Investigating Phenolic Compounds as Markers of Botanical Origin in Raw and Processed Buckwheat Honey Using LC-QTOF-MS

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ABBREVIATIONS

ACN	Acetonitrile
ANOVA	Analysis of variance
CCFI	Canadian center for food integrity
CFIA	Canadian food inspection agency
DA	Discriminant analysis
DAD	Diode array detector
DNA	Deoxyribonucleic acid
ECD	Electrochemical detection
ESI	Electrospray ionization
FN	False negative
FP	False positive
FTIR	Fourier transform infrared spectroscopy
GAE	Gallic acid equivalents
GC	Gas chromatography
HCA	Hierarchical cluster analysis
HFCS	High fructose corn syrup
HHP	High hydrostatic pressure
HMF	Hydroxymethylfurfural
HPLC	High-performance liquid chromatography
HPP	High-pressure processing
HRMS	High-resolution mass spectrometry
IR	Infrared
LC	Liquid chromatography
LDA	Linear discriminant analysis
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LTQ	Linear trap quadrupole
MDL	Method detection limit
MCC	Matthews correlation coefficient
ME	Matrix effect
MQL	Method quantification limit
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m/z	Mass to charge ratio
NA	Not available
ND	Not detected
NMR	Nuclear magnetic resonance
NTA	Non-targeted analysis
PCA	Principal component analysis
PCR	Principal component regression
PDA	Photodiode array
PLS-DA	Partial least squares discriminant analysis
PPR	Positive predictive rate

PTFE	Polytetrafluoroethylene
QA	Quality assurance
QC	Quality control
QE	Quercetin equivalents
QqQ	Triple quadrupole
QTOF	Quadrupole time-of-flight
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
RSD	Relative standard deviation
RT	Retention time
SPE	Solid-phase extraction
SULLE	Sugaring-out assisted liquid-liquid extraction
TA	Targeted analysis
TNR	True negative rate
TOF	Time-of-flight
TP	True positive
TPC	Total phenolic content
TPR	True positive rate
t-SNE	t-Distributed stochastic neighbor embedding
UHPLC	Ultra-high-performance liquid chromatography
USFDA	United states food and drug administration
UV	Ultraviolet

ABSTRACT

Honey is a highly valued natural food product, renowned for its superior nutritional benefits compared to other sweeteners. However, the global honey market faces significant challenges due to widespread honey fraud, which carries profound economic, nutritional, and health implications. Among the various forms of honey fraud, the mislabelling of botanical origin is particularly concerning. The botanical origin of honey greatly influences its market price, making it a prime target for fraudulent practices. Conventional methods such as pollen analysis and sensory evaluation, while traditional, often yield ambiguous results due to the diverse floral sources from which bees collect nectar. Accurately determining honey's botanical origin is crucial not only for maintaining consumer confidence and market integrity but also for safeguarding producers who adhere to stringent quality standards. Therefore, developing reliable and efficient methods to authenticate honey's botanical origin is imperative to address these concerns. The objective of this thesis was to develop an LC-QTOF-MS method for phenolic compounds in honey, focusing on identifying a phenolic marker for buckwheat honey and evaluating its reliability through cross-validation with another LC-MS instrument. Additionally, this study aimed to investigate how storage and thermal processing affect the phenolic profile of honey, specifically assessing the reliability of the buckwheat honey phenolic marker under varying conditions.

Chapter 3 details the development and validation of a multi-targeted direct-injection method using high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (HPLC-QTOF-MS) for 29 phenolic compounds in honey. This method demonstrated robustness, sensitivity, and good precision. The application of this method to 465 honey samples has revealed a unique phenolic profile in buckwheat honey, identifying p-hydroxybenzoic acid as a reliable marker for buckwheat honey with a threshold of 5318 ng/g. Validation using LC-QqQ-MS/MS confirmed the marker's transferability across laboratories, with 99.14% classification consistency.

In Chapter 4, a case study was conducted on six selected honey samples to further assess the marker's reliability in distinguishing buckwheat honey from other botanical origins under various storage and thermal treatment conditions. Thermal treatments minimally affected the phenolic profile, with botanical origin remaining the primary classification determinant. Results indicated

that the p-hydroxybenzoic acid marker threshold of 5318 ng/g remained valid for buckwheat honey stored at temperatures up to 25°C, although its concentration decreased at 65°C, potentially leading to misclassification. Moreover, no significant variation in p-hydroxybenzoic acid concentration was observed across different thermal treatments within the same honey sample, underscoring the robustness of the phenolic marker for botanical origin classification.

Overall, this study demonstrates the feasibility of using p-hydroxybenzoic acid as a marker for authenticating buckwheat honey. This marks the first study to establish a threshold for a phenolic compound marker, enabling a comprehensive assessment of the botanical origin of honey. It also validates the marker's robustness and reliability across different analytical instruments and under various storage and thermal processing conditions. Furthermore, this research is also the first to have explored a broad spectrum of phenolic compounds in honey, analyzing their responses to diverse storage and thermal conditions and investigating their impact on botanical origin assessment. Ultimately, these findings provide essential insights for developing standardized methods to reliably determine honey's botanical origin, advancing analytical strategies in the scientific community. By enhancing the accuracy and reliability of honey authenticity testing, this research supports regulatory initiatives, protects consumer interests, and maintains the integrity of honey products in the market.

RÉSUMÉ

Le miel est un produit alimentaire naturel hautement apprécié, renommé pour ses avantages nutritionnels supérieurs à ceux des autres édulcorants. Toutefois, le marché mondial du miel fait face à d'importants défis en raison de la fraude omniprésente sur le miel, qui a de profondes répercussions sur l'économie, la nutrition et la santé. Parmi les différentes formes de fraude sur le miel, l'étiquetage erroné de l'origine botanique est particulièrement préoccupant. L'origine botanique du miel influence considérablement son prix sur le marché, ce qui en fait une cible privilégiée pour les pratiques frauduleuses. Les méthodes conventionnelles telles que l'analyse pollinique et l'évaluation sensorielle, bien que traditionnelles, produisent souvent des résultats ambigus en raison de la diversité des sources florales sur lesquelles les abeilles récoltent le nectar. Il est essentiel de déterminer avec précision l'origine botanique du miel, non seulement pour préserver la confiance des consommateurs et l'intégrité du marché, mais aussi pour protéger les producteurs qui adhèrent à des normes de qualité rigoureuses. Par conséquent, il est impératif de développer des méthodes fiables et efficaces pour authentifier l'origine botanique du miel afin de répondre à ces préoccupations. L'objectif de cette thèse était de développer une méthode LC-QTOF-MS pour les composés phénoliques dans le miel, en se concentrant sur l'identification d'un marqueur phénolique pour le miel de sarrasin et en évaluant sa fiabilité par validation croisée avec un autre instrument LC-MS. De plus, cette étude visait à examiner l'effet de l'entreposage et du traitement thermique sur le profil phénolique du miel, en évaluant spécifiquement la fiabilité du marqueur phénolique du miel de sarrasin dans des conditions variables.

Le chapitre 3 détaille le développement et la validation d'une méthode d'injection directe multi-ciblée utilisant la chromatographie liquide haute performance couplée à la spectrométrie de masse quadripolaire à temps de vol (HPLC-QTOF-MS) pour 29 composés phénoliques dans le miel. Cette méthode a démontré sa robustesse, sa sensibilité et sa bonne précision. L'application de cette méthode à 465 échantillons de miel a révélé un profil phénolique unique dans le miel de sarrasin, identifiant l'acide p-hydroxybenzoïque comme un marqueur fiable pour le miel de sarrasin avec un seuil de 5318 ng/g. La validation par LC-QqQ-MS/MS a confirmé la transférabilité du marqueur entre laboratoires, avec une cohérence de classification de 99,14%.

Au chapitre 4, une étude de cas a été menée sur six échantillons de miel sélectionnés afin d'évaluer davantage la fiabilité du marqueur pour distinguer le miel de sarrasin des autres origines botaniques sous diverses conditions de stockage et de traitement thermique. Les traitements thermiques ont affecté de manière minimale le profil phénolique, l'origine botanique demeurant le principal critère de classification. Les résultats ont indiqué que le seuil de l'acide p-hydroxybenzoïque de 5318 ng/g demeurait valide pour le miel de sarrasin entreposé à des températures allant jusqu'à 25 °C, bien que sa concentration diminue à 65 °C, pouvant potentiellement entraîner une classification erronée. De plus, aucune variation significative de la concentration d'acide p-hydroxybenzoïque n'a été observée entre les différents traitements thermiques au sein du même échantillon de miel, soulignant la robustesse du marqueur phénolique pour la classification de l'origine botanique.

Dans l'ensemble, cette étude démontre la faisabilité de l'utilisation de l'acide p-hydroxybenzoïque comme marqueur pour authentifier le miel de sarrasin. Il s'agit de la première étude à établir un seuil pour un marqueur de composé phénolique, permettant une évaluation compréhensive de l'origine botanique du miel. Elle valide également la robustesse et la fiabilité du marqueur à travers différents instruments analytiques ainsi que dans diverses conditions d'entreposage et de traitement thermique. De plus, cette recherche est également la première à avoir exploré un large spectre de composés phénoliques dans le miel, analysant leurs réponses à diverses conditions d'entreposage et de traitement thermique et examinant leur impact sur l'évaluation de l'origine botanique. En fin de compte, ces résultats fournissent des informations essentielles pour développer des méthodes standardisées permettant de déterminer de manière fiable l'origine botanique du miel, faisant progresser les stratégies analytiques au sein de la communauté scientifique.

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CONTRIBUTION OF AUTHORS

This thesis is presented in a traditional thesis format and consists of five chapters. In Chapter 1, a general introduction to phenolic compounds in honey is presented, emphasizing their role in assessing the botanical authenticity of honey and outlining the research objectives. Chapter 2 offers a comprehensive literature review overviewing the current issues surrounding honey's botanical origin authenticity, the necessity for reliable assessment methods, and the various factors influencing honey's phenolic profile. Chapter 3 focuses on multi-targeted analysis of phenolic compounds in honey, specifically examining their ability in distinguishing buckwheat honey from other botanical origins using LC-QTOF-MS. In addition, this chapter provides insights into the method's transferability to other laboratories, employing LC-QqQ-MS/MS as a case study. Chapter 4 delves into the impact of storage and thermal processing on honey's phenolic profile. Finally, Chapter 5 presents an overall conclusion of the thesis and offers recommendations for future research endeavors.

Throughout the project, the thesis supervisor, Dr. Stéphane Bayen, and thesis co-supervisor, Dr. Salwa Karboune, oversaw the progression of the experimental work, offering direct advisory input, and critically edited the thesis. Dr. Yixian Wang, the committee member, provided valuable feedback on the experimental advancements. The present author was responsible for the experimental design, laboratory work, data acquisition, and data treatment in all chapters. In Chapter 3, honey samples were mainly collected by Shaghig Bilamjian, with collaboration on sample preparation from Dr. Lei Tian, and Shaghig Bilamjian. Drs. Lan Liu and Lei Tian played pivotal roles in optimizing the method on the LC-QTOF-MS for phenolic compound detection in Chapter 3, with assistance from Dr. Lei Tian, Zhi Hao Chi and Jingyun Zheng in data processing and interpretation. Furthermore, Dr. Shawninder Chahal contributed significantly to data treatment, interpretation, and figure creation in Chapter 3. In this chapter, members of the Canadian Food Inspection Agency, notably Dr. Randy Purves, Bryn Shurmer, and Jana Kinar, played essential roles in method optimization on the LC-QqQ-QTOF-MS/MS instrument. Jingyun Zheng aided in experimental design and statistical analysis of data in Chapter 4, while Dr. Shawninder Chahal created the PCA and heat map for the storage experiment. Special recognition goes to Dr. Lei Tian and Jingyun Zheng for their continuous assistance and unwavering support throughout the entirety of the project.

CONFERENCE PRESENTATIONS

Alexandra Roginski, Shawninder Chahal, Lei Tian, Shaghig Bilamjian, Stéphane Bayen. Data Mining the Honey LC-MS Spectra for Botanical Origin Authenticity Markers. **2024 Future of Food Symposium**, Hotel Omni Mont-Royal, Montreal, May 16th – 17th 2024. (Poster presentation).

Alexandra Roginski, Lei Tian, Shaghig Bilamjian, Shawninder Chahal, Stéphane Bayen. Targeted and Non-targeted Identification of Phenolic Compounds as Markers for Authenticating Honey's Botanical Origin using LC-QTOF-MS. **BÉNÉFIQ 2023**, Centre des congrès de Québec, Quebec City, October 4th - 5th 2023. (Poster presentation, 1st place for best poster presentation award).

Alexandra Roginski, Lei Tian, Shaghig Bilamjian, Tarun Anumol, Daniel Cuthbertson, Stéphane Bayen. Non-Targeted Identification of Phenolic Compounds as Markers for Authenticating Honey’s Botanical Origin using LC-QTOF-MS. **65th International Conference on Analytical Sciences and Spectroscopy (ICASS)**, Ottawa, Delta Hotel Marriott Ottawa City Centre, July 26th to 28th, 2023. (Oral communication).

Alexandra Roginski, Lei Tian, Shaghig Bilamjian, Tarun Anumol, Daniel Cuthbertson, Stéphane Bayen. Identification non ciblée de nouveaux marqueurs pour authentifier l’origine botanique du miel par LC-QTOF-MS. **90e Congrès de l'Acfas**, Université de Montréal, Montreal, Canada. May 10th 2023. (Oral communication).

CHAPTER 1. INTRODUCTION

1.1 General Introduction

Honey can be defined as a viscous liquid made by honeybees (mainly *Apis mellifera*) from the nectar of plants or the secretions of other insects known as honeydew (Salvador et al., 2019). Honey is considered a high-value food product compared to other sweeteners due to its characteristic taste, nutrients, and health-benefiting properties. Studies show that honey has the ability to inhibit around 60 species of bacteria, some species of fungi, and viruses (Eteraf-Oskouei & Najafi, 2013). Honey is also shown to have important antioxidant potential as it has the ability to prevent damage caused by free radicals, thus reducing the incidence of various diseases caused by oxidative stress, including cancer, cardiovascular, neurological, respiratory, and chronic inflammatory diseases (Larsen & Ahmed, 2022). In addition to honey having an antioxidant capacity, studies reveal that honey has many other healing capacities, including antimicrobial, antiviral, antiparasitic, anticancer, and antidiabetic activities.

Honey is regarded as a high-value food commodity compared to other types of sweeteners currently present in the market owing to its superior nutritive value. The combination of health benefits and the high cost of honey production makes this product a highly vulnerable food to fraud as fraudsters consider this food commodity as a good opportunity to gain economic advantage by adulterating or mislabelling honey. In fact, honey is among the top five food products subject to fraud (García, 2018). Studies indicate that about 90% of all cases of sweetener-related adulteration involve honey fraud (Sobrino-Gregorio et al., 2019). Specifically, the botanical origin of honey is mainly targeted by fraudsters, accounting for 44% of honey fraud cases, as it has a significant impact on its marker price, making it particularly susceptible to misleading labeling (Mădaş et al., 2019; Tsagkaris et al., 2021). Particularly, monofloral honey is targeted as it is a type of honey that has a distinctive flavor, aroma, or other attributes as it originates predominantly from the nectar of a single plant species. Among the various monofloral types, buckwheat honey is especially targeted in honey fraud due to its high nutritional and health benefits, second only to the renowned manuka honey (Deng et al., 2018). Buckwheat honey is highly sought after for its unique floral undertones, rich malty flavor, dark amber color, and distinctive aroma. It has gained attention for its high antioxidant activity and antibacterial potential, attributed to its substantial phenolic content (Brudzynski et al., 2012; Deng et al.,

2018). These attributes not only make buckwheat honey a valuable food product but also a lucrative target for adulteration. The combination of its unique sensory properties and health benefits underscores the importance of ensuring the authenticity of buckwheat honey, highlighting why it is often the focus of honey fraud.

Currently utilized methods for assessing the botanical origin of honey are inadequate for ensuring the accurate assessment of the botanical authenticity of honey. Pollen analysis (melissopalynology), the most common method, is highly time-consuming and requires appropriate equipment and specialists to perform these tests (Tian et al., 2024). In addition, honey processing methods such as filtration can remove pollen partially or entirely, resulting in the pollen analysis method providing inconclusive results (Battesti & Goeury, 1992; Bryant, 2017). Furthermore, variations in pollen size, production, and floral morphology mean that a uniform pollen percentage threshold cannot be applied to all monofloral honey (Ruoff & Bogdanov, 2004). Therefore, pollen analysis is frequently employed as a benchmark to validate the outcomes derived from contemporary instrumental techniques (Balkanska et al., 2020). This inconsistency further complicates the accurate determination of honey's botanical origin. Moreover, testing the physicochemical properties of honey, such as pH, water content, sugar composition, or electrical conductivity, is another method used to assess the botanical authenticity of honey (El Sohaimy et al., 2015; Tsagkaris et al., 2021). However, these tests offer indirect measurements that only aid in classification but still require complementary techniques to confirm the botanical origin. Consequently, they are insufficient on their own for accurate botanical classification. The primary issue is that these methods are not only inadequate for accurately determining honey’s botanical origin but there are also no standardized approaches for this purpose, except for Manuka honey (Li et al., 2024). This lack of standardized assessment methods highlights the critical need to develop reliable and accurate techniques for verifying the botanical authenticity of honey, especially given the prevalence of honey fraud. Developing such methods is essential to protect consumers and ensure the integrity of honey products.

1.2 Research Hypothesis

The present study was conducted with the hypothesis that:

Hypothesis 1: Methods based on high-performance liquid chromatography using a direct injection technique can detect a wide range of phenolic compounds in honey from various

botanical origins. The types and concentrations of these phenolic compounds differ depending on the honey's botanical source, allowing them to act as discriminators of botanical origin, with certain compounds serving as markers of botanical authenticity. These markers are transferable to other LC-MS instruments, enabling the targeted assessment of honey's botanical origin across different laboratories.

Hypothesis 2: Honey storage for extended periods below room temperature is anticipated to maintain its phenolic profile, whereas higher temperatures may lead to degradation. Thermal processing of honey is likely to decrease phenolic compound concentrations due to degradation, possibly resulting in misclassification of the honey's botanical origin.

1.3 Research Objectives

The overall aim of this research is to explore the feasibility of utilizing phenolic compounds as indicators of floral origin in both raw and processed honey. More specifically, this paper will address the following points:

Objective 1: Develop and validate an LC-QTOF-MS method for analyzing phenolic compounds in honey and compare the phenolic content of buckwheat honey with other botanical origins to identify unique phenolic markers for differentiating buckwheat honey. Employ LC-QqQ-MS/MS as a case study to assess the transferability of the direct injection method for the buckwheat honey phenolic marker onto alternative analytical instruments.

Objective 2: Investigate the impact of storage and thermal processing on the phenolic profile of honey, particularly focusing on the buckwheat honey phenolic marker, to determine its reliability as an indicator of buckwheat honey authenticity under different storage and thermal conditions.

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

Food fraud is a significant and growing problem that affects consumer trust and food safety worldwide. The COVID-19 pandemic significantly exacerbated the issue, leading to an increase in adulteration and mislabeling of food products (Brooks et al., 2021; Cane & Primrose, 2021). Consumers are increasingly concerned about food authenticity, wanting assurance that the products they purchase genuinely reflect the claims made on their packaging. A food product is considered to be authentic or genuine when the content corresponds to what is described on the label (CFIA, 2022b). In recent years, food fraud has become a major issue, with consumers demanding stricter government policies and enhanced surveillance to ensure the safety and quality of market-available foods. A 2017 study by The Canadian Center for Food Integrity (CCFI) revealed that about 55% of Canadians lack confidence in the safety of both imported and domestic foods, while 59% are particularly concerned about imported foods (Kelly, 2021). This comes to show that strengthening the safety of the food supply chain is essential to allow Canadians to feel more secure in the products they are purchasing.

Food fraud, while not new, has gained more attention due to the development of novel technologies that detect adulterants in food products. Fraud is particularly prevalent in high-value foods like oil, fish, meat, spices, dairy products, and honey, as the potential for financial gain is substantial (Aslam et al., 2023). Honey, a common target for fraud due to its perceived health benefits and natural sweetness, has gained research interest as incidents of honey fraud have increased over the years. Such fraud can involve various forms of adulteration, including diluting the product with cheaper sweeteners, mislabeling the botanical or geographical origin, and making false claims on the label about being raw, filtered, or organic (Tsagkaris et al., 2021). All these deceptive practices mislead consumers and undermine their trust. As a result, rigorous testing methods are essential to verify honey's authenticity and ensure that consumers receive a genuine product. These methods help maintain market integrity, protect consumer health, and preserve the reputation of legitimate producers. The main goals of this review are to (i) provide an overview of honey, (ii) emphasize the significance of addressing the issue of honey fraud, (iii) identify which phenolic compounds are present in honey from different botanical origins, (iv)

understand what parameters impact the phenolic content in honey and (v) provide an update on the current analytical methods used to assess the botanical authenticity of honey.

2.2 Honey

2.2.1 Honey introduction

Honey holds the distinction of being civilization's first sweetener source. Researchers have uncovered evidence suggesting that humans in Asia utilized honey for both culinary and medicinal purposes as far back as roughly 8000 years ago (Eteraf-Oskouei & Najafi, 2013). Today, amid a surge in health-conscious trends, honey is experiencing a resurgence in popularity, owing to its perceived therapeutic properties and natural origin. Produced and processed by honeybees (*Apis mellifera*), honey is derived from either the nectar of plants (blossom honey) or honeydew, an insect secretion (Salvador et al., 2019). To obtain the viscous liquid, known as honey, honeybees laboriously transform nectar or honeydew into honey, utilizing their elongated proboscis to gather nectar, which they then ingest. Enzymes in the bee's digestive system subsequently break down the nectar's complex sugars (sucrose) into simpler forms (fructose and glucose) through a process called inversion, altering the product's chemical composition and pH to facilitate long-term storage (Nicolson, 2011). Once collected and transported to their hives, the bees deposit the nectar or honeydew into hexagonal beeswax cells known as honeycombs. Since nectar and honeydew contain approximately 70% water, honeybees must reduce the moisture content to around 18% by creating a warm breeze within the hive through wing fanning, resulting in a concentrated sugar solution known as honey (Lazutin, 2020). The honeycomb is then sealed with beeswax, enabling the honey to be stored for extended periods due to its inherent stability. Harvesting honey involves beekeepers removing wax caps from honeycomb cells and extracting the honey using centrifugal force (Pereira et al., 2023). To simplify the filtering process, raw honey is often heated to approximately 55°C to reduce viscosity (Aydoğan Coşkun et al., 2020; Escriche et al., 2008). Depending on membrane filter pore sizes, filtration methods can remove wax, pollen, bee parts, and bacteria (Subramanian et al., 2007). Ultrafiltration, for instance, removes desirable enzymes and proteins, while microfiltration, with pore sizes around 200 nm, eliminates viable microorganisms, including yeast cells (~4 - 6 µm), potentially resulting in sterile honey (Barhate et al., 2003). Pasteurization, a common practice, is employed to prolong shelf life by eliminating yeast cells. This heating process, typically

performed at temperatures of 70-78°C or higher, reduces moisture content and slows crystallization while promoting a brown coloration through the Maillard reaction (Eshete & Eshete, 2019; Subramanian et al., 2007).

Once honey is produced and packaged, the label must accurately represent its contents. The label should include the common name (“honey” alone or accompanied by the blossom name), geographical location, net quantity (g or kg), color class, name and principal place of business, and honey grade (CFIA, 2022c). Honey can only be labeled as "honey" if it contains no other ingredients. If blended with other ingredients like flavoring or sweetening agents, it cannot be labeled as "honey," as this would constitute mislabeling (CFIA, 2022c). Honey can also be labeled as “raw” or “unfiltered” when it has not undergone heat treatments or processing. Raw honey is particularly valued by consumers for its higher vitamin and phenolic compound content, which are often reduced by pasteurization (Aydoğan Coşkun et al., 2020). Honey is also graded to inform consumers about its origin: local, imported, or traded interprovincially. Canadian honey grades include Canada No 1, Canada No 2, and Canada No 3. Blended honey grades are No. 1, No. 2, and No. 3, based on consistency, moisture content, and flavor, which determine the honey's quality (CFIA, 2022a). Honey is also classified by color and optical density using the Pfund scale, with six color classes: “Extra White,” “White,” “Golden,” “Light Amber,” “Dark Amber,” and “Dark.” Honey’s colour is greatly influenced by the botanical origin of the honey and will impact its flavour (CFIA, 2022a). Furthermore, honey is categorized by variety, such as monofloral and polyfloral. The definitions for ‘monofloral’ and ‘polyfloral’ honey vary by country regulations and guidelines. Monofloral honey predominantly contains nectar from a single plant species, although it may also include nectar from other flowers. Pollen count, determined through pollen analysis, also known as melissopalynology, is often used to categorize monofloral honey. This analysis measures the pollen composition in honey, indicating the plant sources the bees visited (Addi & Bareke, 2021). For honey to be considered monofloral, it must contain a minimum threshold level of pollen from a single plant source; otherwise, it is classified as polyfloral. The minimum required pollen count for monofloral honey depends on floral morphology, pollen size, and pollen production. Some plants produce more pollen than nectar or very small pollen grains, leading to overrepresentation or underrepresentation in pollen analysis (Swiatly-Blaszkiewicz et al., 2021). Due to the difficulty of harvesting pure monofloral honey,

along with its distinct aroma, taste, color, fragrance, and therapeutic properties of this type of honey makes it generally more expensive.

