024). D-allulose 3-epimerase for low-calorie D-allulose synthesis:microbial production, characterization, and applications. *Critical reviews in biotechnology*, *45*(2), 353–372.

Yang, J., Fan, D., Zhao, F., Lin, Y., Zheng, S., & Han, S. (2022). Characterization of D-Allulose-3-Epimerase From Ruminiclostridium papyrosolvens and Immobilization Within Metal-Organic Frameworks. *Frontiers in bioengineering and biotechnology*, *10*, 869536.

Yang, J., Tian, C., Zhang, T., Ren, C., Zhu, Y., Zeng, Y., . . . Ma, Y. (2019). Development of food-grade expression system for d-allulose 3-epimerase preparation with tandem isoenzyme genes in Corynebacterium glutamicum and its application in conversion of cane molasses to D-allulose. *Biotechnology and Bioengineering*, 745-756.

Zhang, W., Chen, D., Chen, J., Xu, W., Chen, Q., Wu, H., . . . Mu, W. (2023). D-allulose, a versatile rare sugar: recent biotechnological advances and challenges. *Critical Reveiws in Food Science and Nutrition*, 5661-5679.

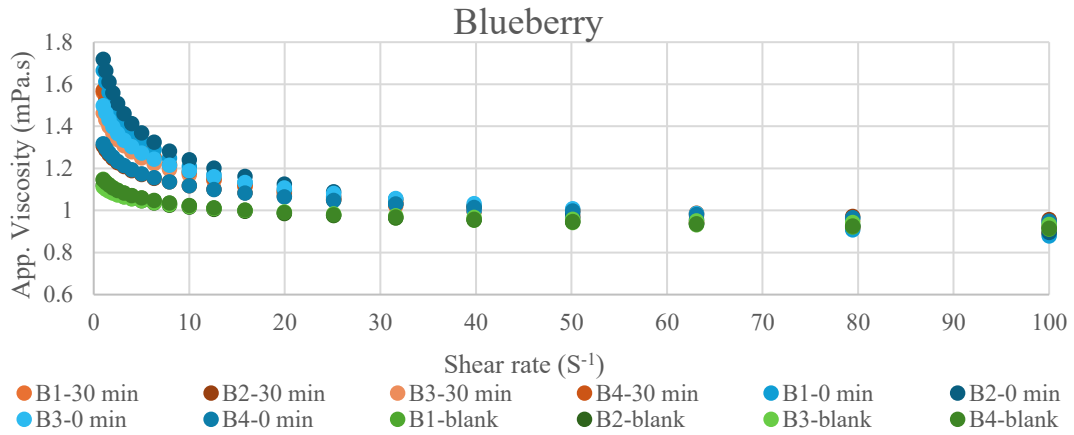
Zhang, W., Li, H., Zhang, T., Jiang, B., Zhou, L., & Mu, W. (2015). Characterization of a d-psicose 3-epimerase from Dorea sp. CAG317 with an acidic pH optimum and a high specific activity. *Journal of Molecular Catalysis B: Enzymatic*, 68-74.

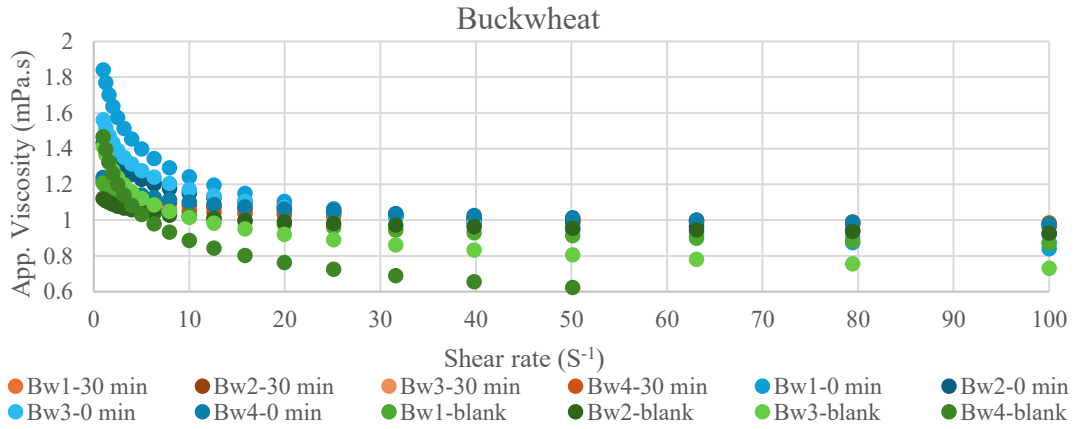
Zhang, W., Zhang, Y., Huang, J., Chen, Z., Zhang, T., Guang, C., & Mu, W. (2018). Thermostability Improvement of the d-Allulose 3-Epimerase from Dorea sp. CAG317 by Site-Directed Mutagenesis at the Interface Regions. *Joural of Agricultural and Food Chemistry*, 5593-5601.