Table 2.1. Minimum percentage of pollen required for the characterization of monofloral honeys in five European countries according to their national legislations: or provisions, decisions or guidelines. Reprinted from (Thrasyvoulou et al., 2018).

Pollen grains	Croatia (%)	Greece (%)	Germany (%)	Italy (%)	Serbia (%)
Arbutus unedo	10				
Brassica napus	60	-	80		
Calluna vulgaris	20	-	-		20
Castanea sativa	85	87	90		85
Citrus spp.	10 (5*)	3	10	10	
Gossypium		3			
Erica spp.		45	45		
Eucalyptus spp.		45	85		
General monofloral	45	45	45		
Medicago sativa					>30
Lavandula spp.	10 (5*)				
Phacelia tanacetifolia	60				
Robinia pseudoacacia	20		-		20
Rosmarinus officinalis					20
Salvia officinalis	15 (10*)				
Satureja montana	20				
Taraxacum officinalis					20
Thymus spp.	-	18		15	
Tilia spp.	25 (10*)		20		25
Trifolium, melilotus			70		
Helianthus		20	50		40

*With characteristic organoleptic properties of honey for particular plant species (smell, taste, color).

2.2.2 Chemical properties & composition of honey

Honey's chemical composition is significantly influenced by several factors, including its botanical origin, degree of maturity, climatic conditions, processing methods, and storage conditions. Research indicates that honey typically contains approximately 200 substances, with sugars comprising the majority (80-85%), followed by water (15-17%), proteins (0.1-0.4%), and various other compounds such as organic acids, vitamins, minerals, enzymes, and phenolic compounds, all contributing to its sensory characteristics (Becerril-Sánchez et al., 2021; Eteraf-

Oskouei & Najafi, 2013). The primary sugars found in honey are fructose (about 38-55%) and glucose (about 31%), along with fructo-oligosaccharides (Cheung et al., 2019). These carbohydrates, derived from the nectar collected by honeybees, are responsible for honey's viscosity, hygroscopicity, and crystallization tendencies. Furthermore, the monosaccharide concentration is also responsible for other properties of honey such as flavor, and texture. Fructose is slightly sweeter than sucrose whereas glucose is less sweet than sucrose. Therefore, depending on the sugar distribution in honey (fructose/glucose ratio), the sweetness level may vary. In addition, the glucose-to-water ratio in honey will affect its crystallization and consistency (Hunter et al., 2021). Honey with a low glucose/water ratio will generally not easily crystalize, thus enhancing the smooth texture of honey. When the supersaturated solution is at room temperature, glucose precipitates into small solid granules which results in the honey being more viscous (Escuredo et al., 2014). Other articles report that the fluid, viscous, or crystalline consistency of honey is dependent on not only the quantity and type of sugars present but also the moisture content and storage temperature (Becerril-Sánchez et al., 2021). Moreover, water content significantly impacts honey's shelf life, with lower moisture levels associated with longer storage capabilities. Factors affecting water content include hive humidity, nectar origin, and processing treatments (Bellik & Iguer-ouada, 2013). Additionally, honey contains a wide range of flavonoids and phenolic acids, which exhibit antioxidant effects, along with vitamins (such as ascorbic acid, pantothenic acid, niacin, and riboflavin), minerals (such as calcium, copper, iron, magnesium, manganese, phosphorus, potassium and zinc), organic compounds (such as tocopherols, acetic, citric, formic, glutaric, fumaric, succinic, and many others), enzymes (mostly catalase, diastase, invertase, superoxide dismutase, and glucose oxidase), and Maillard reaction products (Ajibola et al., 2012; Khan et al., 2018; Rossano et al., 2012; Suto et al., 2020). These chemical components influence various properties of honey, including its color, consistency, as well as flavor and aroma, which are contributed by different volatile compounds, primarily aldehydes, alcohols, ketones, acids, and esters. Phenolic acids, flavonoids, minerals, and sugar composition determine honey's color, with darker honey typically exhibiting higher total flavonoid content (Becerril-Sánchez et al., 2021). Furthermore, the organic acid content and composition will affect honey’s acidity and greatly impact the characteristic taste of honey (Becerril-Sánchez et al., 2021). In summary, honey's physical, biochemical, and sensory properties are primarily influenced by its botanical origin, geographical area of collection,

environmental factors, plant maturity, processing techniques, and storage conditions. Moreover, honey's rich nutritive profile makes it highly valuable, not only to consumers but also to fraudsters seeking to exploit its high value.

2.2.3 Production and consumption of honey in Canada and globally

In recent years, as the trend towards healthier eating has led to a decline in the consumption of sweet goods, honey has emerged as a notable exception. Its extensive list of health benefits, coupled with the increasing preference for natural and high-quality ingredients, has propelled it to become one of the most sought-after sweeteners globally. Notably, Canadian honey production has reached record levels, with approximately 91,807 pounds produced in 2023 alone (Government of Canada, 2024). Predominantly produced in the prairies, Alberta leads with 40.3% of total Canadian honey production, followed by Saskatchewan (21.8%) and Manitoba (18.8%), while Quebec contributes 3.8% (Government of Canada, 2024). However, challenges stemming from the COVID-19 pandemic have caused Canadian honey production to decline by 4.8% compared to the previous year. Despite this decrease, the total value of honey sold saw a significant increase of 14.9%, attributed to a decline in international supply, resulting in Canadian honey producers receiving 20.1% more revenue (Government of Canada, 2021b). Although the majority of honey produced in Canada is consumed domestically, imports amounted to approximately $57 million in 2023, while exports totaled $46 million (Government of Canada, 2024). Despite its global significance, Canada accounts for only 1% of total world honey production and ranks 20th in terms of volume. Notably, China leads as the world's largest honey exporter and producer by volume, while the United States holds the title of the largest honey importer (Government of Canada, 2024). In conclusion, the increasing preference for reduced sugars and natural sweeteners has profoundly impacted honey's consumption worldwide, with consumers increasingly demanding authenticity and adherence to label claims.

2.2.4 Vulnerability of honey to fraud

Honey is a high-value food commodity due to its superior nutritive value and complex production process, making it significantly more expensive to produce than other plant-based sweeteners like corn syrup or cane sugar. This combination of health benefits and high costs makes honey highly susceptible to fraud. The major types of honey fraud include mislabeling its botanical or geographical origin, diluting it with cheaper syrups, harvesting it before it matures,

artificially feeding bees with syrups, and reducing or removing hazardous components such as hydroxymethylfufural (HMF) produced during thermal treatment (Wang et al., 2022). Fraudsters primarily exploit honey's value through adulteration and mislabeling (CFIA, 2022a). Adulteration typically involves the unlawful practice of unethically substituting or partially mixing honey with cheaper substances like low-grade honey, sugars, and syrups. According to *Codex Alimentarius*, honey is the natural sweet substance produced by honeybees from plant nectar or insect secretions (Codex-Alimentarius, 2019). Therefore, adding any other substances is considered adulteration, which not only lowers the product's quality and deceives consumers but can also pose health risks. Common honey adulterants include low-cost sugar syrups such as corn syrup, high fructose corn syrup (HFCS), cane syrup, glucose syrup, and inverted syrup (Fakhlaei et al., 2020). Adulterating honey with these syrups reduces its concentration of beneficial compounds like phenolics, flavonoids, amino acids, vitamins, and minerals. Traditional analytical methods could only detect C4 sugars (from corn or sugar cane), but not C3 sugars (from rice, sugar beets, or wheat), allowing fraudsters to manipulate honey undetected (Sobrino-Gregorio et al., 2019). Mislabelling is another type of honey fraud that involves false claims about the honey's botanical or geographical origin and its nature (e.g., raw, processed, organic). For example, acacia honey is often partially substituted with the less expensive rapeseed honey, negatively impacting the beekeeping industry (Wang et al., 2022). Another fraudulent practice is harvesting honey too early, resulting in 'unripe' honey with a high water content that can ferment and spoil. This practice is common in some Asian countries, where additional aroma compounds are used to mask the spoiled taste (García, 2018). Although this type of fraud reduces harvest time and costs, it results in inferior quality honey with reduced health properties. All in all, with the advancements in technology, testing methods have evolved, allowing food scientists to better detect honey fraud. Despite the development of new and more sophisticated analytical equipment (such as DNA-based, chromatography, and spectrometry methods, as well as a combination of these methods with chemometrics), fraudsters appear to be even more creative in their fraudulent activities, devising sophisticated methods to circumvent the system (Hong et al., 2017).

2.2.5 Application of honey in the food industry

Honey is a highly valued natural sweetener with numerous beneficial properties, making it a popular choice in the food industry. Studies indicate that honey has desirable antioxidant, antimicrobial, and antifungal properties, which are highly sought after by consumers. These health benefits drive the food industry to incorporate honey into various processed goods. Honey is often consumed in its pure form, such as liquid, crystallized, or in a comb state. However, from January 2016 to October 2020, Canada introduced over 1,200 products containing honey as an ingredient. Of these, 1,108 were categorized as food products, 82 were beverages, and 10 were pet foods. The top categories of new food products containing honey included snacks (323), breakfast cereals (153), bakery items (147), sauces and seasonings (102), and processed fish, meat, and egg products (89) (Government of Canada, 2021a). The Canadian food industry has embraced honey in new products for several reasons. Honey is a healthier alternative to refined sweeteners like white sugar or high fructose corn syrup, often used in foods and drinks. It can also replace unrefined sugars, such as brown sugar, in recipes where moisture content is crucial for texture and flavor. As a humectant, honey provides moisture in baked goods, making the baking industry the largest user of honey in the food sector (Bellik & Iguer-ouada, 2013). Honey's pure nature, fine flavor, and distinctive color make it highly desirable for food processing. For companies developing healthy, biological, and “all-natural” food products, honey offers a way to add sweetness without refined sweeteners. Additionally, honey's resistance to spoilage makes it valuable for products requiring extended shelf life (Subramanian et al., 2007). Its nutritional and health-enhancing properties further increase its appeal in high-value food products. Despite its advantages, using honey in food products has limitations. The high cost of honey can make it less accessible for some industries, potentially raising the price of the final product. Natural variations in honey's characteristics, color, and flavor can affect the final product, requiring constant adjustments in formulations (Bellik & Iguer-ouada, 2013).

2.3 Phenolic compounds

Phenolic compounds are a diverse group of secondary metabolites found in plants, characterized by the presence of one or more hydroxyl groups attached to an aromatic ring (Becerril-Sánchez et al., 2021). The phenolic compounds in honey are primarily derived from the nectar and pollen collected by honeybees, and they are categorized into two major groups: phenolic acids and

flavonoids. Phenolic acids consist of at least one hydroxyl group (-OH) and are functional derivatives of cinnamic acid (C6-C3) or benzoic acid (C6-C1) (Kaurinovic & Vastag, 2019). Flavonoids, on the other hand, are comprised of three rings. The first ring is a dehydroxylated phenolic ring, the second one is a monohydroxylated phenolic ring, and finally the third ring is a heterocyclic ring containing pyran oxygen. Flavonoids are classified based on the position of the secondary ring and the oxidation state of the third ring. When the phenolic ring is located on position 2, it generates flavones, flavanols, flavans, catechins, and anthocyanidins. However, if the phenolic ring is placed in position 3, the compound is an isoflavonoid, and when it is found in position 4, it is a 4-phenyl-coumarin or neoflavonoid (Becerril-Sánchez et al., 2021). These structural differences between phenolic acids and flavonoids not only influence their chemical properties but also their biological activities and their roles in the health benefits attributed to honey.

Phenolic compounds have gained significant attention over the years, mainly due to their extensive health benefits. These substances are recognized as the primary contributors to the antioxidant properties of honey (Samarghandian et al., 2017). Polyphenols can stabilize free radicals by donating hydrogen atoms from their hydroxyl groups. Numerous studies suggest that the phenolic compounds in honey may offer anti-cancer, anti-microbial, anti-fungal, anti-viral, anti-inflammatory, and antidiabetic effects. It is proposed that the health benefits of honey largely depend on the bioavailability of these phenolic compounds, including their absorption and metabolization by the body (Becerril-Sánchez et al., 2021). Additionally, phenolic compounds in honey have been shown to provide protective effects on the respiratory, cardiovascular, gastrointestinal, and nervous systems (Alvarez-Suarez et al., 2013; Cianciosi et al., 2018).

2.3.1 Main phenolic compounds in honey

Honey contains a wide range of phenolic compounds, which are responsible for its color, aroma, taste, and biological properties, such as antioxidant, radical-scavenging, and antibacterial activities (Becerril-Sánchez et al., 2021). These polyphenols are introduced into honey through the nectar or pollen collected by honeybees from various plants (Olas, 2020). Honeybees mix their body fluids with the nectar and pollen of flowers or with secretions known as honeydew,

which are composed of sugars, proteins, water, and phenolic compounds. These components are incorporated into the honey during its production by the honeybees.

The most abundant phenolic subgroups are phenolic acids and flavonoids. Commonly detected flavonoids in honey include flavanols, flavonols, and flavones (Cianciosi et al., 2018). The primary phenolic compounds found in honey samples are apigenin, caffeic acid, chlorogenic acid, p-coumaric acid, p-hydroxybenzoic acid, myricetin, protocatechuic acid, quercetin, rutin, and vanillic acid (Becerril-Sánchez et al., 2021). A more extensive list of the main phenolic compounds in honey is provided in Table 2.2.

Table 2.2. Summary of commonly reported phenolic compounds identified in honey.

Phenolic Compounds	Group	Molecular Formula	Reference
Phenolic Acids			
(±) 2-cis,4-trans-Abscisic acid	Terpene	$C_{15}H_{20}O_4$	(Koulis et al., 2021)
4-Hydroxybenzaldehyde	Hydroxybenzaldehyde	$C_7H_6O_2$	(Badea & Vamanu, 2023)
2,3,4-Trihydroxybenzoic acid	Hydroxybenzoic acid	$C_7H_6O_5$	(Cheung et al., 2019)
Benzoic acid	Hydroxybenzoic acid	$C_7H_6O_2$	(Olas, 2020)
Caffeic acid	Hydroxycinnamic acid	$C_9H_8O_4$	(Kumar et al., 2017; Seraglio et al., 2016)
Chlorogenic acid	Hydroxycinnamic acid	$C_{16}H_{18}O_9$	(Cittan & Çelik, 2018; Oszmiański et al., 2018)
Cinnamic acid	Hydroxycinnamic acid	$C_9H_8O_2$	(Ibrahim et al., 2015)
Ellagic acid	Hydroxybenzoic acid	$C_{14}H_6O_8$	(Ibrahim et al., 2015)
Ferulic acid	Hydroxycinnamic acid	$C_{10}H_{10}O_4$	(Hossain et al., 2010; Kumar et al., 2017)
Gallic acid	Hydroxybenzoic acid	$C_7H_6O_5$	(Hossain et al., 2010; Kumar et al., 2017)
Gentisic acid (2,5-Dihydroxybenzoic acid)	Hydroxybenzoic acid	$C_7H_6O_4$	(Koulis et al., 2021)
Homogentisic acid	Phenolic acid	$C_8H_8O_4$	(Jurič et al., 2021)
p-Coumaric acid	Hydroxycinnamic acid	$C_9H_8O_3$	(Hossain et al., 2010)
p-Hydroxybenzoic acid	Hydroxybenzoic acid	$C_7H_6O_3$	(Cittan & Çelik, 2018; Zhao et al., 2014)
Protocatechualdehyde (3,4-Dihydroxybenzaldehyde)	Hydroxybenzaldehyde	$C_7H_6O_3$	(Cheung et al., 2019)
Protocatechuic acid (3,4-Dihydroxybenzoic acid)	Hydroxybenzoic acid	$C_7H_6O_4$	(Hossain et al., 2010; Kumar et al., 2017)
Rosmarinic acid	Hydroxycinnamic acid	$C_{18}H_{16}O_8$	(Pauliuc et al., 2020)
Salicylic acid	Hydroxybenzoic acid	$C_7H_6O_3$	(Biluca et al., 2017)

Sinapic acid	Hydroxycinnamic acid	$C_{11}H_{12}O_5$	(Asenstorfer et al., 2006; Cittan & Çelik, 2018)
Syringic acid	Hydroxybenzoic acid	$C_9H_{10}O_5$	(Hossain et al., 2010)
Trans-cinnamic acid	Hydroxycinnamic acid	$C_9H_8O_2$	(Taş-Küçükaydın et al., 2023)
Vanillic acid	Hydroxybenzoic acid	$C_8H_8O_4$	(Hossain et al., 2010; Kumar et al., 2017)
Vanillin	Hydroxybenzaldehyde	$C_8H_8O_3$	(Jiang et al., 2020)
	Flavonoids		
Apigenin	Flavone	$C_{15}H_{10}O_5$	(Seraglio et al., 2016)
Catechin	Flavan-3-ols	$C_{15}H_{14}O_6$	(Kumar et al., 2017; Oszmiański et al., 2018)
Catechol	Flavonoid	$C_6H_6O_2$	(González-Ceballos et al., 2023)
Chrysin	Flavone	$C_{15}H_{10}O_4$	(Kumar et al., 2017; Seraglio et al., 2016)
Epicatechin	Flavan-3-ols	$C_{15}H_{14}O_6$	(Elamine et al., 2021)
Galangin	Flavonol	$C_{15}H_{10}O_5$	(Castro et al., 2014; Seraglio et al., 2016)
Genistein	Isoflavone	$C_{15}H_{10}O_5$	(López-Fernández et al., 2020)
Hesperetin	Flavanone	$C_{16}H_{14}O_6$	(Sergiel et al., 2014)
Isorhamnetin	Flavonol	$C_{16}H_{12}O_7$	(Y. Chen et al., 2015)
Kaempferol	Flavonol	$C_{15}H_{10}O_6$	(Kumar et al., 2017; Seraglio et al., 2016)
Luteolin	Flavone	$C_{15}H_{10}O_6$	(Seraglio et al., 2016)
Morin	Flavonol	$C_{15}H_{10}O_7$	(Lianda et al., 2012)
Myricetin	Flavonol	$C_{15}H_{10}O_8$	(López-Fernández et al., 2020)
Naringenin	Flavanone	$C_{15}H_{12}O_5$	(Nyarko et al., 2023)
Pinobanksin	Flavonol	$C_{15}H_{12}O_5$	(Castro et al., 2014)
Pinocembrin (5,7-Dihydroxyflavanone)	Flavanone	$C_{15}H_{12}O_4$	(Castro et al., 2014; Zhao et al., 2020)
Pinostrobin	Flavonoid	$C_{16}H_{14}O_4$	(Ciucure & Geana, 2019)
Quercetin	Flavonol	$C_{15}H_{10}O_7$	(Castro et al., 2014; Hossain et al., 2010)
Rutin	Flavonol	$C_{27}H_{30}O_{16}$	(M. Chen et al., 2015)

2.3.1.1 Total phenolic content (TPC)

Assessing the total phenolic content (TPC) in honey from different botanical and geographical origins is essential for assessing its quality and health benefits. Phenolic compounds contribute to honey's antioxidant, antimicrobial, and anti-inflammatory properties, making them key indicators of its nutritional value. Additionally, variations in TPC can reveal insights into the floral sources and environmental conditions affecting the honey. According to data gathered across studies, the average TPC in honey is 325.96 mg GAE/kg. This data, summarized in Table 2.3 and determined

using the Folin-Ciocalteu method, highlights the importance of understanding the diverse phenolic profiles in honey, which can aid in verifying its origin and ensuring product consistency for consumers. Across studies, the TPC in honey ranges from 79.2 mg GAE/kg in blueberry honey to 1121.15 mg GAE/kg in manuka honey. Manuka honey has the highest mean TPC among different honey origins, followed by buckwheat honey at 868.3 mg GAE/kg and thyme honey at 512.16 mg GAE/kg.

Table 2.3. Summary of reported total phenolic content (TPC) in honey according to botanical origins.

Botanical origin	Total phenolic content (mg GAE[a]/kg)	Average TPC per botanical origin (mg GAE[a]/kg)	Country	Literature review
Acacia	187	147.77	Poland	(Kędzierska-Matysek et al., 2021)
	52.6		China	(Cheung et al., 2019)
	63.1		South Korea	(Cheung et al., 2019)
	526.4		Malaysia	(Shamsudin et al., 2022)
	33.21		Iran	(Becerril-Sánchez et al., 2021)
	24.3		Hungary	(Becerril-Sánchez et al., 2021)
Buckwheat	211	868.3	Poland	(Becerril-Sánchez et al., 2021)
	1826		Poland	(Puścion-Jakubik et al., 2022)
	567.9		Poland	(Kędzierska-Matysek et al., 2021)
Blueberry	79.2	79.2	Canada	(Becerril-Sánchez et al., 2021)
Eucalyptus	133.4	385.1	Spain	(Cheung et al., 2019)
	957		Australia	(Becerril-Sánchez et al., 2021)
	320		Italy	(Becerril-Sánchez et al., 2021)
	130		China	(Becerril-Sánchez et al., 2021)
Goldenrod	130.61	130.61	Canada	(Cheung et al., 2019)
Honeydew	164.3	400.63	Poland	(Kędzierska-Matysek et al., 2021)
	860		Poland	(Puścion-Jakubik et al., 2022)
	575.74		Brazil	(Seraglio et al., 2016)
	570		Poland	(Puścion-Jakubik et al., 2022)
	640		Greece	(Becerril-Sánchez et al., 2021)
	201		Poland	(Becerril-Sánchez et al., 2021)
	128.3		Algeria	(Becerril-Sánchez et al., 2021)
	65.67		Malaysia	(Becerril-Sánchez et al., 2021)
Lemon	98.5	82.65	Spain	(Cheung et al., 2019)
	66.8		China	(Cheung et al., 2019)
Linden	62.3	162	China	(Cheung et al., 2019)
	224.3		Poland	(Kędzierska-Matysek et al., 2021)

	133.1		Poland	(Becerril-Sánchez et al., 2021)
	292		Poland	(Puścion-Jakubik et al., 2022)
	98.3		Germany	(Cheung et al., 2019)
Manuka	235.5	1121.15	New Zealand	(Cheung et al., 2019)
	1180		New Zealand	(Becerril-Sánchez et al., 2021)
	2170		New Zealand	(Becerril-Sánchez et al., 2021)
	899.09		New Zealand	(Alzahrani et al., 2012)
Mint	237	237	Romania	(Pauliuc et al., 2020)
Multifloral	203	433.21	Romania	(Pauliuc et al., 2020)
	428 - 782		Brazil	(Lianda et al., 2012)
	328.9		Poland	(Kędzierska-Matysek et al., 2021)
	187		Poland	(Puścion-Jakubik et al., 2022)
	320		Poland	(Puścion-Jakubik et al., 2022)
	454 - 750		Ethiopia	(Liben et al., 2018)
	328.9		Poland	(Kędzierska-Matysek et al., 2021)
	1199		Greece	(Becerril-Sánchez et al., 2021)
	170		Mexico	(Becerril-Sánchez et al., 2021)
	141		Poland	(Becerril-Sánchez et al., 2021)
	74.4 – 140.8		India	(Becerril-Sánchez et al., 2021)
	60.5		Algeria	(Becerril-Sánchez et al., 2021)
	0.26		Turkey	(Becerril-Sánchez et al., 2021)
	223.4 – 1027.7		Mexico	(Alma Delia Hernández-Fuentes, 2021)
Orange blossom	340 - 401	278.6	Brazil	(Lianda et al., 2012)
	532		Brazil	(Lianda et al., 2012)
	49.6		Mexico	(Becerril-Sánchez et al., 2021)
	104		Greece	(Becerril-Sánchez et al., 2021)
	57.2		Spain	(Cheung et al., 2019)
Rapeseed	158.4	236.82	Poland	(Kędzierska-Matysek et al., 2021)
	199		Romania	(Pauliuc et al., 2020)
	331		Poland	(Puścion-Jakubik et al., 2022)
	199		Romania	(Pauliuc et al., 2020)
	296.68		India	(Becerril-Sánchez et al., 2021)
Sunflower	211	211	Romania	(Pauliuc et al., 2020)
Thyme	189	512.16	Romania	(Pauliuc et al., 2020)
	195.3		Spain	(Cheung et al., 2019)
	953		Greece	(Becerril-Sánchez et al., 2021)
	1901		New Zealand	(Becerril-Sánchez et al., 2021)
	250		Italy	(Becerril-Sánchez et al., 2021)
	20		Greece	(Becerril-Sánchez et al., 2021)
	76.85		Iran	(Becerril-Sánchez et al., 2021)
Wildflower	76.8	106.6	Spain	(Cheung et al., 2019)
	136.4		Italy	(Cheung et al., 2019)

Wolfberry	139.3	148.55	China	(Cheung et al., 2019)
	157.8		China	(Cheung et al., 2019)

[a] Gallic acid equivalents, = highest TPC within a botanical origin, = lowest TPC within a botanical origin

2.3.1.2 Individual phenolic compounds in buckwheat honey

The composition of individual phenolic compounds in honey is influenced by several factors including botanical and geographical origins, degree of maturity, processing methods, and storage conditions. Section 2.3.2 delves into the impact of each of these factors on the phenolic profile of honey. However, it is widely observed that botanical origin plays a predominant role in shaping the phenolic composition of honey (Becerril-Sánchez et al., 2021). Consequently, this section focuses on analyzing individual phenolic compounds in buckwheat honey. Nonetheless, it is important to note that geographical origin also significantly affects honey's phenolic profile. Nectars from different geographical regions may vary in phenolic composition and concentrations, contributing to the observed variations in phenolic content among honey samples from the same botanical source but different geographical origins (Nyarko et al., 2023).

Buckwheat honey is notably rich in p-hydroxybenzoic acid, with an average concentration of 22.73 mg/kg across 31 samples, ranging from 1.9 to 62.05 mg/kg. Following p-hydroxybenzoic acid, significant average concentrations were observed for ferulic acid (9.52 mg/kg, n=22), p-coumaric acid (8.56 mg/kg, n=36), and (±)-2-cis,4-trans-abscisic acid (7.52 mg/kg, n=13). Although hesperetin and protocatechualdehyde showed high concentrations (23.76 mg/kg and 14.21 mg/kg, respectively), these findings are based on a single sample each and are therefore not representative. The identification of ferulic, p-coumaric, and (±)-2-cis,4-trans-abscisic acids as predominant phenolic compounds in buckwheat honey was determined from samples with sizes of ten or more, ensuring a more accurate representation. The most substantial variations in phenolic content were seen in p-hydroxybenzoic acid, ranging from 1.9 to 62.05 mg/kg, and ferulic acid, ranging from 0.038 to 58.64 mg/kg. Figure 2.1 summarizes the concentrations of individual phenolic compounds in buckwheat honey as reported in the literature, highlighting these significant findings. (Cheng et al., 2015; Drăgănescu et al., 2020; Jasicka-Misiak et al., 2012; Kędzierska-Matysek et al., 2021; Nešović et al., 2020; Pasini et al., 2013; Puścion-Jakubik et al., 2022; Stanek & Jasicka-Misiak, 2018; Wen et al., 2017; Wilczyńska, 2012; Zhou et al., 2012).

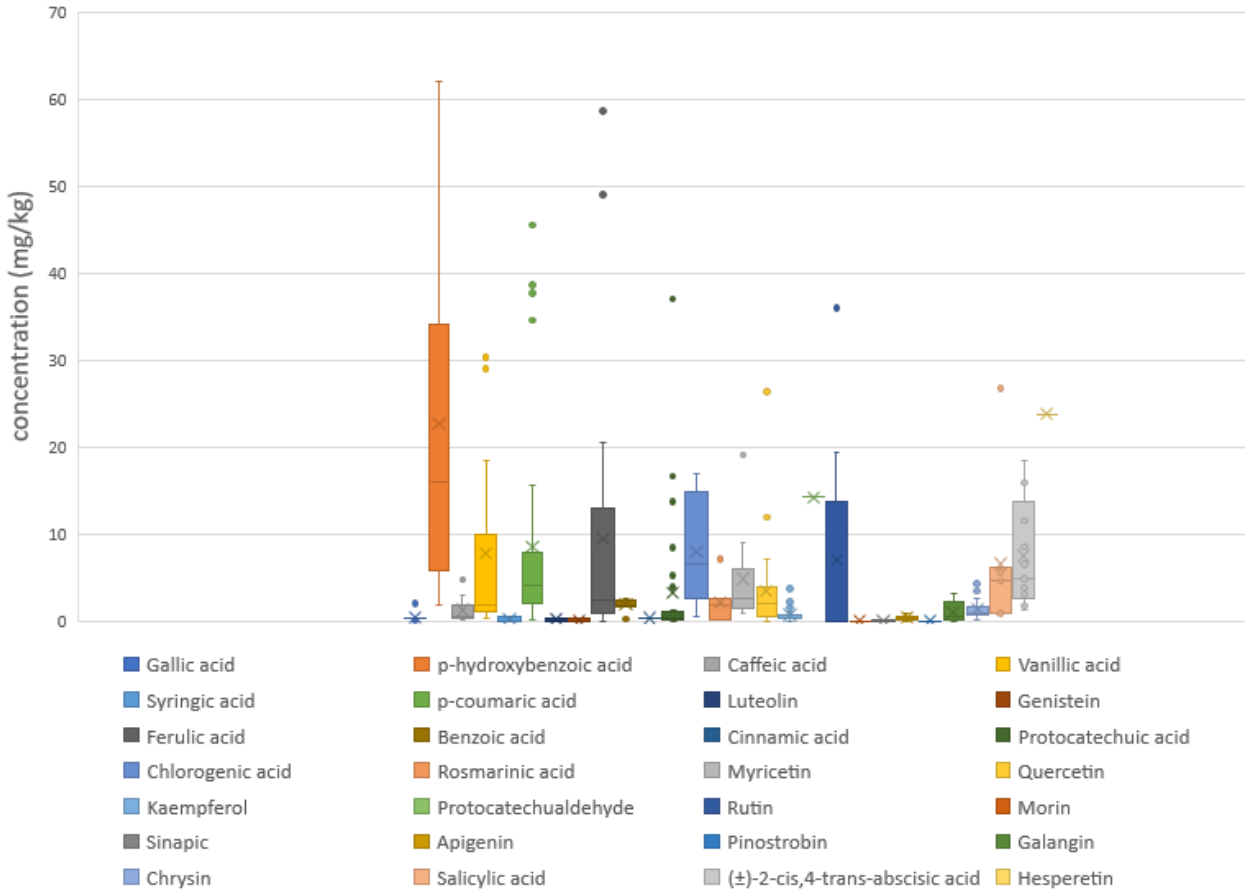

Figure 2.1. Individual phenolic compounds reported in buckwheat honey (botanical origin confirmed with pollen analysis) (Cheng et al., 2015; Drăgănescu et al., 2020; Jasicka-Misiak et al., 2012; Kędzierska-Matysek et al., 2021; Nešović et al., 2020; Pasini et al., 2013; Puścion-Jakubik et al., 2022; Stanek & Jasicka-Misiak, 2018; Y.-Q. Wen et al., 2017; Wilczyńska, 2012; Zhou et al., 2012).

2.3.2 Factors affecting the phenolic profile in honey

The phenolic compound abundance and diversity in honey are dependent on various factors, including the botanical source, climatic and geographical conditions, flower's degree of maturity, honey's season of harvest, processing conditions, interactions with food matrix, and storage conditions. Each of these factors plays a crucial role in determining the phenolic profile of honey, contributing to its unique chemical composition and health benefits. This section will delve into how some of these factors affect the phenolic content in honey. By understanding

these influences, we can better appreciate the complexity and variability of honey's phenolic profile and its implications for quality, authenticity, and health benefits.

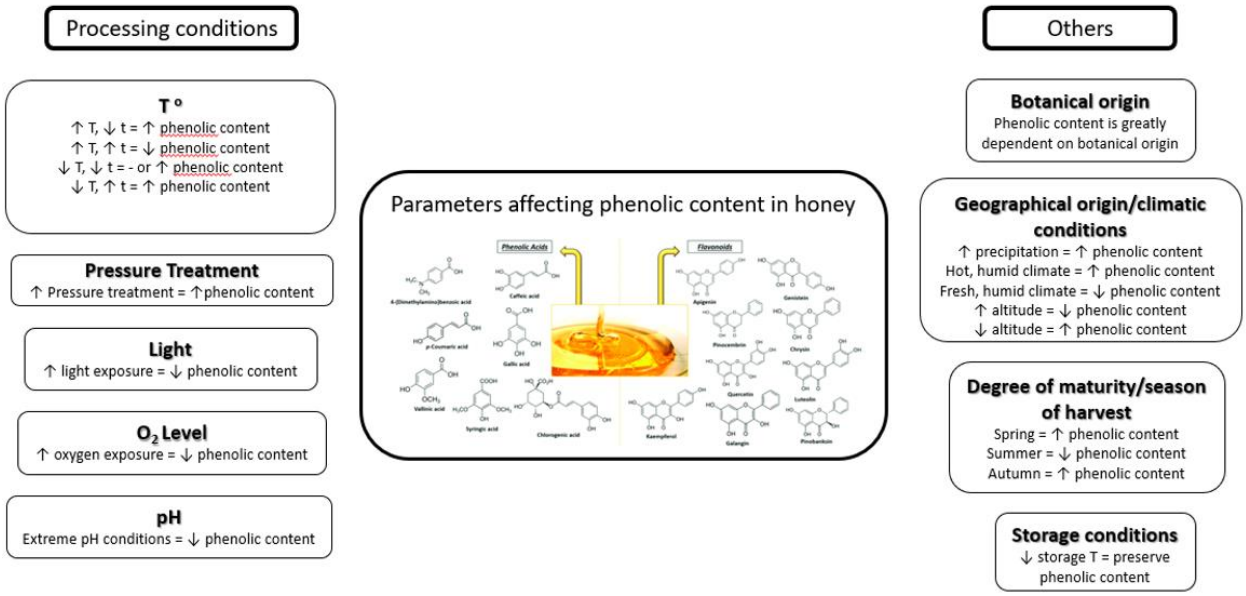

Figure 2.2. Summary of parameters affecting the phenolic content in honey.

2.3.2.1 Botanical origin

Phenolic compounds are strongly related to the botanical origin of honey as it has been reported that the phenolic content differs depending on the floral source. Nectar from different flowers possesses distinct phenolic compositions, contributing to the unique phenolic profile of honey derived from various botanical sources (Becerril-Sánchez et al., 2021). This variation is attributed to the unique chemical composition of nectar produced by various plant species, which influences the phenolic composition of the resulting honey. Plants produce nectar as a reward to attract pollinators such as bees, and the composition of nectar varies among plant species based on factors such as genetics, environmental conditions, and physiological state (Sambangi, 2022). The phenolic compounds found in nectar originate from the plant's metabolism and are synthesized in specialized secretory structures within the flower, such as nectaries and glandular trichomes (Marilia De & Demarco, 2008). Different plant species produce nectar with varying concentrations and types of phenolic compounds, including phenolic acids, flavonoids, and other polyphenols. These compounds serve various functions in plants, such as defense against pathogens and herbivores, attraction of pollinators, and protection against environmental

stressors (Sambangi, 2022). Consequently, the phenolic composition of nectar reflects the unique biochemical profile of each plant species. In addition, when bees forage for nectar, they collect these phenolic-rich floral resources and subsequently introduce them into the hive during honey production. During the honey-making process, enzymes secreted by bees and microorganisms present in the hive can further modify the phenolic compounds, leading to the formation of additional phenolic derivatives. However, the primary source of phenolic compounds in honey remains the nectar collected from flowers (Cianciosi et al., 2018). Consequently, phenolic compounds have garnered attention as potential markers for authenticating honey's botanical origin. Honey originating from a single floral source exhibits unique phenolic profiles, allowing these compounds to serve as distinguishing factors of this particular botanical origin. Specific phenolic compounds, such as galangin, gallic acid, benzoic acid, isorhamnetin, kaempferol, luteolin, and quercetin, to name a few, are commonly found across different honey types, while others, such as p-hydroxybenzoic acid, hesperetin, and naringenin, among others, are unique to specific varieties (Olas, 2020). Table 2.4 in section 2.3.2.1.1 provides an extensive list of proposed phenolic compounds suggested as potential markers for honey from different botanical origins, further highlighting the intricate relationship between the botanical origin and phenolic composition of honey.

2.3.2.1.1 Phenolic compounds suggested as potential markers of honey from different botanical origins

Determining the botanical origin of honey traditionally relies on melissopalynology analysis, which involves studying the pollen content in honey samples to identify the floral sources. However, this method is often impractical due to its time-consuming and labor-intensive nature (Tian et al., 2024). As an alternative, recent research has shifted focus towards phenolic compounds as potential markers for authenticating honey's botanical origin. Phenolic compounds, abundant in honey and closely related to its floral source, offer a promising avenue for origin determination (Becerril-Sánchez et al., 2021). The phenolic profile of honey is influenced by various factors, as discussed in Section 2.3.2, with honey from different botanical sources exhibiting distinct phenolic profiles. Consequently, specific phenolic compounds have emerged as potential chemical markers for identifying the botanical origin of honey. While numerous studies have explored the phenolic composition of various honey types, conclusive results regarding specific marker compounds remain elusive. Table 2.4 provides an overview of

characteristic phenolic compounds proposed as potential markers for honey from different botanical origins. However, further research in this area is crucial for refining the use of phenolic compounds as reliable indicators of honey's botanical origin. Many candidate markers in the literature are not currently reliable due to the variability in study findings, small sample sizes, and the lack of consensus on specific compounds that could serve as markers. Additionally, the absence of clear thresholds for these markers further limits their practical application.

In the realm of food authenticity, a marker is defined as a distinct indicator that helps verify, directly or indirectly, whether a product meets specific claims. These markers, either alone or combined with others, must effectively distinguish between genuine and counterfeit products. It has been proposed that authenticity markers are categorized into three types based on their discriminatory method: "threshold," "binary," and "interval" markers (Bayen et al., 2024). A threshold marker distinguishes authenticity by setting a specific limit that authentic products meet and non-authentic ones do not. A binary marker identifies authenticity through the simple presence or absence of a particular trait. An interval marker uses a range, where authentic samples fall within a defined interval, while non-authentic samples lie outside it, or *vice versa*.

Table 2.4. Proposed phenolic compounds suggested as potential markers of honey from different botanical origins.

Botanical Origin	Phenolic compounds suggested as candidate markers[a]	Geographical origin	Method of detection	References
Acacia honey (*Robinia pseudoacacia*)	- Chlorogenic acid	China	LC-ECD	J. Wang et al. (2014)
Acacia honey (*Acacia Mill.*)	- Chrysin - Pinocembrin	Italy	NMR	Schievano et al. (2013)
Asphodel honey (*Asphodelus microcarpus Salzm. et Viv.)*	- Methyl syringate	Italy	NMR and LC-MS/MS	Tuberoso et al. (2009)
Buckwheat honey (*Fagopyrum esculentum L.*)	- p-Coumaric acid - p-Hydroxybenzoic acid	Poland	HPLC	Puścion-Jakubik et al. (2022)
	- p-Coumaric acid	Lithuania	HPLC-PAD/UV	Ramanauskiene et al. (2012)
	- p-Coumaric acid - p-Hydroxybenzoic acid - Rutin	China	HPLC-MS/MS	Wen et al. (2017)

	- p-Coumaric acid - p-Hydroxybenzoic acid	Poland & Serbia	UHPLC-QqQ-MS/MS	Nešović et al. (2020)
	- p-Coumaric acid - p-Hydroxybenzoic acid	Poland	LC-HRMS	Koulis et al. (2021)
	- Homogentisic acid - (±)-2-cis,4-trans-Abscisic acid - (±)-2-trans,4-trans-Abscisic acid	Italy	HPLC	Wang et al. (2022)
Chestnut honey (*Castanea mollissima BL.*)	- Caffeic acid - Naringenin	Italy	HPLC/DAD	Preti and Tarola (2022)
Citrus honey (*Citrus reticulata Blanco*)	- 8-Hydroxylinalool	Italy	NMR spectroscopy	Schievano et al. (2012)
	- Hesperetin	Spain	HPLC	Escriche et al. (2011)
	- Hesperetin	Italy	HPLC/DAD	Preti and Tarola (2022)
	- Hesperetin	Spain	HPLC	Ferreres et al. (1993)
Dandelion honey (*Taraxacum* sp. Honey)	- Myricetin	Italy	LC/MS	Di Marco et al. (2018)
Eucalyptus (*Eucalyptus robusta Smith*)	- Gallic acid	Lithuania	HPLC-PAD/UV	Ramanauskiene et al. (2012)
Forest honey (specie not specified)	- Protocatecuic acid	Germany	GC/MS	Recklies et al. (2021)
Heather honey (*Calluna vulgaris L.*)	- Caffeic acid - Chlorogenic acid	Italy	LC/MS	Di Marco et al. (2018)
	- Myricetin	Poland	HPLC	Zieliński et al. (2014)
Heather honey (*Erica* sp.)	- Chrysin - Galangin - Kaempferol - Myricetin - Quercetin	Poland	NMR spectroscopy	Jasicka-Misiak et al. (2012)
Honeydew honey	- Vanillic acid	Poland	HPLC	Puścion-Jakubik et al. (2022)
	- Carboxylic acid - Protocatechuic acid	Germany	GC/MS	Recklies et al. (2021)
	- Quercetin - Chlorogenic acid - p-Coumaric acid	Italy	LC/MS	Di Marco et al. (2018)
	- Kaempferol - Chrysin - p-Coumaric acid	Italy	HPLC/DAD	Preti and Tarola (2022)

Jujube honey (*Ziziphus jujuba Mill.)*	- Cinnamic acid	China	HPLC	Zhao et al. (2016)
Linden honey (*Malvaceae*)	- 3,4-Dihydroxybenzoic acid - Syringic acid	Poland	HPLC	Puścion-Jakubik et al. (2022)
	- Vanillic acid - Caffeic acid	China	UPLC-MS/MS	Shen et al. (2018)
	- 3,4-Dihydroxybenzoic acid - p-Coumaric acid - Chlorogenic acid - Caffeic acid	Italy	HPLC/DAD	Preti and Tarola (2022)
Lingonberry (*Vaccinium vitis-idaea Linn.*)	- Acacetin - Cinnamic acid	Finland	HPLC-MS/MS	Salonen and Julkunen-Tiitto (2012)
Longan honey (*Dimocarpus longan Lour.)*	- p-Coumaric acid - Ferulic acid - Syringic acid	China	HPLC	Zhao et al. (2016)
Manuka honey (*Leptospermum scoparium*)	- Methyl syringate	New Zealand	HPLC	Weston et al. (2000)
	- 2-Methoxybenzoic acid - Trimethoxybenzoic acid	New Zealand	LC-MS/MS	Stephens et al. (2010)
	- Kojic acid - Lepton - 2-Methoxybenzoic acid - 4-Methoxyphenyllactic acid - Methyl syringate	New Zealand	UPLC-PDA-MS/MS	Oelschlaegel et al. (2012)
	- Kojic acid - Leptosin	New Zealand	UHPLC-PDA-MS/MS	Beitlich et al. (2014)
Rapeseed honey (*Brassica napus L. var. napus*)	- Ellagic acid	China	LC-ECD	J. Wang et al. (2014)
	- Benzoic acid	China	HPLC-MS/MS	Wen et al. (2017)
	- Syringic acid	China	UPLC-MS/MS	Shen et al. (2018)
Rosemary honey (*Rosmarinus officinalis*)	- Caffeic acid - Chrysin - Kaempferol - Naringenin - Pinocembrin	Spain	HPLC	Escriche et al. (2014)
Sage honey (*Sage officinalis L.)*	- Kaempferol	Croatia	LC-MS/MS	Gasic et al. (2015)
Sativa honey (*C. sativa*)	- Apigenin - Caffeic acid - Quercetin - p-Coumaric acid	Italy	LC-MS	Di Marco et al. (2018)
Strawberry tree honey (*Arbutus unedo L.*)	- Homogentisic acid - (±)-2-cis,4-trans-Abscisic acid	Italy	HPLC-DAD MS/MS	Tuberoso et al. (2010)

	- (±)-2-trans,4-trans-Abscisic acid - Rutin - Luteolin - Syringic acid - Abscisic acid	Italy	HPLC/DAD	Preti and Tarola (2022)
Sunflower honey (*Helianthus annuus*)	- Quercetin - Eriodictyol	Serbia	UHPLC-LTQ	Kečkeš et al. (2013)

[a] Markers identified in the referenced study due to high levels in specific botanical origins.

2.3.2.2 Geographical origin and climatic conditions

The phenolic composition and concentration in honey is also greatly dependent on the geographical origin of honey. Factors such as climate, altitude, and region (rural or urban) may all impact the presence of specific phenolic compounds in pollen. Generally, honey from the same botanical and geographical origins exhibit similar phenolic profiles due to consistent pollen patterns. Conversely, honey from identical floral sources but different geographical locations, or from different floral sources within the same geographical location, display varied phenolic compositions. A study investigating the phenolic compound profile in Sulla (*Hedysarum spp.*) honey produced in different areas of Southern Italy has concluded that concentrations of caffeic acid, chlorogenic acid, p-coumaric, ferulic acid, and gallic acid were greatly influenced by the geographical location of the honey's production site (Gambacorta et al., 2014). Differences in phenolic content among honey samples from the same botanical origin but different geographical origins may be attributed to soil composition, humidity, temperature, altitude, and possible land contamination (e.g., mining activities or automobile exhaust emissions), which can affect the plant's physiological state and consequently influence phenolic biosynthesis (Gambacorta et al., 2014). Another study examining the phenolic compound level in different climates in Kenya has found significant differences amongst samples collected from various regions. Regions with high precipitations had the highest total phenolic content of 141.72 mg GAE/100g compared to samples from hot and humid climates (116.18 mg GAE/100g) and semiarid climates (98.38 GAE/100g). Similar trends were observed for the total flavonoid content, with honey from hot and humid climates showing 35.47 mg QE/100g and those from fresh and humid climates yielding 29.19 mg QE/100g. These differences are likely due to variations in vegetation and the availability of melliferous plants, which are highly dependent on regional climate (Becerril-Sánchez et al., 2021). Moreover, since honeybees collect nectar from plants which contains

varying levels of phenolic compounds depending on the botanical and geographical origins, the phenolic profile is therefore very specific to the floral and geographical origin of honey. For instance, gallic acid and p-coumaric acid are predominant phenolic acids in Polish honeys (Socha et al., 2009), while Italian honey is characterized by chlorogenic acid, p-coumaric acid, and ferulic acid when present in levels of tens of mg/kg (Perna et al., 2013). Serbian honey, on the other hand, is dominant in p-coumaric acid (up to 9.97 mg/kg) and ellagic acid (0.28-8.48 mg/kg), with other phenolic acids generally below 1 mg/kg (Gašić et al., 2014). Overall, the geographical origin of honey appears to greatly impact the phenolic content and composition in honey samples due to various factors, including climate, altitude, and region (rural and urban). It is in fact suggested that both botanical and geographical origins are considered major contributors to the changes in phenolic content in honey.

2.3.2.3 Degree of maturity and season

The maturity level of the flowers used by honeybees to produce honey, as well as the season of honey collection, significantly impact the final phenolic composition in honey. Generally, autumn honey (e.g., buckwheat and thyme honeys) tends to have a higher phenolic content compared to spring honey (e.g., acacia and clover honeys). However, this higher phenolic content in autumn honey is not solely due to the season of harvest; rather, it is because flowers that bloom later in the summer, such as buckwheat and thyme, inherently contain higher levels of phenolic compounds. Additionally, the degree of maturity of the flowers used for honey production influences the nectar's composition available for honey making. The maturity of flowers affects the biosynthesis of the plant’s secondary metabolites, including phenolic compounds (Kekecoglu et al., 2021). Studies suggest that the more homogeneous the maturity level of the nectar collected by honeybees, the higher the stability of the chemical composition of the honey (Becerril-Sánchez et al., 2021; Dżugan et al., 2018). This indicates that the phenolic composition can vary depending on whether the honey is produced from mature or immature flowers. For instance, the levels of gallic acid and caffeic acid differ significantly in honey produced from mature versus immature flowers, suggesting that phenolic compounds can serve as potential markers for distinguishing the maturity level of flowers used in honey production (Zhang et al., 2021). However, it is noteworthy that few studies have specifically investigated the impact of flower maturity on the phenolic composition of honey.