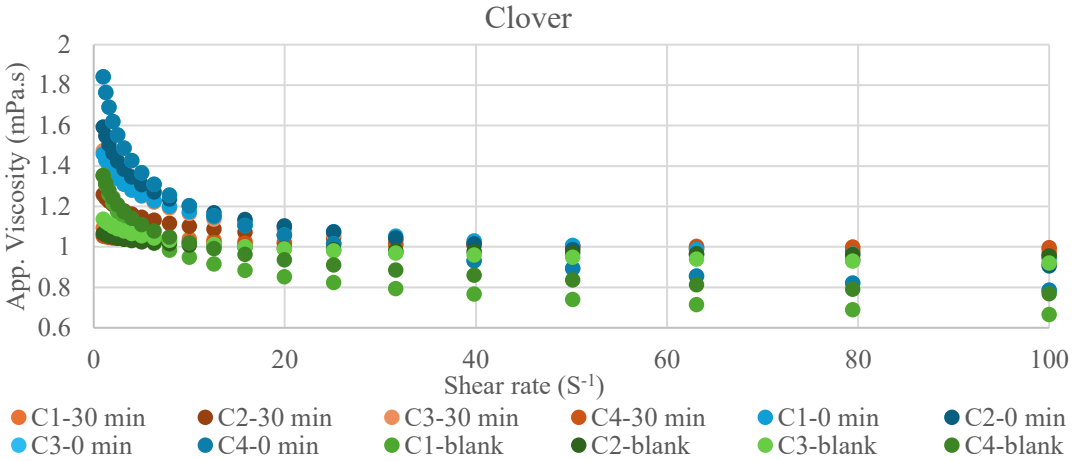
Appendix

Supplementary Table 4.1 Responses of the optima conditions for D-allulose production with *Dorea sp* DAEase in 78% (w/v) honey.

	Predicted value (clover)	Floral type	Experimental responses (10 min)	Experimental responses (30 min)
Selected parameters				
Enzyme unit (U/ml)			7.6	
Honey concentration % (w/v)			78	
Reaction time			10	
Responses				
D-allulose concentration (mg/ml)	44	Blueberry	53.95±10.93	71.06±9.86
		Buckwheat	20.98±11.53	29.08±15.71
		Clover	46.63±18.57	70.74±15.20
Bioconversion yield % (w/w)	14.10	Blueberry	18.31±4.15	23.96±2.15
		Buckwheat	7.66±5.08	9.55±5.55
		Clover	15.42±4.99	23.75±4.76







Supplementary Figure 4.1 Shear-dependent viscosities of blueberry, buckwheat and clover honey samples