2.3.2.4 Temperature

Temperature plays a critical role in influencing the phenolic composition of honey, exerting a significant impact on the stability of phenolic compounds. While the precise mechanisms underlying the effect of temperature on phenolic content remain not fully elucidated, studies suggest that both temperature and exposure time are key factors influencing phenolic levels in honey samples (Braghini et al., 2019; Braghini et al., 2021; Mat Ramlan et al., 2021; Šarić et al., 2013; Zarei et al., 2019). High temperatures during processing, whether for short or long durations, are found to have a more pronounced effect on phenolic content compared to milder heat treatments. Moreover, extended heating periods at lower temperatures have been shown to significantly increase phenolic content in honey. In some instances, thermal treatment of honey leads to the formation of individual phenolic compounds through the hydrolysis or conversion of other honey components. For instance, at temperatures between 55°C and 65°C, compounds such as isoquercetin and rutin were identified after heating for durations as short as 22 minutes (Braghini et al., 2021). Similarly, at higher temperature ranges (85°C to 95°C), phenolic compounds like ferulic acid, chlorogenic acid, and protocatechuic acid were detected after as little as 15 seconds of heating (Braghini et al., 2019). Table 2.5 summarizes the effect of temperature on honey's phenolic composition.

Table 2.5. Summary of the effect of temperature on phenolic content in honey reported in the literature.

Temperature range (°C)	**Time**	**Impact on specific and total phenolic compounds**	**Reference**
[45-55[	10 min	TPC: 22% ↓	(Omar et al., 2021)
	1h	TPC: - p-Coumaric acid: ↑ Ferulic acid: ↑ Chlorogenic acid: ↑ Protocatechuic acid: ↑	(Mat Ramlan et al., 2021)
	470 min	p-Coumaric acid: ↓ Salicylic acid: ↓ Vanillic acid: ↓ Aromadendrin: ↓ Apigenin: ↓ Hispidulin: ↓ Quercetin: ↓	(Braghini et al., 2021)

	48h	Kaempferol: ↓ Galangin: ↓ Myricetin: ↓ p-Coumaric acid: ↓	(Escriche et al., 2014)
	48h	TPC: 13% ↑	(Flanjak et al.)
[55-65[	10 min	TPC: -	(Zarei et al., 2019)
	20 min	TPC: -	(Zarei et al., 2019)
	22 min		(Braghini et al., 2021)
	30 min	TPC: ↓	(Zarei et al., 2019)
	1h	TPC: -	(Mat Ramlan et al., 2021)
	1h	TPC: - p-Coumaric acid: ↑ Ferulic acid: ↑ Chlorogenic acid: ↑ Protocatechuic acid: ↑	(Mat Ramlan et al., 2021)
	1h	p-Coumaric acid: ↓ Salicylic acid: ↓ Vanillic acid: ↓ Aromadendrin: ↓ Apigenin: ↓ Hispidulin: ↓ Quercetin: ↓	(Braghini et al., 2021)
	1h	Protocatechuic acid: 61.3% ↓ Protocatechualdehyde: 5.5% ↑ 4-Hydroxyphenylacetic acid: 11.7% ↑ Chlorogenic acid: 23.5% ↓ Rutin: 54.7% ↓ Ethylvanillin: 29.3% ↓	(Hájek, 2023)
	170 min	p-Coumaric acid: ↓ Salicylic acid: ↓ Vanillic acid: ↓ Aromadendrin : ↓ Apigenin : ↓ Hispidulin: ↓ Quercetin: ↓	(Braghini et al., 2021)
	12h	TPC: ↑	(Aydoğan Coşkun et al., 2020)
[65-75[	0.24 min	TPC: ↑	(Braghini et al., 2021)
	1 min	TPC: ↑	(Braghini et al., 2021)
	3 min	TPC: ↑	(Braghini et al., 2021)

	8 min	TPC: ↑	(Braghini et al., 2021)
	10 min	TPC: 25% ↓	(Omar et al., 2021)
	1h	TPC: -	(Mat Ramlan et al., 2021)
	6h	TPC: 24% ↑	(Flanjak et al.)
[75-85[	4 min	Kaempferol: ↓ Galangin: ↓ Myricetin: ↓ p-Coumaric acid: ↓	(Escriche et al., 2014)
	20 min	Protocatechuic acid: 63.2% ↓ Protocatechualdehyde: 47.5% ↓ 4-Hydroxyphenylacetic acid: 16.2% ↓ Chlorogenic acid: 31.4% ↓ Rutin: 55.2% ↓ Ethylvanillin: 25.9% ↓	(Hájek, 2023)
[85-95]	15sec	TPC: ↑ p-Coumaric acid: ↑ Aromadendrin: ↑ Chrysin: ↓ Carnosol: ↓	(Braghini et al., 2019)
	60sec	TPC: ↑ p-Coumaric acid: ↑ Aromadendrin: ↑ Chrysin: ↓ Carnosol: ↓	(Braghini et al., 2019)
[95-105[	15 sec	TPC: ↑ Caffeic acid: ↓	(Aydoğan Coşkun et al., 2020)
	5 min	TPC: ↑↓	(Šarić et al., 2013)
	10 min	TPC: 29% ↓	(Omar et al., 2021)
	60 min	TPC: ↓	(Omar et al., 2021)

↑ represents an increase in the individual phenolic concentration or TPC, ↓ represents a decrease in the individual phenolic concentration or TPC, - indicates no change in the individual phenolic concentration or TPC.

Multiple studies have proposed various theories to explain the fluctuation of phenolic content during heat treatment. One hypothesis suggests that the increase in phenolic concentration may be attributed to the hydrolysis of specific glycosides into aglycone forms, a process triggered by factors like high acidity, temperature, and the presence of natural enzymes in honey (Morales-de la Peña et al., 2011; Song et al., 2016). Additionally, it is postulated that elevated treatment temperatures could lead to the hydrolysis of polysaccharides, thereby increasing the

concentration of phenolic compounds (T. Wang et al., 2014). Furthermore, high-temperature conditions may facilitate the hydrolysis and conversion of other honey constituents, such as amino acids, resulting in the formation of novel phenolic compounds (da Silva et al., 2016; Nemitz et al., 2017). Another contributing factor to the observed increase in phenolic content following heat treatment is the denaturation of proteins, making phenolics more readily available. Phenolics often form complexes with proteins, rendering them undetectable in their free form during quantification analysis using various analytical methods. However, heat treatment leads to protein denaturation, disrupting these complexes and allowing for the quantification of phenolics (Sęczyk et al., 2019). Conversely, the decrease in phenolic content during heat exposure is typically attributed to changes in the chemical structure of phenolic compounds. Reactions such as isomerization, polymerization, oxidation, and degradation may occur, generating more stable intermediate products that often yield non-phenolic compounds (Larsen & Ahmed, 2022; Maghsoudlou et al., 2019). High temperatures act as catalysts for these chemical reactions, leading to the breakdown of phenolic compounds and rendering them undetectable by analytical techniques due to alterations in their chemical structure. In summary, the influence of heat treatment on phenolic content in honey remains somewhat ambiguous. Nonetheless, both temperature and exposure time emerge as primary factors affecting phenolic levels in honey samples. These findings underscore the complexity of the relationship between heat treatment and phenolic content in honey, warranting further investigation for a comprehensive understanding.

2.3.2.5 Storage conditions

Storage conditions, particularly time and temperature, are additional factors influencing the profile and concentration of phenolic compounds in honey. However, assessing their impact is complex due to the interplay of various factors such as light exposure, honey's botanical and geographical origins, and its inherent components like enzymes, volatile compounds, and pH levels (Chou et al., 2020; Šarić et al., 2020). Studies indicate that during the initial 6 months of storage at room temperature with daytime light exposure, the flavonoid content tends to increase, possibly due to enzymatic reactions or secondary reactions generating new flavonoid compounds (Monggudal et al., 2018; Šarić et al., 2020). Prolonged storage under UV light may also lead to the spontaneous production of new compounds from the existing substrates, further increasing

flavonoid content (Brudzynski & Kim, 2011). Subsequently, between the 6th and 9th months, a decline in flavonoid content is observed, attributed to decreased enzymatic activity and the destabilization of unstable flavonoids into more stable intermediates (Šarić et al., 2020). Lower storage temperatures seem to have minimal impact on phenolic content changes over time, whereas higher temperatures can significantly affect their stability, resulting in greater fluctuations (Braghini et al., 2019; Chou et al., 2020). Overall, while multiple factors influence phenolic profile changes during storage, time and temperature emerge as key parameters. However, given the multitude of factors at play, establishing a direct relationship between storage conditions and phenolic profile remains challenging. Nevertheless, many studies come to the agreement that storing honey at refrigerated temperatures is the best way to preserve the phenolic and flavonoid content in honey as these temperatures allow them to remain stable which is crucial in the context of tracking a marker. Interestingly, if phenolic compounds degrade with storage time, perhaps these degradation products may themselves become useful markers.

2.4 Analytical methods used to assess the authenticity of honey

Currently, available official honey authenticity methods target only a few types of adulteration. These methods rely mostly on targeted analysis (TA), an approach designed to detect specific types of markers, which is an inefficient technique for detecting new types of fraud (Rodionova & Pomerantsev, 2020). Alternatively, non-targeted analysis (NTA) allows for the rapid characterization of thousands of never-before-studied chemicals in complex matrices. NTA fingerprinting has the potential to investigate a wider range of quality attributes simultaneously and chemical fingerprints are virtually impossible to imitate for fraudsters due to their complexity (Rodionova & Pomerantsev, 2020). As a result, TA and NTA are employed for distinct purposes, leading to significantly different outcomes. Both analytical workflows for honey authentication are discussed in this section and the major differences between the two analysis approaches will be described. In addition, specific examples of techniques used for honey authentication will be provided.

2.4.1 Targeted analysis

TA is the detection and/or quantification of one or multiple pre-characterized analytical compound(s) from a standard (Ballin & Laursen, 2019). Due to the fact that the analytes in question are already defined, this method has therefore a greater selectivity and sensitivity as

compared to NTA. This method is often used to confirm the presence or absence of an analyte in a product. In addition, targeted analysis is usually preferred when dealing with primary markers which provide results that directly address a specific authenticity issue and are often used when the food is suspected to be fraudulent (Ballin & Laursen, 2019; Bayen et al., 2024). For example, there are many proposed phenolic compounds suggested as potential markers of honey from different botanical origins (summarized in Table 2.4). By using the TA approach to authenticate honey, these known markers could be used to identify the botanical origin of honey. An issue with TA is that it only focuses on selected analytes which is problematic in authenticity analysis as novel adulterants will not be detected using this method. Moreover, TA requires complex and laborious extraction processes before sample analysis which is not practical for authenticity purposes. In brief, TA is a good tool to use when the adulterant present in the food product is known. This technique is desired for the quantification of these adulterants as it is a highly selective and sensitive method. However, the biggest drawback to this analysis type is that it is unable to detect novel adulterants. This is problematic as it allows fraudsters to manipulate the system and develop new techniques to adulate food without it being detected.

2.4.2 Non-targeted analysis

NTA is the simultaneous detection of several unspecified compounds in a product without having prior information on it. This technique is often used to observe the presence or absence of patterns or describe the fingerprint of a product. It also has the ability to efficiently screen for unexpected compounds present in the food sample using a library. Hence, NTA has become one of the most useful tools for authenticity determination. This type of analysis method is suggested to be very valuable for modern food authentication, especially in complex food matrices, due to its ability to present data as a fingerprint and its particular suitability to discover novel markers (Bayen et al., 2024). Fingerprinting is powerful in authenticity assessment as it has the ability to detect the smallest changes in food and express these changes in a valuable manner through advanced multivariate statistics (Ballin & Laursen, 2019). No matter the technique used by fraudsters to adulterate the food, deviations in the composition of the food products can easily be identified through the examination of the database collected from NTA. Therefore, NTA has the ability to authenticate complex issues such as production methods, botanical and geographical origins through the observation of patterns (Ballin & Laursen, 2019). To sum, as discussed

previously, TA does not have the ability to detect unknown adulterants, thus making this method less efficient in detecting never-seen-before adulterants. However, NTA is a promising method for authenticity verification since fingerprinting is shown to provide a unique signature of the product which cannot be replicated by fraudsters.

2.4.3 Current approaches available to authenticate the botanical origin of honey

To authenticate the botanical origin of honey, several methods are commonly used. One of the first and most common techniques is melissopalynology, which involves the microscopic analysis of the pollen grains contained in honey to assess its floral origin. This technique involves microscopic analysis of the characteristics of pollen grains within honey, comparing their morphological features such as size, shape, surface ornamentation, and apertures to reference datasets to ascertain floral sources (Corvucci et al., 2015). The presence and predominance of specific pollen types indicate the plant sources from which the bees collected nectar to produce the honey. However, melissopalynology has limitations, notably in over- or under-representing pollen in honey. Some plants produce abundant pollen, overshadowing minor nectar contributors, while others with less accessible pollen may be underrepresented, despite being a significant nectar source (Molan, 1998). In addition, commercial honey processing, including filtration to remove debris, can further affect pollen presence, particularly from sources with large pollen grains which may be filtered out and not be present in honey (Battesti & Goeury, 1992; Bryant, 2017). While melissopalynology is valuable, these challenges highlight the need for complementary analytical methods to achieve more accurate and reliable results.

Sensory analysis, often complemented by physicochemical parameters determination, serves as an alternative method for confirming honey's botanical origin. Sensory analysis involves human assessment of taste, aroma, odor, and crystallization. However, sensory analysis lacks standardization, even with trained evaluators (Tsagkaris et al., 2021). Physicochemical analysis emerges as a simpler and cost-effective option, assessing properties like pH, acidity, electrical conductivity, moisture content, and sugar profile. Coupled with chemometric tools like PCA, PLS-DA, regression trees, and LDA, it aids in distinguishing honey's botanical origin (Tsagkaris et al., 2021). Additionally, elemental composition analysis (e.g., Fe, Zn, K, Cu, Mg, ...) offers insights into honey's origin (Squadrone et al., 2020). Minerals in honey originate from plant nectar and pollen grains, reflecting the plant's elements and soil type (Madejczyk &

Baralkiewicz, 2008). Thus, the elemental fingerprint correlates with flower composition and determines honey's botanical origin.

Chemical analysis stands out as one of the most dependable methods for assessing honey's botanical origin, offering the capability to identify and quantify bioactive markers. Separation techniques like liquid chromatography (LC) and gas chromatography (GC) are widely employed, enhancing detectability, accuracy, and precision. LC, favored for its versatility in measuring various compounds, targets specific compounds such as phenolic compounds, organic acids, sugars, amino acids, minerals, and trace elements which are indicative of botanical sources in honey. In contrast, GC excels in profiling volatile organic compounds, such as aldehydes, ketones, acids, alcohols, hydrocarbons, terpenes, benzenes compounds, and their furan and pyran derivatives (Soares et al., 2017). Spectroscopic techniques provide rapid and effective spectral information and allow the discrimination of honey’s botanical origin when compounded with chemometric analysis (Tsagkaris et al., 2021). Nuclear magnetic resonance (NMR) spectroscopy delves into honey's chemical composition at a molecular level, identifying unique metabolites linked to floral sources (Ohmenhaeuser et al., 2013). Fourier Transform Infrared (FTIR) spectroscopy discerns distinctive functional groups and molecular structures that may be unique in honey from different botanical origins (Bunaciu & Aboul-Enein, 2022). Raman spectroscopy detects shifts in the wavelength of scattered light as it interacts with molecular vibrations. This technique provides a different set of molecular information compared to infrared (IR) spectroscopy and is sensitive to molecular structures that can identify specific botanical markers in honey (Svečnjak et al., 2011). Mass spectrometry (MS) is a powerful analytical technique that measures the mass-to-charge ratio of ions. It is highly effective and accurate in identifying and quantifying individual chemical compounds in honey, allowing for the determination of the concentration of specific botanical markers in honey (Jandrić et al., 2017). The advantages of using mass spectrometry are that it can detect trace levels of compounds present in honey and has a high specificity compared to spectroscopic techniques which may be useful when identifying biomarkers in honey (Tsagkaris et al., 2021).

DNA analysis techniques, such as DNA barcoding or metabarcoding, have emerged as potent tools for evaluating the botanical origin of honey (Bruni et al., 2015). These methods entail the extraction and sequencing of target DNA, typically from pollen grains, which serve as the

primary botanical material in honey. This approach offers expedited and more precise taxonomic identification of botanical sources compared to traditional melissopalynology. Notably, DNA analysis can discern plant species with high accuracy, even when pollen grains share morphological similarities (Hawkins et al., 2015). Unlike subjective microscopic analysis which depends on the analyst's expertise, DNA-based methods yield objective results, enhancing reliability (Tsagkaris et al., 2021). However, DNA analysis may face challenges with filtered honey, where lower DNA quantities impede effectiveness, primarily providing qualitative outcomes. Additionally, honey processing and storage conditions can potentially compromise DNA integrity, posing obstacles to extraction and analysis processes (Soares et al., 2017). These nuances underscore the need for careful consideration of various factors when employing DNA analysis techniques in the authentication of honey's botanical origin.

2.4.4 Current analytical techniques available to authenticate the botanical origin of honey using phenolic compounds as markers of authenticity

Phenolic compounds have emerged as valuable indicators in characterizing honey, offering insights into its botanical origins. Recognizing their significance, there is a growing emphasis on developing reliable analytical techniques to leverage this information effectively. Authenticating the botanical origin of honey using phenolic compounds as markers necessitates the deployment of robust analytical techniques. This entails beginning with an efficient extraction process to isolate phenolics from the honey matrix, followed by employing reliable separation and detection methods to identify and quantify these compounds accurately. Subsequently, chemometric analysis assumes a critical role in discerning phenolic markers that reflect the honey's botanical origin. Table 2.6 lists many of the analytical tools used for analyzing phenolic compounds in honey.

Table 2.6. Overview of analytical techniques for analyzing phenolic compounds in honey.

Extraction method	Separation and detection method	Mobile phase	Stationary phase (dimensions)	Separation time	References
Dilute-and-shoot	HPLC–MS/MS	(A) water with 0.1% formic acid (B) acetonitrile with 0.1% formic acid	VENUSIL C18 (100 × 2.1 mm, 3 µm)	17 min	(Seraglio et al., 2016)
LLE	HPLC-TOF/MS	(A) 0.1% formic acetic acid in water (B) acetonitrile	Agilent Poroshell 120 EC-C18 (50 ×4.0 mm, 2.7 µm)	30 min	(Sıcak et al., 2021)
SPE Amberlite XAD-2	HPLC–DAD	(A) Acidified demineralised water-3% formic acid (B) Methanol	LiChrospher 100 RP18 (125 × 4 mm, 5 µm)	60 min	(Šarić et al., 2020)
SPE	GC-QqQ-MS	(helium gas)	HP-5 ms fused silica capillary column (30 m × 0.25 mm × 0.25 µm)	NA	(Kozłowicz et al., 2020)
Strata C18–E	UHPLC–LTQ Orbitrap MS and UHPLC–UV–MS/MS	(A) water containing 0.1% acetic acid (B) 100% acetonitrile.	Syncronis C18 (100 × 2.1 mm, 1.7 µm)	20 min	(Vasić et al., 2019)
LLE	HPLC–MS/MS	(A) 95% methanol in water (B) 0.1% formic acid in water	Synergi column (150 ×2.0 mm, 4.6 µm)	17 min	(Braghini et al., 2019)
SPE C18	UHPLC–PDA–MS/MS (detection λ = 254–372 nm)	(A) 0.1% formic acid in water (v/v) (B) 0.1% formic acid in 40% ACN in water (v/v)	BEH C18 (100 × 2.1 mm, 1.7 µm)	9.5 min	(Dżugan et al., 2020)
LLE	UPLC–QToF-MS	(A) H2O: MeOH (90:10) with 5 mM ammonium acetate (B) MeOH with 5 mM ammonium acetate	Acclaim RSLC C18 column (2.1 × 100 mm, 2.2 µm)	20 min	(Koulis et al., 2021)
QuEChERS	HPLC–DAD (detection λ = 240, 260, 280, 300, 325 and 370 nm)	1.6% MeOH, 3.3% ACN, 1.0% THF and 94.1% formic acid 0.1% changing to10.0% MeOH, 33.3% ACN, 6.0% THF and 50.7% formic acid 0.1%	Zorbax Poroshell 120 C18 (50 × 4.6 mm, 2.7 µm)	25 min	(Silva et al., 2019)
SPE Amberlite XAD-2	HPLC–ECD	(A) methanol (B) 0.1% (v/v) aqueous formic acid	Zorbax SB-C18 (250 × 4.6 mm, 5 µm)	60 min	(Wang et al., 2020)
SULLE	UHPLC–Q Exactive	(A) 0.1% formic acid in H2O (B) 0.1% formic acid in MeCN	Kinetex PFP column (100 × 3.0 mm, 1.7 µm)	14 min	(Labsvards et al., 2021)
LLE	HPLC–ESI–MS/MS	(A) 95% methanol in water (B) 0.1% formic acid in water.	Synergi column (150 × 2.0 mm, 4.6 µm)	17 min	(Biluca et al., 2020)

SPE Amberlite XAD-4	HPLC–ESI-TOF–MS	(A) 0.5 mL/100 mL of acetic acid in deionised water (B) methanol	ZORBAX Eclipse plus reversed-phase C18 (150 × 4.6 mm, 1.8 µm)	34 min	(Ouchemoukh et al., 2017)
SPE Strata-X cartridges	HPLC–ESI–MS/MS	(A) water with 0.1% formic acid (B) methanol with 0.1% formic acid	Zorbax C18 (50 × 2.1 mm, 0.18 µm)	15 min	(Anand et al., 2019)
LLE	HPLC–DAD (detection λ = 320 nm)	(A) 0.1% acetic acid in water (B) acetonitrile	Phenomenex Kinetex Biphenyl (150 × 4.6 mm, 2.6 µm)	NA	(Pauliuc et al., 2020)
SPE Strata-X cartridge	HPLC–DAD–MS (detection λ = 254–360 nm)	(A) 1% aqueous solution of formic acid (B) acetonitrile	Luna C18 (150 × 2.0 mm, 3 µm)	80 min	(Bertoncelj et al., 2011)
SULLE	HPLC–ECD	(A) 0.5% aqueous formic acid (v/v) (B) methanol	Waters XBridge C18 (250 × 4.6 mm, 5 µm)	37 min	(Zhu et al., 2019)
SPE C18	HPLC–DAD–MS/MS (detection λ = 280, 320, 350 nm)	(A) water/ formic acid (99.5:0.5, v/v) (B) acetonitrile/ formic acid (99.5:0.5, v/v)	C18 Synergi Hydro (250 × 4.6 mm, 4 µm)	60 min	(Rodrigues da Silva et al., 2021)
Strata C18–E	UHPLC–QqQ–MS/MS	(A) water with 0.1% formic acid (B) acetonitrile with 0.1% formic acid	Syncronis C18 column (100 × 2.1 mm, 1.7 µm)	14.59 min	(Nešović et al., 2020)
SULLE	UHPLC–Q Exactive	(A) deionised water with 0.1% FA (B) MeCN with 0.1% FA	Kinetex PFP column (100 × 3.0 mm, 1.7 µm)	12 min	(Rusko et al., 2021)
LLE	HPLC–DAD (detection λ = 280, 330 nm)	(A) 2.0% (w/v) acetic acid (B) acetonitrile	Eclipse XDB C18 reversed phase (150 ×4.5 mm, 5 µm)	40 min	(Karabagias et al., 2014)

NA: Not available, LLE: Liquid-Liquid Extraction, SPE: Solid-Phase Extraction, XAD: XAD Resin (a type of adsorbent resin), QuEChERS: Quick, Easy, Cheap, Effective, Rugged, and Safe, SULLE: Sugaring-out assisted Liquid-Liquid Extraction, HPLC: High-Performance Liquid Chromatography, UHPLC: Ultra-High-Performance Liquid Chromatography, GC: Gas Chromatography, MS: Mass Spectrometry, TOF: Time-of-Flight, DAD: Diode Array Detector, QqQ: Triple Quadrupole, LTQ: Linear Trap Quadrupole, UV: Ultraviolet, PDA: Photodiode Array, ECD: Electrochemical Detection, ESI: Electrospray Ionization

An efficient extraction method is paramount for isolating phenolic compounds from honey effectively. Common extraction techniques employed include Liquid-Liquid Extraction (LLE), Solid-Phase Extraction (SPE), Dilute-and-Shoot, Strata C-18, QuEChERS, and Solid-Phase Ultrasound-Assisted Liquid-Liquid Extraction (SULLE). LLE, a conventional approach, separates compounds based on their solubility in different immiscible liquids. While simple and cost-effective, LLE can be time-intensive and necessitates substantial solvent volumes, posing environmental concerns. SPE, on the other hand, employs a solid adsorbent to extract compounds, offering efficiency, reduced solvent usage, and potential automation. However, its reliance on specific cartridges and equipment renders SPE comparatively costly (Istasse et al., 2016). Dilute-and-Shoot involves direct injection of diluted honey samples into the chromatographic system, expediting the process but risking inadequate cleanup and matrix effects (Tian et al., 2024). Strata C-18, a specialized SPE variant utilizing C-18 cartridges, proves highly effective for moderately polar compounds and has the ability to enrich trace phenolic compounds in honey, albeit with additional cartridge expenses (Sun et al., 2016). QuEChERS, designed for simplicity and efficiency, facilitates rapid extraction with minimal solvent usage, yet its suitability for all phenolic compounds may vary (Y. Wang et al., 2023). SULLE combines SPE with ultrasound assistance to enhance extraction efficiency while reducing solvent use. Although effective, SULLE mandates specialized equipment and meticulous optimization (Zhu et al., 2019).

For the separation and detection of phenolic compounds in honey, High-Performance Liquid Chromatography (HPLC) stands out as the primary technique used as it provides high resolution and can handle complex mixtures, making it ideal for phenolic compound analysis (Becerril-Sánchez et al., 2021). Integration with Mass Spectrometry (HPLC-MS and GC-MS) enhances reliability, efficiency, accuracy, and speed (Jibril et al., 2019). While GC-MS excels in characterizing volatile compounds, LC-MS is better for phenolic acid and flavonoid detection (Makowicz et al., 2019). Although time-consuming and costly, LC-MS techniques, when coupled with several advanced detection and analysis techniques such as QTOF, DAD, LTQ Orbitrap, UV, PDA, ECD, and Q-Exactive offer thorough insights into phenolic compound profiles (Hassan et al., 2022; Jibril et al., 2019). For instance, Quadrupole Time-of-Flight (QTOF) combines a quadrupole mass filter with a time-of-flight analyzer, providing high mass accuracy and resolution by selecting ions based on their mass-to-charge ratio (m/z) and measuring their

flight time (Koulis et al., 2021). Diode Array Detector (DAD) is an optical detector that measures the absorbance of the eluent across a range of wavelengths simultaneously, useful for obtaining UV-visible spectra of analytes. LTQ Orbitrap is a hybrid system that integrates a linear trap quadrupole (LTQ) for ion trapping and fragmentation with an Orbitrap mass analyzer. Known for its high-resolution and accurate mass measurements, LTQ is ideal for phenolic acids and flavonoid aglycones analysis (Kečkeš et al., 2013; Vasić et al., 2019). UV (Ultraviolet) detector is commonly used in liquid chromatography and measures the UV absorbance of analytes at a single wavelength, suitable for compounds that absorb UV light. While both UV detectors and DADs are used to measure absorbance in chromatography, a DAD offers additional capabilities by providing UV-visible spectra across a range of wavelengths, making it more versatile for compound identification and analysis (Y. Wang et al., 2023). PDA (Photodiode Array Detector) is also similar to a DAD and captures absorbance data over a spectrum of wavelengths, allowing for detailed spectral analysis of each analyte (Sun et al., 2016). The main difference between DAD and PDA lies in their operational principles and capabilities. ECD (Electrochemical Detector) analyzes electroactive compounds by measuring the current produced from their oxidation or reduction at an electrode, offering high sensitivity (F. Wu et al., 2022). Q-Exactive combines a quadrupole mass filter with an Orbitrap analyzer, enabling precise quantitative and qualitative analysis with high resolution and mass accuracy (Labsvards et al., 2021). These methods offer various options for the minor and major phytochemical profiling of honey. Notably, studies by Koulis et al. have highlighted the efficacy of ultra-high performance liquid chromatography-quadrupole time-of-flight mass-spectrometry (UPLC-QToF-MS) in assessing honey phenolics using both targeted and non-targeted approaches (Koulis et al., 2021). While targeted UPLC-QToF-MS enables the identification and quantification of phenolic compounds, untargeted analysis, coupled with chemometrics, aids in discovering biomarkers linked to honey's botanical origin and discriminating against its botanical origin.

Once phenolic compounds are identified in honey, employing chemometric analysis becomes pivotal in discerning phenolic markers indicative of the honey's botanical origin. It's widely acknowledged in numerous studies that regardless of the analysis method applied to honey samples, significant attention should be directed toward the data processing tools utilized (Drivelos et al., 2021). Principal Component Analysis (PCA) is one of the most frequently used techniques to assess the botanical origin of honey as it serves to reduce data dimensionality while

preserving important information (Makowicz et al., 2019). It also helps in visualizing patterns and identifying clusters in complex datasets of phenolic compound profiles from different botanical sources of honey. However, its interpretation can pose challenges with extensive datasets and might overlook non-linear relationships. Hierarchical Cluster Analysis (HCA) is a method for grouping similar samples together based on their phenolic compound profiles. It helps in identifying similarities and differences between honey samples derived from different botanical sources (Boffo et al., 2012). Discriminant Analysis (DA) proves efficient in classifying samples into predefined categories based on their phenolic compound profiles and is relatively easy to interpret. It helps in building models to differentiate between honey samples originating from different floral sources. Nonetheless, it assumes linear relationships between variables, which may not always be true (Drivelos et al., 2021). Partial Least Squares Discriminant Analysis (PLS-DA) is a regression extension of DA that takes into account the correlation structure between predictor variables (phenolic compounds) and response variables (botanical origin of honey) while managing multicollinearity effectively. Although beneficial for high-dimensional data, PLS-DA may risk overfitting without proper validation, complicating interpretation (Drivelos et al., 2021). Principal Component Regression (PCR) combines PCA and regression analysis, offering resilience against multicollinearity. Yet, it may entail less straightforward interpretation compared to other methods and could sacrifice some predictive power (Keithley et al., 2009). Chemometrics serves as a powerful tool to recognize patterns in multivariate data and uncover relationships between samples and variables within a dataset. Each chemometric technique employs distinct algorithms to extract pertinent information from the data, utilizing sophisticated statistical mathematics to construct models revealing variations in the dataset (Oliveri & Simonetti, 2016). By integrating these advanced analytical and chemometric techniques, researchers can accurately identify phenolic compounds in honey and establish reliable markers to authenticate botanical origin, thereby mitigating honey fraud and ensuring product authenticity.

To sum, a range of analytical techniques can be used for authenticating honey using phenolics as markers of authenticity. Current approaches demonstrate the capability to accurately discriminate between the botanical origins of honey. However, to enhance detection accuracy, it is imperative to ensure thorough extraction of phenolic compounds from the honey matrix and develop methods for honey authenticity assessment that preserve the chemical composition of honey

during sample preparation. Additionally, the application of chemometrics proves highly beneficial, offering a chemical fingerprint of the honey sample that aids in distinguishing adulterated from authentic honey. Furthermore, while analytical techniques generate vast amounts of complex data, chemometrics is a very useful tool to interpret this data effectively, extracting meaningful information about the phenolic composition of honey and allowing it to be used to discriminate between the botanical origins in honey.

2.5 Key literature/data confirming the novelty of this experiment

Phenolic compounds in honey, while extensively studied for their health benefits, have only recently been investigated for their potential to authenticate honey's botanical origin. However, few studies have analyzed the phenolic profiles of Canadian honey. Exploring these compounds in honey produced in Canada is beneficial, as it may reveal a unique set of phenolic compounds that could differentiate Canadian honey from honey produced in other countries. Additionally, developing robust analytical methods tailored to Canadian honey will lay the groundwork for future research and standardization efforts, aiding in the creation of regulatory guidelines specific to the Canadian honey industry and ensuring consumers receive authentic, high-quality products. To the best of our knowledge, this will be the first study to investigate a wide array of phenolic compounds in honey with a substantial sample size (n = 465) from various botanical origins. While studies have identified phenolic markers for botanical authenticity, no quantitative threshold levels for these markers in honey have been established. Moreover, despite numerous studies suggesting phenolic compounds as reliable indicators of honey’s botanical authenticity, there is a limited understanding of how thermal processing and storage affect honey’s phenolic profile and, consequently, its botanical classification. While honey authentication in its pure form is relatively well understood, the increasing use of honey in processed food products presents a challenge for maintaining its authenticity after thermal exposure. There is a critical need to further investigate the effects of heat treatment on the phenolic content of honey, as its impact remains unclear. Specifically, the current literature indicates that phenolic compounds are influenced by different temperature conditions, but it does not adequately assess whether these changes significantly affect the ability of phenolic markers to accurately determine the botanical origin of honey. Although many studies have investigated the impact of heating and storage on the phenolic profile of honey, few have simultaneously examined a comprehensive array of

phenolic compounds, and the findings have often been inconsistent. By addressing these gaps, this research will contribute significantly to the field of honey authentication, providing essential data for the development of standardized methods and enhancing the reliability of botanical origin determination even after honey has undergone thermal processing.

2.6 Conclusion

In conclusion, honey is attracting both consumer interest and the attention of fraudsters due to its growing popularity as a natural food commodity rich in nutrients and bioactive compounds, particularly phenolic compounds. This review has delved into the multifaceted role of phenolic compounds in honey, elucidating their significance in conferring various health benefits and their potential as markers for honey's botanical authenticity. Phenolic compounds, encompassing phenolic acids and flavonoids, have emerged as pivotal constituents in honey, contributing to honey's antioxidant, antimicrobial, and anti-inflammatory effects. As consumers increasingly prioritize health-conscious choices, the consumption of honey has surged, amplifying the need to ensure its authenticity and quality. Recently, research has focused on investigating phenolic compounds as potential markers of the botanical authenticity of honey, given their strong relationship to the floral origin of honey. While botanical origin exerts a profound influence on phenolic profiles, this review also highlighted the importance of considering other factors such as geographical origin, climate, maturity, storage, and processing conditions in shaping honey's phenolic profile. Understanding the mechanisms governing phenolic compound stability and transformation is essential for utilizing them effectively as authenticity markers. Furthermore, despite the numerous studies suggesting phenolic compounds as reliable indicators of honey's botanical authenticity, there remains a limited understanding of how thermal processing and storage impact honey's phenolic profile and subsequent botanical classification. Moreover, exploring phenolic profiles in honey from diverse geographical regions, including understudied regions like Canada, holds promise for identifying unique phenolic signatures and enhancing traceability. Ultimately, as honey continues to captivate consumer interest and face challenges in authenticity, unraveling the intricacies of phenolic compounds as markers of botanical authenticity of honey holds the key to safeguarding its integrity and promoting consumer confidence. By advancing the understanding of phenolic compound dynamics and their role in

authenticity assessment, the path is paved for a more resilient and transparent honey industry, ensuring that consumers confidently enjoy the diverse benefits of this natural "*golden elixir*".

CONNECTING PARAGRAPH I

Chapter 3, which focused on the multi-targeted analysis of phenolic compounds in honey and the identification of a discriminating marker for buckwheat honey using LC-QTOF-MS, builds upon the foundational knowledge established in Chapter 2. The extensive literature review in Chapter 2 underscored the complexity of honey's chemical composition and the critical role of phenolic compounds in its authentication. Key challenges highlighted the need for further research in this area, such as the limited investigation of phenolic compounds in Canadian buckwheat honey, the absence of threshold levels for current authenticity markers, and the lack of cross-instrumental validation of these markers.

To address these gaps, Chapter 3 delves into developing a multi-targeted LC-QTOF-MS method for the analysis of phenolic compounds in honey. This approach not only advanced the scientific understanding of honey authentication but also proposed a practical solution for the routine verification of buckwheat honey authenticity. By bridging the theoretical insights from Chapter 2 with empirical data, Chapter 3 significantly contributes to the broader goal of establishing standardized methodologies for honey quality assurance in the food industry. This chapter's advancements lay the groundwork for more robust and reliable honey authentication practices, ensuring product integrity and consumer trust.

CHAPTER 3. MULTI-TARGETED ANALYSIS OF PHENOLIC COMPOUNDS IN HONEY AND IDENTIFICATION OF A PHENOLIC MARKER FOR ITS ABILITY TO DISCRIMINATE BUCKWHEAT HONEY FROM OTHER BOTANICAL ORIGINS USING LC-QTOF-MS

3.1 Abstract

Honey, valued for its sweetness and health benefits, is frequently targeted by food fraud, with the majority of cases involving misrepresentation of its botanical origin. This type of fraud significantly impacts honey's market price due to the unique qualities of monofloral honeys. Traditional verification methods, such as pollen analysis and sensory tests, are time-consuming and often inadequate, underscoring the need for more reliable approaches. This study introduces a dilute-and-shoot LC-QTOF-MS method for comprehensive phenolic profiling and marker discovery in honey. The method's robustness was validated for 29 targeted phenolic compounds through instrument linearity ($r^2 \geq 0.98$), precision (RSD $\leq$ 15%), and recoveries (69.4 – 128.1%). Applied to 465 honey samples collected from 2021 to 2023, results revealed buckwheat honey's distinct phenolic profile, identifying p-hydroxybenzoic acid as a reliable marker with a threshold of 5318 ng/g, achieving a 96.7% true positive rate and a 92.6% positive predictive rate. Cross-instrument validation using LC-QqQ-MS/MS confirmed the marker's transferability, ensuring broader applicability. The method achieved a 99.14% classification agreement with initial LC-QTOF-MS results, showcasing its potential for routine application in honey authentication. This study advances the understanding of honey authentication and proposes a practical approach for routine verification of buckwheat honey, contributing to standardized methodologies for honey quality assurance in the food industry, thereby enhancing product integrity and consumer trust.

3.2 Introduction

As honey fraud cases are on the rise, more effort is needed to identify and report adulterated honey to halt this illegal activity. With fraudsters becoming more innovative in their fraudulent activities and devising sophisticated methods to exploit the system, researchers need to develop new techniques to validate the authenticity of honey. Since honey fraud is primarily impacted by the mislabelling of its botanical origin, more effort is necessary to find tools to discriminate honey from different botanical origins (Tsagkaris et al., 2021). Monofloral honey, known for their refined and unique flavor, are perceived as high-quality products and are consequently the

most susceptible to adulteration through incorrect labeling and fraudulent admixing with lower-quality honey (Soares et al., 2017).

Buckwheat honey, derived from the flowers of buckwheat (*Fagopyrum esculentum*), is particularly important in botanical origin assessment and honey fraud for several reasons. It displays a higher phenolic content than other honey types and has a distinct and robust flavor profile and a unique dark color, making it a valuable and sought-after product in both culinary and medicinal contexts, thus increasing its market value (Deng et al., 2018; Drăgănescu et al., 2020). However, these distinctive characteristics also make it a prime target for adulteration and mislabeling, as producers may blend it with cheaper honey to capitalize on its higher price. Accurately authenticating buckwheat honey ensures that consumers receive genuine products and maintain trust in the market. Additionally, studying buckwheat honey's phenolic profile can provide insights into the unique bioactive compounds that contribute to its health benefits, further emphasizing the need for robust methods to verify its botanical origin. As honey fraud becomes a global concern, establishing reliable markers and analytical techniques for authenticating buckwheat honey can serve as a model for protecting the integrity of other high-value honey.

The current tools used to assess the botanical authenticity of honey, such as testing its physicochemical properties, melissopalynology, DNA analysis, and sensory evaluation, have proven inadequate for accurately determining its floral origin (Soares et al., 2017; Tian et al., 2024; Tsagkaris et al., 2021). Therefore, advanced analytical techniques coupled with chemometric tools are necessary for a more precise assessment. These advanced methods play a crucial role in identifying specific markers that can systematically authenticate the botanical origin of honey. What is crucial are not just the tools themselves, but the markers they can detect. By employing advanced analytical techniques alongside chemometrics, these markers can be identified. Subsequently, routine analytical methods are needed to confirm the presence of these markers in honey samples. Among the various chemical components studied for this purpose, phenolic compounds have garnered significant attention. Many studies suggest individual phenolic compounds as markers of botanical origin based on their high concentrations in specific botanical sources, summarized in Table 2.4. However, several issues undermine the reliability of these markers. Firstly, the lack of established threshold levels for these markers makes it

challenging to determine their significance. Without clear thresholds, it is impossible to assess whether the concentration of a marker in a honey sample is sufficiently high to confirm its botanical origin. Secondly, when establishing whether a phenolic compound could be considered a marker and its threshold level, it is crucial to consider the sample size and diversity of botanical origins. Small sample sizes or limited botanical diversity can lead to unrepresentative thresholds, reducing the reliability of these markers. A larger sample size ensures that the matrix captures a wide variety of instances and reduces the impact of random variation, leading to more reliable and generalizable conclusions about the marker's ability to discriminate between different botanical origins (Foody, 2009). In addition, many factors beyond botanical origin influence the phenolic composition and concentration in honey, including geographical origin, climate conditions, seasonal variations, processing conditions, and storage. With a limited sample size, the identified thresholds may not account for these variables, affecting the accuracy of phenolic markers. The dynamic nature of phenolic compounds, which can undergo various chemical transformations, poses challenges to their stability and consistency over time. This instability can lead to changes in phenolic profiles, complicating the establishment of permanent markers for botanical authenticity. Another issue is the variability in analytical methods used to identify and quantify these markers. Different laboratories may employ varying techniques and instruments, resulting in inconsistencies in data and making it difficult to compare results across studies.

Several studies have proposed phenolic compounds as potential markers for determining the botanical origin of honey. Despite their identification, there remains a notable gap in validating these markers for a robust and accurate assessment of honey authenticity. Validating a marker involves several crucial steps to ensure its reliability in detecting fraudulent practices in the honey industry. Initially, rigorous optimization of analytical methods is crucial to enhance the detection efficiency of the marker. Parameters such as sensitivity, selectivity, specificity, and reproducibility are meticulously assessed during method development to ensure consistent and reliable detection capabilities (ISO, 2017). Establishing the limit of detection (LOD) and limit of quantification (LOQ) for the marker sets sensitivity thresholds, ensuring its detectability even at trace concentrations. Furthermore, evaluating accuracy (trueness) and precision (repeatability) through spiked samples containing the identified marker validates its quantitative reliability. An indispensable aspect of marker validation includes testing across multiple analytical instruments. This cross-instrument validation verifies the marker's transferability, robustness, and reliability

across different platforms and laboratory settings (Bayen et al., 2024). Without such comprehensive validation, accrediting these methodologies for routine application in honey authenticity verification becomes challenging, potentially compromising their effectiveness in combating honey fraud.

The objective of the present study was to develop and validate a dilute-and-shoot LC-QTOF-MS method for analyzing phenolic compounds in honey, specifically focusing on buckwheat honey. By investigating the phenolic content of buckwheat honey, the study aimed to determine whether it possesses distinctive characteristics compared to other botanical origins. A candidate phenolic marker was identified, and its threshold level was established to authenticate this specific botanical origin. To assess the transferability of the marker to other instruments, cross-instrument validation was performed. Buckwheat honey was selected as a case study to demonstrate the feasibility of this approach, ultimately aiming to develop standardized methods for determining the botanical authenticity of honey in future research.

3.3 Methodology

3.3.1 Chemicals and reagents

The native analytical standards (±)-2-cis,4-trans-abscisic acid (≥ 98%), 2,3,4 trihydroxybenzoic acid (≥ 98%), benzoic acid (≥ 99.5%), chrysin (≥ 97%), gallic acid (≥98%), galangin (CAS# 548-83-4), homogentisic acid (≥ 98%), luteolin (≥ 98%), quercetin (CAS# 117-39-5), trans-cinnamic acid (≥ 99%), p-coumaric acid (≥ 98%), protocatechuic acid (3,4-dihydroxybenzoic acid) (CAS# 99-50-3), protocatechualdehyde (3,4-dihydroxybenzaldehyde) (≥97%), salicylic acid (≥ 99%), sinapic acid (≥ 98%), p-hydroxybenzaldehyde (≥ 99%), rosmarinic acid (CAS# 20283-92-5), rutin (CAS# 153-18-4), morin (CAS# 480-16-0), and vanillin (≥ 99%) were obtained from Sigma Aldrich (Oakville, Canada). The analytical standards 2,5-dihydroxybenzoic acid (≥ 98%), chlorogenic acid (CAS# 327-97-9), ellagic acid (≥ 98%), epicatechin (≥ 98%), genistein (≥ 97%), hesperetin (≥ 98%), myricetin (≥ 95%), caffeic acid (≥ 98%), syringic acid (≥ 95%), vanillic acid (≥ 97%), p-hydroxybenzoic acid (≥ 99%), kaempferol (≥ 97%), ferulic acid (CAS# 537-98-4), and apigenin (≥ 95%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HPLC and analytical grade solvents (water, methanol, and acetonitrile) were obtained from Fisher Chemicals (Pittsburgh, PA, USA) and LC/MS grade formic acid from Agilent

Technologies (Santa Clara, CA, USA). All glassware was baked for four hours at 325°C before use.

3.3.2 Standards and working standards

Stock solutions of all analytical standards were prepared fresh at 1 mg/mL in LC/MS grade MeOH and stored at -20°C in amber vials. A 10 µg/mL, 1 µg/mL, and 100 ng/mL 33 phenolic standard mix containing all analytical standards listed above, with the exception of p-hydroxybenzaldehyde, was prepared in LC/MS grade MeOH which was used for spiking honey samples to test the recovery and preparing calibration curves. The 33 phenolic standard mixtures were diluted in 1:1 (*v/v*) ACN:H_2O to make 22 calibration levels of 0.0005, 0.001, 0.0025, 0.005, 0.0075, 0.01, 0.02, 0.05, 0.075, 0.1, 0.2, 0.5, 0.75, 1, 1.2, 1.5, 1.75, 2, 2.5, 3, 4, 5 µg/mL. These calibration curve levels were selected to encompass the anticipated different concentration ranges of these compounds in honey. To quantify the phenolic compound concentrations in honey, three sets of matrix-matched calibration curves were prepared. Since the phenolic content in honey correlates with its color, the honey samples were categorized into three relative groups: "low," "medium," and "high" phenolic content. The "low" calibration curve was generated using a mixture of ten honey samples classified as white according to the *Pfund* color grading system. For the "medium" category, a blend of ten amber or golden-colored honey samples was utilized, while the "high" category involved a combination of ten dark honey samples. Each matrix-matched calibration curve comprised 23 levels, ranging from unspiked to 5 µg/mL spiked honey mixtures, using the same incremental concentrations as the solvent calibration curve. These concentrations were prepared by dilution in water to mimic the 9:1 (*v/v*) H_2O:honey ratio used in the honey sample preparation.

3.3.3 Honey samples

A total of 465 honey samples were collected from 2021 to 2023 from various suppliers and markets in Montreal, Canada, and imported from numerous countries. The collection process was particularly focused on obtaining samples of buckwheat (n=90), blueberry (n=64), and clover (n=89) honey (based on the self-reporting floral origin on the product label) given their prominence as primary honey varieties produced in Quebec. Additionally, samples representing 33 other botanical origins of honey were gathered, with notable categories including linden (n=24), goldenrod (n=16), acacia (n=10), polyfloral (n=89), and those labeled as unknown

(n=18) or other monofloral honey (n=65), based on the identification declared on the label. The botanical origin of all honey samples was determined using melisopalynology and sensory evaluation, which included assessments of color, odor, flavor characteristics, and overall appearance and consistency. Approximately 30 mL of each sample was transferred into stained-glass amber vial and stored at -20˚C until subsequent analysis.

3.3.4 Sample preparation

Honey samples were prepared based on the "dilute-and-shoot" method outlined in von Eyken et al. (2019). Briefly, approximately 0.2 g (± 0.01g) of honey was weighed in a 15-mL polypropylene tube. Subsequently, two mL of a mixture consisting of acetonitrile and water (1:1 *v/v*) was added, and samples were vortexed for around 5 minutes, or until complete dissolution of the honey was achieved. Following this, samples underwent filtration through a 0.22 µm PTFE filter (Agilent Technologies) and were further diluted with water to reach a final concentration equivalent to 1% of honey. All samples were stored at -20°C until analysis.

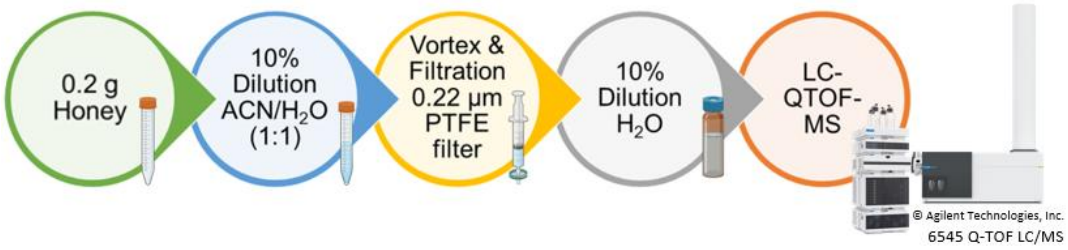

Figure 3.1. "Dilute and shoot" method used for the analysis of honey samples. Adapted from von Eyken et al. (2019).