CHAPTER V. GENERAL CONCLUSION, CONTRIBUTION TO KNOWLEDGE, AND FUTURE WORK

5.1 General Conclusion

The identification of the botanical origin of honey through the examination of enzymatic activity and carbohydrate profiling was the primary objective of the research. The goal was to establish a reliable and robust authentication method of honey that can be integrated with other quantifiable characteristics, such as phenolic compounds and physicochemical properties. Five major enzymes present in honey, namely diastase, invertase, acid phosphatase, glucose oxidase, and catalase, were assessed and quantified. HPAEC-PAD and LC/MS-QToF were used for the identification and quantification of carbohydrates present in honey, with total sample preparation and sample running time of less than 1 hour. While the diastase activity was found to have a significant ($p<0.0001$) negative correlation with the HMF content, the validity of the use of this enzyme as a freshness indicator was confirmed with HMF. Notably, the two plant-originated enzymes, acid phosphatase and catalase, were identified as two features that contribute to the clustering of samples according to their floral types. Buckwheat honey expressed a significantly higher acid phosphatase activity with an average of 683.202 mg P/100g honey/24 hr, whereas the activity in clover, blueberry and goldenrod honey ranged between 3.91-119.73, 0-240.87, and 15.76-738.34 mg P/100g honey/24 hr, respectively. Lower catalase activity was found in clover honey at an average level of 12.03 μg H_2O_2/g honey/min, followed by buckwheat honey with an average of 23.60 μg H_2O_2/g honey/min, and blueberry honey possessed the highest average level of catalase activity at 27.63 μg H_2O_2/g honey/min. Aside from fructose and glucose, 6 disaccharides (trehaose, isomatose, sucrose, maltose, nigerose, and gentiobiose) and 1 trisaccharide (erlose) were identified and quantified. Moreover, unknown 5 disaccharides, 6 trisaccharides, 3 tetrasaccharides, and 2 pentasaccharides were detected. Additionally, significant ($p<0.0001$) negative correlations were observed between glucose content and isomaltose, gentiobiose or nigerose content as well as between the HMF content and the diastase or invertase activity. Both the multivariate statistical analysis and the prediction model, generated based on samples without outliers from the quantified variables, suggest the contribution of acid phosphatase and catalase as biomarkers for the authentication of clover, buckwheat and blueberry honeys. The XGB model provided promising results in differentiating buckwheat honey from the clover and blueberry honeys, and an overall accuracy of 90% in predicting all three types of monofloral honey.

The second objective of this study aimed at the investigation of the use of DAEase from wildtype *Dorea sp.*, to produce a functional honey. This was accomplished through the bioconversion of the intrinsic D-fructose present in honey into D-allulose, a C-3 epimer of D-fructose that possesses inert caloric content. The DAEase sequence was obtained from *Dorea sp*, as it is reported to have an optimal pH of 6, which is close to the slightly acidic pH of honey. The effect of temperature and honey as the food matrix on the epimerization of fructose was first evaluated. Although the same allulose concentration was produced at both 70°C and 40°C, a much shorter reaction time was needed at 70°C. The enzymatic activity was higher by up to 17% in diluted honey solutions that contained 5% (w/v) fructose when compared to that reacted in 5% (w/v) pure fructose solution. An average of 102.07 mg/ml allulose concentration was achieved after 20 min reaction at 70°C in 70% (w/v) honey solution; this was dropped to 87.65 mg/ml after 4 hours. However, when incubating at 40°C, the allulose content reached and maintained a concentration of 94.29 mg/ml after 12 hours, while no significant decrease in the allulose concentration was observed. With the reaction temperature set at 70°C, the effects of biotransformation parameters were further examined to optimize the process. RSM was used to generate predictive models and determine the significance of factors, such as honey concentration, reaction time and enzyme units. The initial honey concentration and the reaction time are the most significant factors influencing the enzymatic synthesis of D-allulose. The optimized conditions were applied for the biotransformation of selected monofloral honeys (blueberry, buckwheat and clover) by DAEase. Along with the assessment of the end-product concentration and bioconversion rate, color differences, rheological properties and pH changes were measured. A low bioconversion rate of 9.55% (w/w) was observed with buckwheat honey and a maximum bioconversion rate of 29.5% (w/w) was achieved in clover honey, providing potential in producing fortified functional honey containing up to 84.65 mg/ml of allulose.

5.2 Contribution to Knowledge

The major contributions to knowledge of this study are captured here:

1. This is the first study to conduct carbohydrate and enzymatic activity profiling on Canadian monofloral clover, buckwheat, blueberry and goldenrod honeys, and notably used a large sample size of 163 honeys.

2. For the first time correlations between carbohydrate composition and 5 enzymes in honey were collectively investigated for the botanical origin authentication of honeys.
3. This is the first study to focus on the application of DAEase catalyzed endogenous biogeneration of D-allulose in honey.
4. The effect of honey floral types on the endogenous biogeneration of D-allulose using *Dorea sp.* DAEase was studied for the first time, further confirming the applicability of producing D-allulose fortified honeys.

5.3 Future Work

To further advance the field of honey authentication, future work is recommended with larger sample size and using broader botanical origin variations beyond the 4 types of monofloral honeys. By expanding the diversity of botanical origins of the samples, we will be able to build a larger database, check the validity of biomarkers identified and potentially establish an authentication model with higher inclusivity.

The identification and qualification of several unknown oligosaccharides present in honeys will provide an insight into a more complete carbohydrate profiling of honey and potentially discover other reliable biomarkers. Case study can be conducted to evaluate the effectiveness of the prediction models in differentiating buckwheat honey from more varieties of monofloral and polyfloral honeys.

The current work demonstrated the application of DAEase in honey to produce D-allulose fortified functional product. Future work could focus on discovering and implementing DAEases from other microbial sources that possess higher thermal stability and lower pH optimal.

Reference

Abuelgasim, H., Albury, C., & Lee, J. (2021). Effectiveness of honey for symptomatic relief in upper respiratory tract infections: a systematic review and meta-analysis. *BMJ evidence-based medicine*, *26*(2), 57–64.

CBI. (2024, April 10). *What is the demand for honey on the European market?* Retrieved from CBI Ministry of Foreign Affairs: https://www.cbi.eu/market-information/honey/what-demand

Eteraf-Oskouei, T., & Najafi, M. (2013). Traditional and Modern Uses of Natural Honey in Human Diseases: A Review. *Iranian journal of basic medical sciences*, 731-742.

Fortune Business Insight. (2024a). *Honey Market Size, Share & Industry Analysis, By Type (Alfalfa, Buckwheat, Wildflower, Clover, Acacia, and Others), By Application (Food & Beverages, Personal Care & Cosmetics, Pharmaceuticals, and Others), By Packaging (Glass Jar, Bottle, Tub, Tube, and*. Retrieved from Fortune Business Insight.

Fortune Business Insight. (2024b). *Manuka Honey Market Size, Share and COVID 19 Impact Analysis, By Type (UMF 5+, UMF 10+, UMF 15+ and UMF 20+), By Distribution Channel (Supermarkets/Hypermarkets, Specialty Stores, Online Stores, and Convenience Stores), and Regional Forecast, 2023-2030*. Retrieved from Fortune Business Insight.

Gillespie, K. M., Kemps, E., White, M. J., & Bartlett, S. E. (2023). The Impact of Free Sugar on Human Health-A Narrative Review. *Nutrients*, *15*(4), 889.

Islam, M. K., Lozada Lawag, I., Green, K. J., Sostaric, T., Hammer, K. A., Lim, L. Y., & Locher, C. (2022a). An investigation of the suitability of melissopalynology to authenticate Jarrah honey. *Current research in food science*, *5*, 506–514.

Oryan, A., Alemzadeh, E., & Moshiri, A. (2016). Biological properties and therapeutic activities of honey in wound healing: A narrative review and meta-analysis. *Journal of Tissue Viability*, 98-118.

Ranneh, Y., Akim, A. M., Hamid, H. A., Khazaai, H., Fadel, A., Zakaria, Z. A., Albujja, M., & Bakar, M. F. A. (2021). Honey and its nutritional and anti-inflammatory value. *BMC Complementary Medicine and Therapies*, *21*(1), 30.

Tsagkaris, A. S., Koulis, G. A., Danezis, G. P., Martakos, I., Dasenaki, M., Georgiou, C. A., & Thomaidis, N. S. (2021). Honey authenticity: analytical techniques, state of the art and challenges. *RSC Advances*, 11273–11294.

Walker, M. J., Cowen, S., Gray, K., Hancock, P., & Burns, D. T. (2022). Honey authenticity: the opacity of analytical reports - part 1 defining the problem. *NPJ science of food*, *6*(1), 11.

Wang, S., Qiu, Y., & Zhu, F. (2024). An updated review of functional ingredients of Manuka honey and their value-added innovations. *Food Chemistry*, *440*, 138060.

Xia, Y., Cheng, Q., Mu, W., Hu, X., Sun, Z., Qiu, Y., Liu, X., & Wang, Z. (2021). Research Advances of D-allulose: An Overview of Physiological Functions, Enzymatic Biotransformation Technologies, and Production Processes. *Foods*, *10*(9), 2186.