3.3.5 Instrumental analysis

3.3.5.1 Instrumental analysis on LC-QTOF-MS

Samples were analyzed using an Agilent 1290 Infinity II LC system coupled to a 6545 Quadrupole-Time of Flight (Q-TOF)-MS (Agilent Technologies, Santa Clara, USA) operating in negative (ESI-) electrospray ionization mode. LC separation was carried out using an InfinityLab Poroshell 120 EC-C18 column (2.7µm x 3.0 x 100mm) which was fitted to an InifinityLab Poroshell 120 EC-C18 guard column, both supplied by Agilent Technologies (Santa Clara, USA). The method was optimized based on the work of P. Wang et al. (2023). The separation was conducted at a flow rate of 0.4 mL/min using water (mobile phase A) and methanol (mobile phase B), both containing 0.1% formic acid. The mobile phase gradient is as follows: 0 min:

70% A; 0-3 min: B increased from 30 to 100%; 3-6 min: 100% B; 6-8 min: B decreased from 100% to 30%, with a one-minute post-column run. The column temperature was set at 30 °C and the injection volume was 20 µL. Nitrogen was used as the drying gas (110°C) with a gas flow rate maintained at 12 L/min, a sheath gas temperature of 150°C with a flow of 10 L/min was used, and the pressure nebulizer was set at 40 psig. The capillary voltage was maintained at 4000 V, the nozzle voltage was 500V, the fragmentor voltage was 150 V, and the skimmer voltage was 55 V. To prevent sugars from entering the column, the first 1.4 minutes of the elution were discarded. The samples were stored in the multi-sampler compartment at a temperature of 4°C. MS data were acquired in the *m/z* 100 – 1700 range and the samples were run in a full scan mode.

3.3.5.2 Instrumental analysis on LC-QqQ-MS/MS

An ultra-high performance liquid chromatographic system was coupled to a Thermo Scientific TSQ Altis Plus triple quadrupole (QqQ) mass spectrometer to analyze honey samples. The LC-MS method was optimized based on the method described in Section 3.2.5.1. The column was an InfinityLab Poroshell 120 EC-C18 column (2.7µm x 3.0 x 100mm) which was fitted to an InifinityLab Poroshell 120 EC-C18 guard column, both supplied by Agilent Technologies (Santa Clara, USA). The column temperature was set to 30°C and the autosampler was maintained at 4°C. The mobile phases were composed of water (mobile phase A) and methanol (mobile phase B), both containing 0.1% formic acid. The gradient was set as: 0 min: 30% B; 0-5.5 min: B increased from 30 to 95%; 5.5-7 min: 95% B; 7-9.5 min: B decreased from 95% to 30%; 9.5 – 10.0 min: 30% B, with a one-minute post-column run. The separation was conducted at a flow rate of 0.4 mL/min. The Thermo TSQ Altis plus mass spectrometer used heated electrospray ionization in negative ion mode (2500 V) with ion transfer tube temperature of 325 °C and a vaporization temperature of 350 °C. The sheath gas, aux gas, and weep gas flows were set to 60, 15, and 1 Arb, respectively. The MRM conditions are also given in Table 1, in Appendix.

3.3.6 Method validation

Five procedural blanks were prepared using an extraction procedure identical to that used for honey sample analysis but without the addition of the honey matrix. Instead of adding the honey matrix, a 9:1 (*v/v*) water: acetonitrile mixture was utilized. Additionally, a solvent blank was prepared using the same solvents employed in the honey sample preparation process (acetonitrile

and water (1:9 *v/v*)). Both procedural blanks and solvent blanks were analyzed several times throughout the sample analysis process, occurring after every analysis of 50 samples. To monitor instrumental variability and ensure consistent instrument performance, a laboratory QC standard mix consisting of a 100 ng/mL 33 phenolic standard mixture in solvent (9:1 (*v/v*) water: acetonitrile mixture) was analyzed every 25 samples throughout the run and a pooled QC containing a mixture of all honey samples was analyzed every 50 samples, and their response was closely monitored. Furthermore, to assess the recovery and procedural precision, three honey samples representing diverse floral origins and exhibiting color variations were carefully selected. This selection aimed to encompass a broad spectrum of honey matrices in terms of phenolic composition and concentration. To establish the recovery, the selected honey samples were spiked before the extraction process at three different levels (20 ng/mL, 100 ng/mL, and 500 ng/mL, corresponding to the concentrations of 2000, 10000, and 50000 ng/g of honey) utilizing the 33 phenolic standard mixture, with triplicate analyses conducted for each spiking level for each honey. Spiking levels were determined based on the lowest, mid, and highest concentrations of each phenolic in honey. To evaluate the precision of the method employed, the three honey samples selected were spiked at 100 ng/mL (with 5 real replicates) and subjected to the same extraction process as the honey samples. Additionally, the matrix effect was evaluated by spiking three honey mixtures at 23 levels, as outlined in Section 3.2.2.

3.3.7 Data treatment and statistical analysis

The concentration of phenolic compounds in samples was determined by using the Agilent Mass Hunter Workstation Software – Quantitative Analysis B.07.01, with a m/z extraction window value set at ± 20 ppm and a retention time window of ± 0.5 min. The 100 ng/mL 33 phenolic standard mixture, analyzed every 25 samples, was used to evaluate the retention time shift and mass accuracy of the instrument. The linearity of the instrument was assessed by calculating the RSD % of the response of the pooled QC. For each phenolic compound, the MDL, MQL, LOD, LOQ, calibration curve linearity, matrix effect, recovery, and method precision were assessed. The procedural blanks served to derive the method detection limit (MDL) (calculated as 3σ/slope of matrix-matched calibration curve) and method quantification limit (MQL) (calculated as 10σ/slope of matrix-matched calibration curve). Meanwhile, the solvent blank facilitated the determination of the limit of detection (LOD) (computed as 3σ/slope of solvent calibration

curve), and the limit of quantification (LOQ) (computed as 10σ/slope of solvent calibration curve). The calibration curve linearity was evaluated from the coefficient of determination (r^2) identified from the three matrix-matched calibration curves. The matrix effect was assessed by comparing the slope of the matrix-matched calibration curve with the slope of the solvent calibration curve (matrix effect (%) = (1-B/A) × 100), where A corresponds to the slope of the solvent calibration curve and B corresponds to the slope of the matrix-matched calibration curve (Chambers et al., 2007). The recovery was evaluated by comparing the measured concentration of each phenolic compound in honey to the theoretical spiked level (recovery (%) = ([spiked honey] – [non-spiked honey]/ [theoretical spiking]) *100). The recovery for each phenolic compound was assessed based on spiking concentrations that matched the average levels of each compound in honey. Recoveries of the phenolic compounds were considered acceptable when in the 70-130% range (Steiner et al., 2020). The method precision was determined by calculating the RSD% for each of the three honey matrices spiked at 100 ng/mL (n = 5 replicates) (RSD (%) = (std deviation/mean) *100).

The data processing, including the generation of PCA, t-SNE, threshold determination, and creation of a confusion matrix, was executed using Python 3.10.13 and several open-source libraries including: mathplotlib 3.8.0, numpy 1.26.0, pandas 2.1.1, scikit-learn 1.3.0, and scipy 1.11.3. Principal component analysis (PCA) and t-distributed stochastic neighbor embedding (t-SNE) analysis were conducted on the standardized log2-scaled peak volume response data of 34 targeted phenolic compounds. In other words, the log2-scaled data was standardized such that each feature had its mean subtracted and was then divided by its standard deviation to put all features on the same scale before dimensionality reduction. The 34 phenolic compounds targeted were composed of the 29 phenolic compounds outlined in Table 3.2, in addition to compounds such as benzoic acid, ellagic acid, p-hydroxybenzaldehyde, syringic acid and vanillin. Confirmation of these compounds was achieved through analytical standards, verifying their m/z ratio and retention time. To establish the threshold for buckwheat honey, the scikit-learn class Decision Tree Classifier (max depth = 1, class weight = “balanced”, random state = 0) was used to implement a single-node decision tree. This model finds a threshold by minimizing the Gini impurity score. This tree was trained on the 358 monofloral honey samples to generate the threshold shown in Figure 3.3 and a 5-fold cross-validation was done using these samples to calculate the MCC score as a mean ± standard deviation. The confusion matrix was generated

using the actual (i.e. botanical origin identified on label) and predicted values in the test set of each of the 5-folds during cross-validation.

3.4 Results and discussion

3.4.1 Method validation

Table 3.2 provides a summary of the method validation parameters for 29 targeted phenolic compounds. Only the phenolic compounds with acceptable recoveries were included in the summary table and selected for quantitative purposes. The precision and accuracy of the instrument are summarized in Table 3.1. The mean relative standard deviation (RSD) for retention time precision across all targeted phenolic compounds was found to be 0.82%, ranging from 0.52% for (±)-2-cis,4-trans-abscisic acid to 1.91% for gallic acid. This low mean RSD and narrow range of RSD values indicate high precision and consistency in retention time measurements, which are crucial for reliable and accurate targeted analysis of phenolic compounds in honey. Furthermore, a mean mass accuracy of -2.48 ppm was observed for all targeted phenolic compounds, with protocatechualdehyde exhibiting the highest mass error at 5.14 ppm and vanillic acid the lowest at -5.90 ppm. This mean mass accuracy suggests that, on average, the measured mass of the targeted phenolic compounds is slightly lower than their expected masses.

Table 3.1. The precision of retention time and mass accuracy of targeted phenolic compounds

Targeted phenolic compounds	Retention time (RSD%)	Average mass error (ppm)
(±)-2-cis,4-trans-Abscisic acid	0.52	-1.85
2,3,4-Trihydroxybenzoic acid	0.96	-3.24
2,5-Dihydroxybenzoic acid	0.93	-2.97
Apigenin	0.68	-0.78
Caffeic acid	0.85	-3.26
Chlorogenic acid	0.71	-1.58
Chrysin	0.53	-5.28
Epicatechin	0.59	-3.20
Ferulic acid	0.72	-1.87
Galangin	0.55	-2.34
Gallic acid	1.91	-0.86
Genistein	0.62	-0.08
Hesperetin	0.56	-1.12
Homogentisic acid	1.17	-3.53

Kaempferol	0.93	-2.58
Luteolin	0.59	-1.60
Morin	0.60	-4.28
Myricetin	1.27	-5.16
p-Coumaric acid	0.76	-3.09
p-Hydroxybenzoic acid	1.09	-3.57
Protocatechualdehyde	0.73	5.14
Protocatechuic acid	0.80	-2.81
Quercetin	0.88	-3.40
Rosmarinic acid	0.53	-3.88
Rutin	0.73	-1.42
Salicylic acid	0.68	-0.36
Sinapic acid	0.63	-4.07
trans-Cinnamic acid	0.61	-2.92
Vanillic acid	1.70	-5.90
Average	0.82	-2.48

3.4.1.1 LOD, LOQ, MDL, MQL

The LOD for the phenolic compounds in this study ranged from 0.01 to 1.62 ug/g of honey, and the LOQ ranged from 0.04 to 5.41 ug/g of honey, with vanillic acid exhibiting the highest LOD and LOQ values. These values are comparable to those reported in the literature (Kocyigit et al., 2019). Silva et al. (2019) reported LODs of 0.25–2.2 µg/g and LOQs of 0.83–7.33 µg/g for 18 phenolic compounds using QuEChERS and HPLC/DAD. Tanleque-Alberto et al. (2020) found LODs of 0.5–1.0 µg/g and LOQs of 2.0–5.0 µg/g for 15 phenolic compounds using SPE and HPLC/DAD. Koulis et al. (2021) reported lower LODs and LOQs, ranging from 0.03–0.33 µg/g and 0.09–0.99 µg/g, respectively, for 21 phenolic compounds using LLE and HPLC-QTOF-MS. The MDL and MQL were calculated for all three honey matrices: white, golden, and dark. The MDL identified in this study ranged from 0.02 to 2.35 µg/g for white honey, 0.03 to 2.81 µg/g for golden honey, and 0.03 to 2.43 ug/g for dark honey. The MQL ranged from 0.08 to 7.83 µg/g for white honey, 0.10 to 9.36 µg/g for golden honey, and 0.09 to 8.09 µg/g for dark honey. The highest MDL and MQL values were found for kaempferol for white and dark honey and quercetin for golden honey. Although the literature does not provide specific MDL or MQL values for phenolics in honey, the MDL and MQL values obtained in this study are comparable to the LOD and LOQ values reported in the literature. Given that MDL and MQL are theoretically expected to be higher than LOD and LOQ, the fact that our study finds similar

MDL and MQL values to the literature's LOD and LOQ values indicates that our method is effective in identifying phenolic compounds in honey.

3.4.1.2 Calibration curve and instrumental linearity

The calibration curve linearity was evaluated from the coefficient of determination (r^2) identified from the three matrix-matched calibration curves. For quantification purposes, the concentration range at which each phenolic compound exhibited a linear range ($r^2 \geq 0.98\%$) was selected. This high correlation coefficient ensures a linear relationship between the concentration of the phenolic compounds and the corresponding response values, thus allowing accurate quantification of phenolic compounds in honey. The linearity of the instrument was deemed acceptable, with the RSD% of the response for the pooled QC remaining below 20% for all phenolic compounds, except for chlorogenic acid and quercetin, which had RSD% values of 20.5% and 23.6%, respectively. With the RSD % being below 20% for all phenolic compounds, the instrument demonstrated good linearity, ensuring the accuracy of the quantified values.

3.4.1.3 Matrix effect

A matrix effect (ME) value below 0% indicates matrix suppression, while a value above 0% indicates matrix enhancement. MEs are generally considered ‘soft’ when they range between -20% and 20%, ‘medium’ between -50% and -20% or 20% and 50%, and ‘strong’ when below -50% or above 50% (Kim et al., 2023; Kmellár et al., 2008). The matrix effect results for the targeted phenolic compounds in white, golden, and dark honey revealed significant variability, with some compounds showing enhancement and others suppression. Matrix enhancement was observed for most compounds, such as protocatechuic acid (77.12%, 83.22%, and 81.1% for white, golden, and dark honey, respectively) and trans-cinnamic acid (84.23%, 83.36%, and 69.43% for white, golden, and dark honey, respectively), which showed the highest effects across all honey types. Compounds like ferulic acid, genistein, p-coumaric acid, and p-hydroxybenzoic acid also exhibited strong matrix enhancement, with values exceeding 60% in all honey types. On the other hand, several phenolic compounds experienced substantial matrix suppression. Notably, 2,3,4-trihydroxybenzoic acid (-81.11% in white honey), galangin (-78.01% in golden honey), and gallic acid (-69.06% in dark honey) had strong suppression effects. The matrix effect findings in the literature show considerable variability across different studies (Koulis et al., 2021; Silva et al., 2019). However, the results of the present study align more closely with the

findings reported by Koulis et al. (2021). All in all, the variability in matrix effects underscores the complexity of phenolic compound analysis in honey. These results highlight the necessity for careful consideration of matrix effects in method development and validation to ensure accurate quantification and reliable results.

3.4.1.4 Recovery

The recovery, also known as trueness, was assessed for each phenolic compound based on spiking concentrations that corresponded to the average levels of each compound in the three honey matrices. Out of the 33 phenolic compounds tested, 29 had recoveries within the acceptable range of 70-130% (Steiner et al., 2020), indicating reliable accuracy for most compounds. However, hesperetin had a recovery of 69.4% ± 2.0 for golden honey, which is slightly below the acceptable range. Additionally, four phenolic compounds, namely benzoic acid, ellagic acid, syringic acid, and vanillin did not meet the acceptable recovery criteria and are not displayed in Table 3.2. These results highlight that while the method demonstrates good overall trueness for the majority of phenolic compounds, certain compounds may require further optimization to achieve accurate recovery rates across different honey matrices.

3.4.1.5 Method precision

The precision of the method, also known as repeatability, employed for the analysis of honey samples was assessed by calculating the relative standard deviation (RSD %) of the five true replicates of the three honey matrices (white, golden, and dark) spiked at 100 ng/mL. The average method precision was 5.0%, 4.3%, and 6.6% for white, golden, and dark honey, respectively. The RSD % ranged from 0.8 to 11.3% for white honey, 1.1 to 14.2% for golden honey, and 1.0 to 13.2% for dark honey which falls within the acceptable range of 15% (Peters et al., 2007). For most phenolic compounds, the variability in repeatability among the different honey colors remained below 10%. However, 2,3,4-trihydroxybenzoic acid showed the greatest difference in precision, with an RSD % of 11.4% for dark honey and 1.1% for golden honey, resulting in a 10.3% difference. These results indicate that the method generally exhibits good precision across the different honey matrices. However, the significant variability observed for 2,3,4-trihydroxybenzoic acid across different honey colors underscores the need to consider honey color in the analysis of phenolic compounds.

Table 3.2. Method performance for 29 targeted phenolic compounds in honey, using LC-QTOF-MS ESI -.

	m/z	RT[a] (min)	LOD[b] (µg/g honey)	LOQ[c] (µg/g honey)	MDL[d] (µg/g honey)	MQL[e] (µg/g honey)	Calibration curve linearity (r^2 [f])	Linear range (µg/g honey)	Matrix effect (%)	Method precision (RSD[g] %) (n=5)	Recovery (n=3) (%)
(±)-2-cis,4-trans-abscisic acid	263.1289	3.319	0.0119	0.0395	W=0.0626 G=0.0530 D=0.0594	W=0.2087 G=0.1768 D=0.1980	S=0.999 W=0.995 G=0.999 D=0.992	0.05 – 10	W= 39.78 G= 40.01 D= 39.69	W=3.78 G=1.17 D=4.02	W=111.49±10.05 G=117.21±18.80 D=122.86±4.68
2,3,4-trihydroxybenzoic acid	169.0142	2.160	0.0722	0.2407	W=0.0228 G=0.0304 D=0.0258	W=0.0760 G=0.1013 D=0.0861	S= 0.992 W=0.995 G=0.995 D=0.991	0.05 – 50	W= -81.11 G= -38.52 D= -62.38	W=5.62 G=1.12 D=11.37	W=78.72±3.02 G=90.04±24.76 D=72.81±1.56
2,5-dihydroxybenzoic acid	153.0193	2.483	0.0636	0.2121	W=0.0401 G=0.0424 D=0.0472	W=0.1337 G=0.1414 D=0.1573	S=0.992 W=0.992 G=0.993 D=0.987	0.05 – 20	W= 7.02 G= 11.87 D= 17.30	W=4.45 G=1.21 D=7.18	W=86.29±4.21 G=96.18±32.39 D=105.12±2.79
Apigenin	269.0455	3.709	0.4595	1.5318	W=0.6163 G=0.6105 D=0.6806	W=2.0545 G=2.0348 D=2.2687	S=0.993 W=0.990 G=0.995 D=0.998	0.5 – 10	W= 60.18 G= 58.98 D= 63.58	W=2.78 G=8.04 D=10.28	W=71.19±1.88 G=73.56±21.02 D=85.64±5.16
Caffeic acid	179.0350	2.466	0.0350	0.1168	W=0.0509 G=0.0646 D=0.0673	W=0.1697 G=0.2155 D=0.2244	S=0.993 W=0.988 G=0.997 D=0.999	0.05 – 10	W= 55.51 G= 64.54 D= 64.57	W=5.15 G=1.50 D=10.08	W=81.92±2.28 G=74.09±6.18 D=79.51±8.36
Chlorogenic acid	353.0878	2.110	0.1137	0.3789	W=0.0533 G=0.1047 D=0.0652	W=0.1777 G=0.3490 D=0.2173	S=0.995 W=0.998 G=0.998 D=0.989	0.05 – 10	W= 32.93 G= 65.35 D=43.80	W=4.55 G=1.48 D=7.86	W=82.53±2.68 G=112.58±15.23 D=70.73±2.78
Chrysin	253.0544	4.110	0.0395	0.1317	W=0.1878 G=0.1896	W=0.6261 G=0.6320	S=0.998 W=0.986	0.5 – 10	W= 15.47 G= 16.27	W=3.05 G=1.50	W=84.52±3.41 G=76.54±20.98

					D=0.3212	D=1.0706	G=0.997 D=0.989		D= 15.88	D=2.28	D=92.21±1.41
Epicatechin	289.0718	2.292	0.0793	0.2644	W=0.1799 G=0.2290 D=0.2280	W=0.5998 G=0.7634 D=0.7598	S=0.980 W=0.996 G=0.999 D=0.996	0.25 – 10	W= -56.95 G= -30.75 D= -27.77	W=1.22 G=3.67 D=3.58	W=88.47±5.56 G=88.11±2.01 D=96.59±5.00
Ferulic acid	193.0506	2.814	0.3124	1.0414	W=0.3271 G=0.3374 D=0.3604	W=1.0902 G=1.1248 D=1.2012	S=0.996 W=0.996 G=0.998 D=0.999	0.5 – 10	W= 60.60 G= 60.48 D= 63.77	W=2.33 G=1.31 D=4.13	W=70.13±2.73 G=93.95±0.67 D=77.47±1.34
Galangin	269.0456	4.156	0.0727	0.2424	W=1.7078 G=1.4090 D=1.5841	W=5.6927 G=4.6965 D=5.2802	S=0.995 W=0.985 G=0.990 D=0.994	2 – 100	W= -46.86 G= -78.01 D= -58.34	W=10.89 G=10.65 D=13.19	W=125.56±5.35 G=121.49±6.24 D=85.81±4.98
Gallic acid	169.0142	1.588	0.0242	0.0805	W=0.1682 G=0.1360 D=0.1373	W=0.5605 G=0.4533 D=0.4576	S=0.995 W=0.997 G=0.998 D=0.998	0.25 – 10	W= -60.43 G= -63.32 D= -69.06	W=4.16 G=3.23 D=4.35	W=100.92±8.28 G=118.54±6.02 D=128.78±4.78
Genistein	269.0455	3.557	0.1273	0.4242	W=0.1682 G=0.2038 D=0.2770	W=0.5607 G=0.6793 D=0.9233	S=0.993 W=0.996 G=0.999 D=0.995	0.25 – 5	W= 65.67 G= 72.46 D= 80.54	W=1.64 G=4.50 D=4.76	W=74.94±4.45 G=75.67±0.92 D=71.94±2.90
Hesperetin	301.0718	3.510	0.0496	0.1653	W=0.0490 G=0.0512 D=0.0615	W=0.1632 G=0.1706 D=0.2051	S=0.994 W=0.988 G=0.994 D=0.984	0.05 – 5	W= 49.41 G= 52.91 D= 59.81	W=3.28 G=3.53 D=3.48	W=70.77±6.34 G=69.44±1.96 D=70.62±4.83
Homogentisic acid	167.0350	1.729	0.0361	0.1202	W=0.0445 G=0.0508 D=0.0547	W=0.1484 G=0.1693 D=0.1824	S=0.984 W=0.997 G=0.998 D=0.992	0.05 – 200	W= 27.11 G= 35.73 D= 38.91	W=2.83 G=1.21 D=5.82	W=77.00±4.24 G=107.65±17.46 D=95.16±4.53
Kaempferol	285.0405	3.651	0.0475	0.1583	W=2.3494	W=7.8314	S=0.990	5 – 10	W= 44.99	W=4.84	W=90.39±2.42

					G=2.3772 D=2.4272	G=7.9241 D=8.0905	W=0.981 G=0.994 D=0.999		G= 44.48 D= 47.46	G=14.19 D=4.17s	G=123.08±16.43 D=105.95±1.78
Luteolin	285.0405	3.502	0.1168	0.3895	W=0.1514 G=0.1242 D=0.1376	W=0.5045 G=0.4139 D=0.4586	S=0.992 W=0.988 G=0.990 D=0.993	0.75 – 10	W= 18.02 G= 1.60 D= 10.56	W=10.21 G=12.35 D=10.35	W=70.35±7.71 G=72.59±5.03 D=82.22±7.54
Morin	301.0354	3.237	0.7984	2.6613	W=1.0217 G=1.1319 D=1.3731	W=3.4056 G=3.7730 D=4.5769	S=0.987 W=0.995 G=0.991 D=0.989	2 – 75	W= 39.10 G= 47.76 D= 55.95	W=6.13 G=5.34 D=5.85	W=79.05±4.60 G=79.99±11.77 D=82.14±4.62
Myricetin	317.0306	3.092	0.8365	2.7884	W=0.6850 G=0.6670 D=0.7271	W=2.2834 G=2.2233 D=2.4235	S=0.982 W=0.984 G=0.989 D=0.985	2 – 75	W= -33.15 G= -17.48 D= -9.69	W=11.34 G=1.52 D=7.04	W=94.55±28.99 G=89.70±11.20 D=72.23±8.55
p-coumaric acid	163.0401	2.798	0.0126	0.0420	W=0.0455 G=0.0497 D=0.0655	W=0.1518 G=0.1656 D=0.2183	S=0.994 W=0.992 G=0.993 D=0.988	0.05 – 20	W= 70.42 G= 72.07 D= 75.62	W=2.74 G=1.42 D=3.34	W=104.59±8.45 G=128.05±1.18 D=73.12±2.63
p-hydroxybenzoic acid	137.0244	2.441	0.23067	0.7689	W=0.2009 G=0.2467 D=0.2087	W=0.6697 G=0.8224 D=0.6956	S=0.992 W=0.983 G=0.993 D=0.981	0.25 – 20	W= 68.48 G= 74.70 D= 77.25	W=3.18 G=2.84 D=4.02	W=84.20±17.97 G=127.39±2.48 D=80.08±8.52
Protocatechualdehyde	137.0244	2.259	0.0287	0.0957	W=0.0490 G=0.0585 D=0.0667	W=0.1633 G=0.1950 D=0.2224	S=0.993 W=0.999 G=0.997 D=0.998	0.05 – 2	W= 16.49 G= 32.25 D= 41.39	W=0.80 G=1.53 D=0.87	W=93.16±1.26 G=104.38±1.90 D=111.92±2.56
Protocatechuic acid	153.0193	2.044	0.0158	0.0525	W=0.1127 G=0.1540 D=0.1375	W=0.3757 G=0.5132 D=0.4584	S=0.998 W=0.993 G=0.995 D=0.995	0.25 – 10	W= 77.12 G= 83.22 D= 81.10	W=1.55 G=2.84 D=7.22	W=74.62±5.86 G=84.65±5.61 D=88.67±4.71

Quercetin	301.0354	3.390	1.0577	3.5255	W=2.2725 G=2.8092 D=2.1598	W=7.5748 G=9.3641 D=7.1993	S=0.9885 W=0.988 G=0.993 D=0.993	5 – 120	W= -17.77 G= 9.36 D= -25.24	W=11.24 G=9.58 D=13.16	W=78.88±12.61 G=78.62±8.78 D=88.49±9.68
Rosmarinic acid	359.0772	2.938	0.2028	0.6760	W=0.3573 G=0.3687 D=0.4364	W=1.1909 G=1.2291 D=1.4548	S=0.994 W=0.999 G=0.999 D=0.997	0.5 – 75	W= 26.47 G= 28.76 D= 38.20	W=6.76 G=1.29 D=9.70	W=105.01±3.89 G=92.77±7.17 D=93.56±3.99
Rutin	609.1461	2.880	0.1902	0.6339	W=0.3599 G=0.3806 D=0.4341	W=1.1995 G=1.2685 D=1.4470	S=0.992 W=0.998 G=0.998 D=0.996	0.5 – 75	W= 31.18 G= 34.43 D= 40.93	W=7.49 G=2.01 D=7.16	W=93.88±4.72 G=97.11±7.05 D=89.91±5.21
Salicylic acid	137.0244	3.402	0.1115	0.3717	W=0.0332 G=0.0341 D=0.0404	W=0.1107 G=0.1136 D=0.1347	S=0.995 W=0.994 G=0.994 D=0.998	0.05 – 10	W= 31.31 G= 33.06 D= 38.04	W=3.72 G=4.63 D=5.22	W=87.69±3.74 G=89.44±7.27 D=109.14±4.38
Sinapic acid	223.0612	2.765	0.2074	0.6912	W=0.3334 G=0.3330 D=0.3210	W=1.1113 G=1.1102 D=1.0701	S=0.990 W=0.997 G=0.999 D=0.996	0.5 – 10	W= 10.50 G= 5.29 D= 1.30	W=4.09 G=2.13 D=5.40	W=96.16±5.63 G=125.46±6.23 D=102.09±0.96
trans-Cinnamic acid	147.0452	3.543	0.7518	2.5060	W=1.7575 G=1.6320 D=0.9056	W=5.8585 G=5.4401 D=3.0185	S=0.994 W=0.991 G=0.986 D=0.991	2 – 20	W= 84.23 G= 83.36 D= 69.43	W=8.33 G=9.92 D=8.46	W=103.55±3.57 G=84.46±2.27 D=70.50±19.83
Vanillic acid	167.0350	2.490	1.6227	5.4089	W=1.5113 G=1.5065 D=1.7697	W=5.0377 G=5.0215 D=5.8991	S=0.994 W=0.986 G=0.989 D=0.987	2 – 10	W= 18.38 G= 17.41 D= 34.91	W=7.98 G=8.52 D=7.27	W=99.80±5.65 G=123.69±8.56 D=119.35±23.91

RT[a] is retention time; LOD [b] is the limit of detection; LOQ [c] is the limit of quantification; MDL [d] is the method limit of detection; MQL [e] is the method limit of quantification; r^2 [f] is the coefficient of variation of the calibration curve; RSD [g] is the relative standard deviation. S= solvent W= white honey; G= golden honey; D=dark honey.

3.4.2 Multi-targeted analysis of phenolic compounds in honey samples

In the present study, to verify the discriminatory potential of phenolic compounds in distinguishing the botanical origins in honey, a multi-targeted analysis of phenolic compounds in 465 honey samples was conducted, constituting a notably vast and intricate dataset. To effectively interpret this large and complex data and ascertain the utility of phenolic compounds in distinguishing honey's botanical origins, employing dimensionality reduction and visualization techniques is paramount. PCA and t-SNE are two powerful tools that can aid in this process.

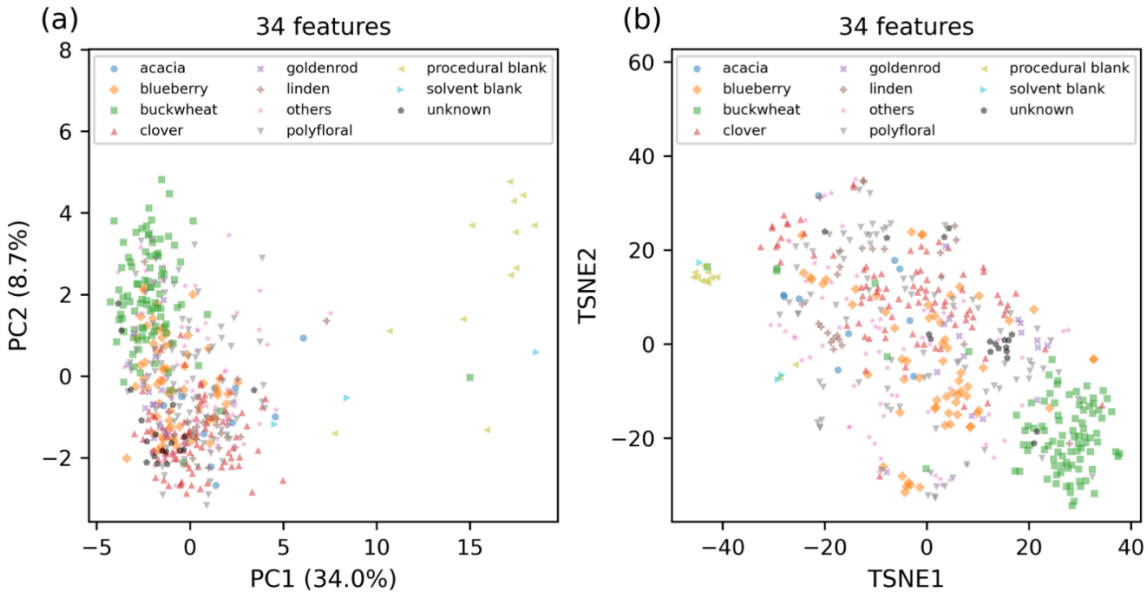

Figure 3.2. Dimensionality reduction ((a) PCA and (b) t-SNE) of phenolic compounds (using peak volume response data) in 465 honey samples and blanks.

PCA is a statistical technique used to reduce the dimensionality of a dataset while preserving maximal variance. It transforms the original variables into a new set of uncorrelated variables called principal components. The reduced dimensions allow for visual inspection of clustering and separation between different botanical origins (Maione et al., 2019). In Figure 3.2 (a), depicting the PCA of the dataset, the signal of honey distinctly diverges from that of blanks on PC1, representing the primary principal component accounting for the majority of the total variance (34.0%). Therefore, PC1 seems to primarily reflect the phenolic concentration in samples, while PC2 could account for both the phenolic composition and concentration in samples, given its role in separating botanical origins in honey. This discernible distinction between the honey and blank samples underscores the sensitivity of the employed analytical

method, suggesting the likely presence of phenolic compounds in honey samples while absent in blanks. This sensitivity is pivotal for precisely identifying and quantifying phenolic compounds within honey. Moreover, it also indicates that phenolic compounds contribute significantly to the overall variance observed in the dataset.

t-SNE is a non-linear dimensionality reduction technique specifically designed for the visualization of high-dimensional data. It maps high-dimensional data to a lower-dimensional space in a way that preserves local relationships between data points and excels at revealing clusters in the data (X. Wu et al., 2022). When investigating Figure 3.2 (b), similarly to the PCA, the procedural and solvent blanks appear to cluster separately, validating that the signal of the blanks is distinct from the honey samples. In addition, buckwheat honey, the botanical origin of interest in this study, forms a distinct cluster in the t-SNE plot, suggesting phenolic compounds as useful markers for its discrimination. This finding aligns with the literature, which highlights buckwheat's unique phenolic profile characterized by elevated phenolic compound concentrations compared to other botanical origins (Deng et al., 2018; Socha et al., 2011). The enhanced clustering pattern of buckwheat honey in t-SNE as compared to PCA could be explained by their differences in handling non-linear relationships and preserving local structure. PCA is sensitive to outliers and noise in the data, which can sometimes obscure clustering patterns, especially in high-dimensional datasets. t-SNE, on the other hand, is generally more robust to noise and can effectively filter out irrelevant variations, leading to clearer clustering results (Perez & Tah, 2020). To sum up, PCA and t-SNE revealed that buckwheat honey has a distinct phenolic profile, facilitating its differentiation from other botanical origins.

3.4.3 Quantification of phenolic compounds in buckwheat honey

The concentrations of 26 phenolic compounds were quantified in 90 labeled buckwheat honey samples and compared with values reported in the literature (Table 3.3). The phenolic compounds exhibited a wide range of detection frequencies and concentration levels, highlighting the complexity in authenticating monofloral honey.

Table 3.3. Comparison of the concentration (ng/g of honey) of phenolic compounds quantified in honey labeled as buckwheat (n=90) with values reported in the literature.

Compound name	Maximum	Median	Average	Detection frequency	Literature (min – max)
(±)-2-cis,4-trans-Abscisic acid	5994	1467	1862	90/90	1320.0 – 18460.0
2,3,4-Trihydroxybenzoic acid	139	< MDL	24	24/90	NA
2,5-Dihydroxybenzoic acid	5148	1257	1458	90/90	NA
Apigenin	1747	328	314	53/90	30.0 – 890.0
Caffeic acid	5258	966	1063	88/90	202.0 – 5160.0
Chlorogenic acid	3298	589	668	90/90	560.0 – 17030.0
Chrysin	3773	894	1213	90/90	220.0 – 4280.0
Epicatechin	575	< MDL	155	38/90	NA
Ferulic acid	3372	1676	1724	86/90	38.0 – 58640.0
Galangin	6720	< MDL	1125	30/90	120.0 – 3080.0
Gallic acid	2628	395	488	82/90	39.0 – 2020.0
Hesperetin	728	< MDL	48	12/90	23760.0*
Homogentisic acid	4058	1358	1550	90/90	NA
Kaempferol	7306	1924	2255	88/90	19.0 – 3740.0
Luteolin	1494	< MDL	124	17/90	40.0 – 420.0
p-Coumaric acid	27038	8663	9299	90/90	190.0 – 45510.0
p-Hydroxybenzoic acid	40005	16410	16661	90/90	1900.0 – 62050.0
Protocatechualdehyde	340	< MDL	21	11/90	14210.0*
Protocatechuic acid	1752	791	879	90/90	80.0 – 36990.0
Quercetin	64951	15387	17618	76/90	46.0 – 26400.0
Rosmarinic acid	835	< MDL	113	29/90	190.0 – 7080.0
Rutin	1930	681	730	89/90	50.0 – 35940.0
Salicylic acid	4989	824	1009	90/90	800.0 – 26750.0
Sinapic acid	1193	< MDL	47	4/90	30.0 – 80.0
Trans-cinnamic acid	26464	6733	7305	70/90	290.0 – 29170.0
Vanillic acid	49102	14169	15544	90/90	430.0 – 30340.0

*: only one study reported this compound in buckwheat honey, hence no presence of range; NA: not available as no previous studies have quantified these compounds in buckwheat honey. All values reported from the literature were obtained from the following sources: (Cheng et al., 2015; Drăgănescu et al., 2020; Jasicka-Misiak et al., 2012; Kędzierska-Matysek et al., 2021; Nešović et al., 2020; Ongalbek et al., 2024; Pasini et al., 2013; Puścion-Jakubik et al., 2022; Stanek & Jasicka-Misiak, 2018; Wen et al., 2017; Wilczyńska, 2012; Zhou et al., 2012).

The detection frequencies for many phenolic compounds in buckwheat honey were high, often exceeding 85%, indicating their consistent ubiquity in this type of honey. However, some phenolic compounds exhibited low detection rates, which can be attributed to seasonal changes, geographical differences, and environmental conditions. Factors such as soil type and climate where buckwheat plants are grown can influence the phenolic composition in the nectar collected by bees, resulting in variations in phenolic profiles in honey (Cheung et al., 2019; Moniruzzaman et al., 2013; Vazquez et al., 2021). Buckwheat honey produced in different geographical regions may also exhibit distinct phenolic profiles due to variations in local environmental conditions, agricultural practices, and surrounding vegetation (Ciucure & Geana, 2019). These factors can lead to the presence of certain phenolic compounds in some samples but not others. Additionally,

the foraging behavior of bees and the availability of other floral sources during the collection period can affect the phenolic composition of the honey. Buckwheat blooms in mid to late summer and early fall, around July to September in many regions (Domingos & Bilsborrow, 2021), coinciding with the blooming season of plants like lavender, goldenrod, and sunflower (Walker & Wixted, 2020). If bees have access to a diverse range of plants, the phenolic profile of the honey can become more varied. Moreover, it can be observed that for most compounds with low detection frequencies (e.g., 2,3,4-trihydroxybenzoic acid, protocatechualdehyde, luteolin, hesperetin, and sinapic acid), their average and maximum concentrations in honey are also relatively low. This suggests that some compounds might be present in concentrations below the detection limit, resulting in lower detection frequencies. All these factors might also explain why some phenolic compounds exhibit important differences between the maximum and average concentrations. For instance, quercetin's average concentration in buckwheat honey is 64951 ng/g, while its median concentration is 17618 ng/g, resulting in a difference of 47331 ng/g. These substantial differences in concentration could also be explained by the method of calculating averages. The average concentration was calculated by including all values, with concentrations below the minimum detection limit (MDL) set to 0 ng/g. This approach tends to lower the average, particularly for compounds with many samples below the MDL, potentially misrepresenting the central tendency of the data. Consequently, the median was also reported, as it represents the middle value of the dataset and is not influenced by extreme values or outliers, providing a more robust measure of central tendency, especially in datasets with significant variability or skewness (Lubbe et al., 2021; Lydersen, 2020).

Furthermore, when examining the maximum, median, and average concentrations of phenolic compounds in buckwheat honey, quercetin stands out with the highest maximum and average concentrations (64951 ng/g and 17618 ng/g, respectively), followed by vanillic acid (49102 ng/g and 15544 ng/g, respectively), and p-hydroxybenzoic acid (40005 ng/g and 16661 ng/g). The highest median concentration was observed for p-hydroxybenzoic acid (16410 ng/g). Although quercetin is found at high levels in buckwheat honey, it is not exclusive to this botanical origin and can also be present in high levels in other types of honey. For instance, across all 465 honey samples with varying botanical origins, the average and median concentrations of quercetin were 19484 ng/g and 15769 ng/g, respectively, which are higher to the concentrations in buckwheat

honey (14392 ng/g and 10398 ng/g, respectively). This indicates that quercetin is prevalent in many botanical origins, not just buckwheat honey. In contrast, p-hydroxybenzoic acid exhibits significantly higher average and median levels in buckwheat honey (16661 ng/g and 16410 ng/g, respectively) compared to the overall average and median concentrations across all 465 samples (5529 ng/g and 2605 ng/g, respectively). In addition, p-hydroxybenzoic acid was detected in all buckwheat honey samples tested, underscoring its potential as a marker for buckwheat honey. This high detection frequency and its distinct concentration profile in buckwheat honey suggest that p-hydroxybenzoic acid could serve as a reliable indicator for authenticating buckwheat honey.

Moreover, the comparison of phenolic compound concentrations with literature values indicates that the phenolic profiles of the buckwheat honey samples in this study generally align with reported ranges. Most phenolic compounds, such as p-hydroxybenzoic acid, (±)-2-cis,4-trans-abscisic acid, p-coumaric acid, rutin, and ferulic acid, have maximum, median, and average concentrations within the literature range. However, some phenolic compounds exhibit considerable variability in their concentrations. For instance, protocatechualdehyde and hesperetin show significantly lower concentrations compared to certain literature values, while apigenin, caffeic acid, galangin, gallic acid, kaempferol, luteolin, quercetin, sinapic acid, and vanillic acid have maximum concentrations identified in this study that are higher than those reported in the literature. The differences in concentrations observed in this study compared to the literature can be attributed to several factors. Buckwheat grown in different regions may have varying phenolic profiles due to environmental factors such as soil type, climate, and surrounding vegetation (Ciucure & Geana, 2019; Vazquez et al., 2021). Additionally, differences in analytical methods used for quantification, including extraction, separation, and detection methods, can lead to variations in reported concentrations (Hassan et al., 2022). The processing and storage conditions of honey also affect its phenolic content. Factors such as exposure to light, temperature, and duration of storage can influence the stability and concentration of phenolic compounds (Becerril-Sánchez et al., 2021; Braghini et al., 2020). Furthermore, the literature values often correspond to only a few buckwheat honey samples, making the sample size relatively small and potentially unrepresentative. The large sample size (90 samples) in this study provides a more comprehensive representation, which might include samples with

unusually high concentrations of certain phenolic compounds not captured in previous studies with smaller sample sizes. This extensive sampling offers a more robust understanding of the phenolic profile of buckwheat honey, highlighting both typical concentrations and the potential for variability.

In summary, the extensive quantification of phenolic compounds across numerous buckwheat honey samples provides a thorough insight into the diverse phenolic profile characteristics of this honey variety. This knowledge not only aids in the authentication of buckwheat honey but also deepens our understanding of its complex botanical composition. The notable detection frequency and unique concentration profile of p-hydroxybenzoic acid indicate its potential as a reliable marker for distinguishing authentic buckwheat honey. However, ongoing advancements in analytical methods and broader-scale investigations will be crucial in refining our comprehension of phenolic compounds in honey, thereby furthering our ability to accurately assess its botanical authenticity.

3.4.4 Identification of a buckwheat honey phenolic marker

While the entire phenolic profile can serve as a marker for botanical authenticity, individual compound markers may be practical for routine analysis or transferable to other analytical platforms. Comprehensive profiling requires a large sample size representing diverse botanical origins and advanced chemometric tools to accurately classify honey based on its botanical origin. This process is both time-consuming and resource intensive. A more feasible alternative is to identify specific phenolic compounds that can act as reliable markers for botanical origin. Among the 29 phenolic compounds, outlined in Table 3.2, that were quantified in all 465 honey samples, p-hydroxybenzoic acid emerged as a potential threshold marker due to its significantly higher concentrations in buckwheat honey compared to other botanical origins, as shown in Figure 3.3.

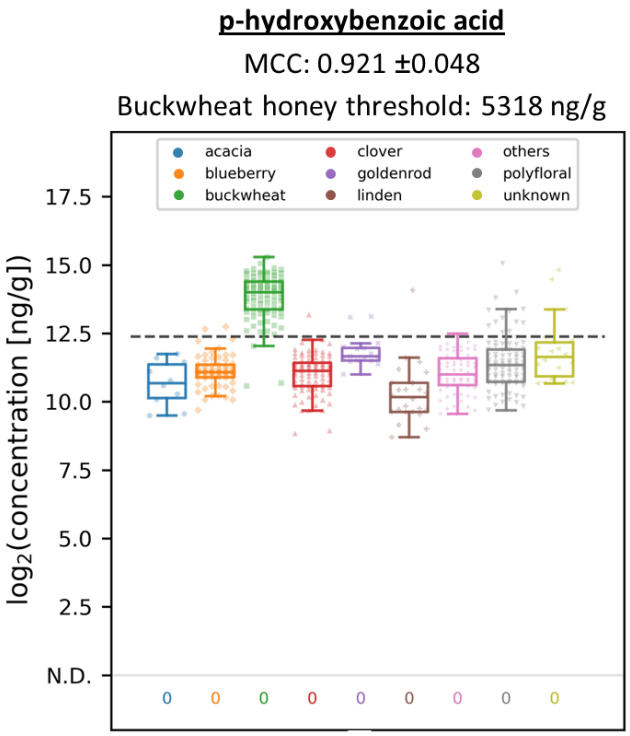

Figure 3.3. Threshold marker p-hydroxybenzoic acid identified for buckwheat honey with a 5318 ng/g threshold level established based on a single-node decision tree conducted on honey samples (n=465).

This compound was selected as the marker for buckwheat honey because it has the highest Matthews Correlation Coefficient (MCC) score of 0.92 ± 0.05. The MCC is a metric used to evaluate the performance of binary classification models, taking into account true positives, true negatives, false positives, and false negatives. The MCC score ranges from -1 to +1, where +1 indicates perfect prediction, 0 indicates no better than random prediction, and -1 indicates total disagreement between prediction and observation (Lubatti et al., 2023). An MCC score of 0.92 ± 0.05 indicates a strong positive correlation between the presence of this phenolic compound and the classification of honey as buckwheat honey. The 0.92 implies that the classifier correctly identifies buckwheat honey with a high degree of accuracy, with minimal errors (false positives and false negatives). The ± 0.05 is the standard deviation, reflecting the variability in the MCC score due to 5-fold cross-validation. This low standard deviation indicates that the MCC score is stable, with only minor variations, suggesting that the classifier's performance is consistently strong across different samples and conditions.

Although p-hydroxybenzoic acid has been reported in numerous studies (sample size ranging from 28 to 105) as a marker for buckwheat honey due to its elevated concentration compared to other honey types (Koulis et al., 2021; Nešović et al., 2020; Puścion-Jakubik et al., 2022; Wen et al., 2017), no studies have previously identified a specific threshold level for this compound as a marker. This study is the first to establish a threshold level for p-hydroxybenzoic acid to authenticate buckwheat honey. To determine this threshold, a single-node decision tree was trained using different thresholds, with Gini impurity as the criterion to find the best concentration threshold of p-hydroxybenzoic acid. The threshold that minimized the impurity or disorder in classifying honey samples was selected, which turned out to be 5318 ng/g. *Gini impurity* is a metric used to measure the degree of impurity or disorder within a set of items, particularly in decision trees. It quantifies how often a randomly chosen element from the set would be incorrectly classified if it was randomly labeled according to the distribution of labels in the set (Zollanvari, 2023). For each potential threshold, the *Gini impurity* was calculated to assess how well the threshold separated the samples into two classes (buckwheat honey and non-buckwheat honey). Lower *Gini impurity* indicates a more effective threshold, resulting in a cleaner split between the classes with fewer misclassifications (Bodine & Hochbaum, 2020).

To assess whether the threshold level of 5318 ng/g for p-hydroxybenzoic acid is suitable for buckwheat honey not analyzed in this study, its level was compared to the concentrations reported in buckwheat honey from the literature. Many studies have found p-hydroxybenzoic acid levels in buckwheat honey exceeding the established threshold (Kędzierska-Matysek et al., 2021; Puścion-Jakubik et al., 2022; Wen et al., 2017; Wilczyńska, 2012; Zhou et al., 2012). However, some studies have found p-hydroxybenzoic acid levels below this threshold in certain buckwheat honey samples (Nešović et al., 2020; Pasini et al., 2013). It is important to note that Nešović et al. (2020) confirmed the botanical origin of their buckwheat honey samples using only melisopalynology analysis and Pasini et al. (2013) relied on color, odor and flavor attributes, as well as overall appearance and consistency as the only factors to confirm the botanical origin of their honey samples. These findings highlight that while the threshold of 5318 ng/g for p-hydroxybenzoic acid is a useful marker for most buckwheat honey, there is variability. This variability can be attributed to regional differences, such as soil type and climate, and storage conditions emphasizing the need to consider additional markers to accurately validate the botanical origin of buckwheat honey. Thus, this threshold can help classify honey with unknown

botanical origin as either buckwheat or non-buckwheat. However, identifying additional markers is crucial to further validate its botanical origin.

3.4.5 Predicted classification of buckwheat honey using p-hydroxybenzoic acid as the marker

A confusion matrix was created to assess the predictive ability and reliability of p-hydroxybenzoic acid in distinguishing between buckwheat and non-buckwheat honey (Figure 3.4).

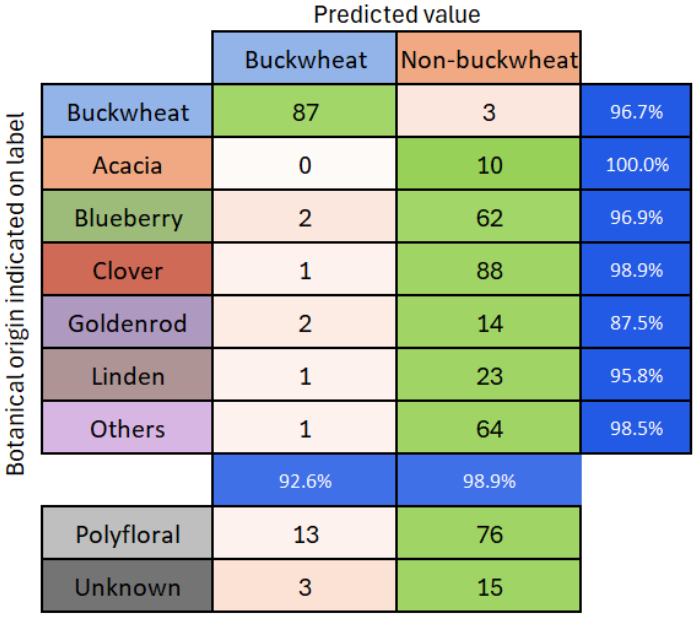

Figure 3.4. Confusion matrix showing the accuracy of p-hydroxybenzoic acid in predicting buckwheat and non-buckwheat honey using LC-QTOF-MS results with a threshold of 5318 ng/g.

The confusion matrix reveals that p-hydroxybenzoic acid has a true positive rate (TPR) of 96.7%, indicating that the model accurately identifies 96.7% of labeled buckwheat honey samples as such. This TPR, also known as sensitivity or recall, measures the proportion of actual positives (true buckwheat honey samples) correctly identified by the model (Yusro et al., 2019). The three samples labeled as buckwheat but classified by the model as non-buckwheat honey were confirmed as polyfloral honey through melisopalynology and sensory evaluation. Pollen analysis detected 0% buckwheat pollen in all three honey samples, highlighting the model's effectiveness in identifying mislabeled buckwheat honey. However, evaluating the model's accuracy solely based on the TPR is insufficient as assessing the model's performance requires more than just sensitivity. It is also crucial to evaluate how accurately the model can predict non-

buckwheat honey (such as acacia, blueberry, clover, goldenrod, linden and other monofloral honey) as truly non-buckwheat. This is measured by the positive predictive rate (PPR), also known as precision (Yusro et al., 2019). In this case, the positive predictive rate of p-hydroxybenzoic acid is 92.6%, indicating that 92.6% of the time, it accurately predicts that a sample labeled as non-buckwheat honey is indeed correct. This high precision is essential for ensuring that non-buckwheat honey are not mistakenly classified as buckwheat honey. It is also important to note that honey labels cannot always be fully trusted. A recent report by the USFDA highlighted that around 10% of honey samples were found to be fraudulent (USFDA, 2024). This means that some monofloral honey might actually contain significant amounts of buckwheat honey, potentially influencing the p-hydroxybenzoic acid levels and leading to misclassification by the model.

Furthermore, the confusion matrix also reveals that among the polyfloral honey, 13 samples were predicted as buckwheat honey, while 76 were predicted as non-buckwheat. These 13 false positive results could be explained by the fact that producers who are uncertain of the botanical origin of their honey might label it as polyfloral to cover this uncertainty (Ruoff, 2006). However, these polyfloral samples might contain significant levels of buckwheat nectar, leading the classification model to predict them as buckwheat honey. Alternatively, high p-hydroxybenzoic acid levels in polyfloral honey could come from mixing with other honey containing this compound in high concentrations. Regarding the unknown samples, three were predicted as buckwheat and 15 as non-buckwheat. These results suggest that the unknown samples might indeed include true buckwheat honey that was not labeled as such. The presence of p-hydroxybenzoic acid in these samples indicates a high likelihood of them being buckwheat honey, even if not explicitly labeled.

3.4.6 Transferability of the method developed and the buckwheat honey phenolic marker identified on the LC-QTOF-MS to other laboratories (using LC-QqQ-MS/MS as a case study)

As a proof of concept of the transferability and the entire authentication tool (dilute-and-shoot method and identified marker with threshold), a method was set in an independent laboratory to quantify p-hydroxybenzoic using LC-QqQ-MS/MS. The MDL for p-hydroxybenzoic acid was determined to be 0.05, 0.06, and 0.07 µg/g for white, golden and dark honey, respectively. The MQL was measured to be 0.17, 0.19, 0.22 µg/g for white, golden and dark honey, respectively.

These values are significantly below the previously established classification threshold, making them applicable. Method precision and recovery were acceptable across all three honey matrices, with precision RSD% of 1.35%, 2.88%, and 7.32%, and recoveries of 125.03 ± 11.28%, 104.59 ± 22.56%, and 72.30 ± 1.63% for white, golden, and dark honey, respectively. Calibration curve linearity was also deemed acceptable as the solvent (r^2 = 0.999%), white (r^2 = 0.998%), golden (r^2 = 0.999%), and dark (r^2 = 0.996%) calibration curves had high correlation coefficients. The successful validation on both the LC-QTOF-MS and LC-QqQ-MS/MS demonstrates that the dilute-and-shoot approach is reliable and versatile, making it well-suited for routine analysis of phenolic compounds in honey authentication.

Moreover, to further validate the identified marker and threshold on its applicability, cross-instrumental validation was conducted using LC-QqQ-MS/MS as a case study. Figure 3.5 illustrates the classification prediction for buckwheat honey based on LC-QqQ-MS/MS data employing p-hydroxybenzoic acid as the marker.

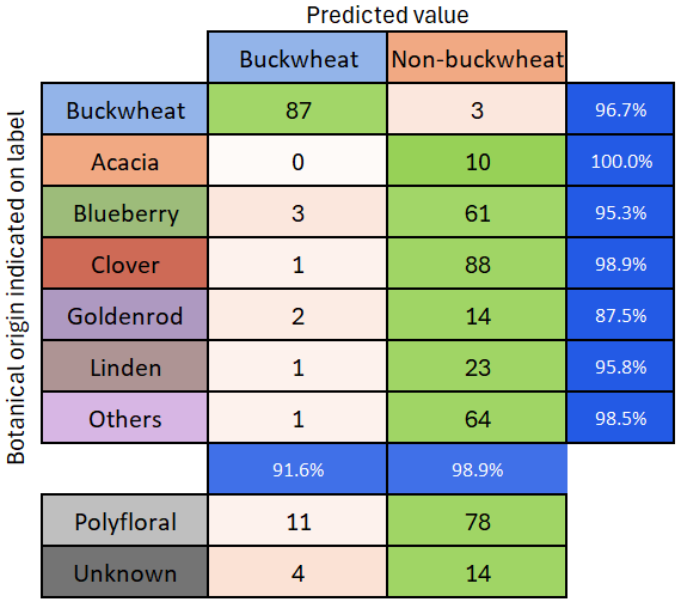

Figure 3.5. Confusion matrix showing the accuracy of p-hydroxybenzoic acid in predicting buckwheat and non-buckwheat honey using LC-QqQ-MS/MS results (5318 ng/g threshold applied).

In the 465 honey samples analyzed, 99.14% were classified in agreement with the results obtained via LC-QTOF-MS, utilizing a threshold of 5318 ng/g. This high level of agreement

underscores the robustness of the proposed marker, as confirmed by a confusion matrix that demonstrates consistent performance across various analytical conditions and equipment. Both LC-MS instruments exhibited equivalent TPR at 96.7%, and a slightly lower PPR (91.6%) was obtained on the LC-QqQ-MS. Despite this minor variation, the results affirm the robustness and reliability of p-hydroxybenzoic acid as a marker for buckwheat honey across different analytical platforms. Cross-instrument validation is a crucial step in the comprehensive validation of analytical markers. It ensures that the marker performs consistently across various instruments, accounting for differences in sensitivity, resolution, and detection limits (Bayen et al., 2024; Chen et al., 2023; FDA, 2018). This process is essential for confirming the marker's reliability and applicability in diverse laboratory environments, thereby promoting broader adoption and standardization. This study stands as the first investigation to assess the transferability of a phenolic compound marker for the botanical authenticity of honey to different laboratories. This research has demonstrated that the marker is efficient and reliable beyond its initial discovery on a single instrument. Such systematic studies should be more widely conducted in scientific literature to enhance the implementation and standardization of robust analytical strategies. In addition, by validating markers across different instruments, we can ensure consistent and accurate detection, which is critical for maintaining the integrity of honey products and protecting consumer interests.

3.5 Conclusion

To conclude, the developed dilute-and-shoot HPLC-QTOF-MS technique for analyzing 29 phenolic compounds in honey has proven to be effective, showcasing the method's robustness and sensitivity. Validation of the method has shown comparable LOD and LOQ values to existing literature, along with good precision, recovery, and linearity. Applying this method to 465 honey samples from different botanical origins unveiled unique phenolic profiles, particularly for buckwheat honey, where p-hydroxybenzoic acid was identified as a potential marker with a threshold level of 5318 ng/g. Cross-instrument validation confirmed this marker's reliability, making it transferable across laboratories with consistent results. These findings highlight the practical usefulness of phenolic compounds, particularly p-hydroxybenzoic acid, in distinguishing buckwheat honey from other botanical sources, providing valuable insights for authenticating honey. In the broader context of food authenticity, this study underscores the

potential of advanced analytical techniques in ensuring the integrity and authenticity of various food products. By identifying specific chemical markers, such methods can combat food fraud, protect consumer interests, and support regulatory frameworks. The success of this study provides a valuable model for authenticating other food products, emphasizing the need for robust, transferable, and precise analytical methods in the ongoing effort to maintain food quality and safety. Future research should prioritize similar systematic studies to facilitate the adoption of such a marker by regulatory bodies for authenticating honey. By expanding cross-instrument validations and ensuring that the marker performs consistently across various instruments, we can significantly improve the reliability and accuracy of honey authenticity testing. This approach will not only strengthen the scientific basis for honey authentication but also support regulatory efforts, protect consumer interests, and maintain the integrity of honey products in the market.

CONNECTING PARAGRAPH II

Chapter 3 represented a significant leap forward in understanding the phenolic composition of honey, particularly buckwheat honey, utilizing the multi-targeted LC-QTOF-MS method. This research enriched the scientific comprehension of the unique phenolic profile in buckwheat honey, enabling its differentiation from other botanical sources. Key findings included the identification of p-hydroxybenzoic acid as a robust indicator of buckwheat honey authenticity. To validate this marker's reliability and transferability across different laboratories, cross-instrumental validation was conducted using LC-QqQ-MS/MS, ensuring consistent performance despite variations in instrument sensitivity, resolution, and detection limits. This chapter not only advanced the standardization of authenticity markers in food but also proposed its potential application in routine honey verification.

Chapter 4 extended this investigation by exploring the stability of the buckwheat honey marker under various storage and thermal processing conditions. By assessing how these factors influenced the phenolic profile, particularly the integrity of the identified marker, the study provided critical insights into its robustness in practical scenarios. These insights are pivotal for evaluating the marker's efficacy in verifying buckwheat honey authenticity post-storage or processing, offering a comprehensive understanding of its reliability across different environmental contexts.

CHAPTER 4. IMPACT OF STORAGE AND THERMAL PROCESSING ON THE PHENOLIC PROFILE OF HONEY

4.1 Abstract

Honey, a sweet and viscous syrup produced by honey bees (Apis mellifera), is revered for its natural sweetness and health benefits, largely attributed to its rich content of phenolic compounds. These compounds not only contribute to honey's antioxidant properties but also serve as crucial markers for authenticating its botanical origin. This study explores how storage and thermal processing affect the phenolic profile of honey, essential for maintaining its quality and authenticity. Specifically, it examines the stability and reliability of phenolic markers, such as p-hydroxybenzoic acid used in classifying buckwheat honey, under various conditions. Six honey samples underwent controlled storage at different temperatures and thermal treatments to assess changes in phenolic composition and marker concentration. The findings underscore the critical role of storage conditions in preserving phenolic integrity, ensuring accurate botanical classification and consumer trust in honey products. Storage temperatures up to 25°C showed no significant ($p > 0.05$) alteration in phenolic profiles or marker concentrations, maintaining their effectiveness for honey classification. However, storage at 65°C notably decreased p-hydroxybenzoic acid concentration, potentially leading to misclassification of true buckwheat honey. Thermal treatment experiments indicated minimal alteration in phenolic profiles between heat-treated and non-heat-treated honey samples, supporting classification based on botanical origin. These results emphasize the importance of proper storage conditions, particularly temperatures at or below 25°C, in preserving honey's phenolic integrity for accurate botanical authentication. While thermal processing had negligible impact on phenolic markers, further research with larger sample sizes is recommended to confirm these findings and enhance the reliability of honey authentication methods.

4.2 Introduction

Phenolic compounds present in honey, including phenolic acids and flavonoids, are sensitive to various environmental factors such as heat, light, pressure treatment, oxygen, pH, and storage conditions (Becerril-Sánchez et al., 2021; Braghini et al., 2019; Braghini et al., 2021; Chaaban et al., 2017; Leyva-Daniel et al., 2017; Mat Ramlan et al., 2021; Yalçın, 2021). Amongst these parameters, heat is reported to have the highest impact on phenolic compound degradation, with

temperature and exposure time identified as critical factors affecting the phenolic profile in honey. Understanding the dynamic relationship between temperature and the phenolic profile of honey is paramount for various reasons. Firstly, phenolic compounds are not only vital for honey's sensory attributes but also contribute significantly to its health-promoting properties, including antioxidant and antibacterial activities (Biluca et al., 2017). Any alteration in the phenolic composition due to storage conditions could compromise these beneficial attributes, potentially diminishing the perceived quality and authenticity of honey. Moreover, as phenolic compounds are increasingly recognized as markers for honey's botanical origin, any deviation in their profile induced by storage temperature variations could lead to inaccurate assessments of honey's authenticity. Therefore, in the context of honey's botanical origin authentication, it becomes crucial to store honey under temperatures that will preserve its phenolic profile for a more accurate assessment of its authenticity.

Similarly, understanding the impact of thermal processing on honey's phenolic composition is essential for assessing its potential implications on the botanical origin authentication of honey. Liquefaction and pasteurization are two types of thermal processing that honey may undergo in an industrial manufacturing setting, each with its own effects on the phenolic profile. Liquefaction typically involves heating honey to approximately 55°C to dissolve crystallized sugars, facilitating easier handling and pouring (Aydoğan Coşkun et al., 2020; Escriche et al., 2014; Eshete & Eshete, 2019). On the other hand, pasteurization involves heating honey to a specific temperature, typically carried out between 70-78°C in commercial settings, to eliminate harmful microorganisms while preserving its liquid consistency (Eshete & Eshete, 2019). This process also destroys yeast, which could lead to undesirable fermentation during the product's shelf-life, and disperses crystallization nuclei responsible for honey solidification, thereby prolonging its retention in a liquid state (Escriche et al., 2014). Both processes involve exposure to heat, which, as mentioned earlier, is the primary parameter affecting the phenolic composition of honey. Therefore, understanding how liquefaction and pasteurization impact the phenolic profile is crucial for accurately assessing the botanical origin of honey.

Moreover, the importance of understanding the impact of temperature on the phenolic profile of honey is becoming increasingly significant, given its growing use in processed food products. Honey, prized for its unique combination of sweetness, flavor, and functional properties, serves

as a natural alternative to artificial sweeteners and high fructose corn syrup, often linked to health concerns. Honey is believed to possess several health-promoting properties, particularly its antioxidant potential, which is largely attributed to its phenolic content (Cianciosi et al., 2018). With the growing demand for natural and healthier food products, honey's use in processed foods has become attractive for food manufacturers. In Canada alone, over 1200 honey-containing products entered the market between January 2016 and October 2020 (Government of Canada, 2021a). This trend is driven by increasing consumer demand for natural, healthier, and organic ingredients, along with the recognized health benefits of honey. Consequently, many food manufacturers now use honey in a wide array of products, ranging from breakfast cereals, cough lozenges, baked goods, and energy bars to dressings, beverages, and marinades (Larsen & Ahmed, 2022). This trend is expected to grow as consumers become more health-conscious and seek natural, wholesome ingredients in their diets. However, the fate of phenolic compounds in honey after processing into food products has not been extensively studied. Given the health-promoting properties of phenolic compounds, understanding their fate in processed foods is essential for evaluating the potential health benefits of these products.

Furthermore, honey not only enhances products' functional properties but also significantly impacts taste, with its botanical origin identified as a key factor in aroma and flavor (Kortesniemi et al., 2018). Buckwheat honey, characterized by its dark amber color and strong odor offers an acquired taste unsuitable for general consumption alone. However, when incorporated into beverages or baked goods, buckwheat honey provides a taste and aroma that adds depth and complexity to the final product. Its malty sweetness complements teas and coffees, while its robustness enhances breads and muffins with an earthy note. Floral undertones add nuance, making it a coveted ingredient for culinary experimentation. Moreover, buckwheat honey has gained attention for being the most nutritious and health-benefiting honey type following manuka honey, a world-famous honey for its healing properties (Deng et al., 2018). Studies demonstrate that buckwheat honey contains a high antioxidant activity and antibacterial potential owing to its high phenolic content (Brudzynski et al., 2012; Deng et al., 2018). Therefore, it becomes important to study the fate of phenolic compounds in buckwheat honey to ensure that storage or thermal processing of honey does not compromise its phenolic composition, thereby preserving its beneficial attributes. Furthermore, considering the distinctive flavor profile and numerous health benefits associated with buckwheat honey, it is essential to verify its

authenticity. Authenticity studies are critical for safeguarding the quality and integrity of food products, meeting consumer expectations, and ensuring transparency in the marketplace.

Despite the numerous papers that have investigated the impact of heating or storage on the phenolic profile in honey, few research papers have simultaneously examined a wide array of phenolic compounds. Additionally, investigating the influence of storage and thermal treatment on honey's phenolic composition can provide deeper insights into how these conditions modify the phenolic profile of honey. This research not only enhances our understanding of the changes in honey's phenolic profile under various conditions but also contributes to our comprehension of the parameters to consider when assessing the classification of honey's botanical origin, particularly in the context of buckwheat honey. Moreover, based on the available information in the literature, while certain papers explore the impact of liquefaction and pasteurization on the phenolic profile of honey, there is a notable lack of research on how these processes influence honey's phenolic composition and subsequently affect the assessment of its botanical origin.

The objective of this study was to assess the effect of storage and thermal treatment on the phenolic profile of honey. In addition, this study aims to assess whether storage and heat treatment conditions effectively preserve p-hydroxybenzoic acid, previously identified as a candidate marker for authenticating buckwheat honey. This case study will bring some understanding of the effect of thermal processing on markers of authenticity.

4.3 Methodology

4.3.1 Honey samples

Six raw or unpasteurized honey samples (Table 4.1) were selected for this study, including (i) two honey samples labeled and confirmed by pollen analysis as being buckwheat honey, (ii) two samples labeled as buckwheat and confirmed by pollen analysis as polyfloral honey, and (iii) two other monofloral honey samples confirmed to contain no buckwheat pollen (<0.1%). Raw or unpasteurized honey were selected to mitigate any potential alteration in the phenolic profile that could have arisen from prior exposure to heat treatment.

Table 4.1. Characteristics of the honey samples selected for the case study examining the impact of storage and thermal processing on the phenolic profile of honey.

Honey class	Internal ID	Floral type (indicated on jar)	Buckwheat pollen %	Processing type	Color class	Pollen analysis conclusion
2 honeys labeled buckwheat and confirmed by pollen analysis as monofloral buckwheat honey	Buck 1	Buckwheat	5.70%	Raw	Dark	Very low buckwheat pollen percentage. It has all the characteristics of monofloral buckwheat (characteristic taste, dark color, slow crystallization). The reason for little buckwheat pollen found could be due to filtration since buckwheat pollen is larger than others.
	Buck 2	Buckwheat	3.80%	unpasteurized unfiltered	Dark	Monofloral buckwheat honey
2 honeys labeled as buckwheat and confirmed by pollen analysis as polyfloral honey	Poly 1	Buckwheat	0.60%	unpasteurized	Dark	Polyfloral honey
	Poly 2	Buckwheat	0.30%	unpasteurized	Dark	According to the percentages, it would be a leguminous honey by adding the clovers and trefoil. The trefoil, even if it is under-represented, is not present in sufficient quantity to make it a monofloral honey.
2 other monofloral honeys not containing buckwheat pollen	Other 1	Blueberry	ND (<0.1%)	unpasteurized	White	Legume honey
	Other 2	Clover	ND (<0.1%)	unpasteurized	White	Monofloral clover honey

4.3.2 Storage experiment sample preparation

Approximately 10 g of honey samples were individually transferred from their original jars into 40 mL amber glass vials. Samples were capped and placed in -80°C and -20°C freezers, a 4°C refrigerator, room temperature (25°C), and a water bath maintained at 65°C. The lower temperatures (-20°C, and 4°C) were chosen to replicate conditions typically utilized in experiments aiming to preserve the chemical composition of honey. Room temperature was included to simulate the storage conditions found in industrial facilities and grocery stores or those adopted by consumers. A temperature of 65°C was selected to evaluate the impact of more extreme conditions, such as sunlight exposure or elevated temperatures experienced during the period between harvest and industrial processing or transportation (Visquert et al., 2014). Samples were stored under these conditions for 1, 3, and 6 months, after which they were analyzed according to the method outlined in Chapter 3, section 3.3.4. Samples stored at -80°C were used as control on the assumption that metabolite profiles had minimal changes at this temperature over 6 months. The same instrumental analysis was applied to these samples as specified in Chapter 3, section 3.3.5. Procedural and solvent blanks were run as described in Chapter 3, section 3.3.6.

4.3.3 Thermal treatment experiment sample preparation

Initially, 0.2 g (± 0.01g) of honey was weighed into a 15-mL glass tube, with the opening of the tubes sealed with aluminum foil and tape to maintain a closed system and prevent water entrance during heating. The samples were subject to heating in a water bath preheated to temperatures of 55°C, 74°C, 85°C, and 100°C, selected to replicate various thermal processing conditions experienced by honey. Upon submersion of the honey samples in the water bath, the timer was initiated, and the water bath temperature was closely monitored and maintained within a +/- 1°C range of the set temperature throughout the experiment. Samples were removed from the water bath at intervals of 5, 10, 30, 60, and 90 minutes and transferred to a cold water bath to halt heating. Unheated honey samples that were kept at -80°C served as controls. Samples were analyzed using LC-QTOF-MS as described in Chapter 3, section 3.3.4. Honey samples were run on the LC-QTOF-MS in a randomized order, with unheated samples distributed evenly throughout the 5-day instrumental run. Moreover, a total of six procedural blanks were prepared (1 mL of a mixture of acetonitrile and water, 1:9 *v/v*). Among these procedural blanks, three were

unexposed to any heat treatment, while the remaining three were subjected to the most extreme thermal condition tested of 100°C for 90 minutes. A solvent blank was prepared using the same solvents as those employed in the preparation of honey samples, namely acetonitrile and water (1:9 *v/v*).

4.3.4 Data treatment and statistics

The quantification of phenolic compounds in honey samples relied on the utilization of both white and dark matrix-matched calibration curves, as detailed in Section 3.3.2 of Chapter 3. These calibration curves were specifically chosen to accommodate the characteristics of the six honey samples selected for this case study, which encompassed both white and dark-colored varieties. Data analysis was conducted using Agilent Mass Hunter Workstation Software – Quantitative Analysis B.07.0, applying a retention time window of ± 0.5 min and a *m/z* window of ± 20 ppm. To evaluate the precision and accuracy of the instrument, the retention time shift and mass measurement error were computed for a laboratory QC standard mix. This mix, comprising a 100 ng/mL 33 phenolic standard mixture, underwent analysis every 20 samples during the run. The limit of detection (LOD) and method detection limit (MDL) were determined as reported in Chapter 3. Both heated (n=3) and non-heated (n=3) procedural blanks were employed to assess any statistical variance between them, thereby evaluating the suitability of a matrix-matched calibration curve prepared with unheated honey for quantification purposes.

4.4 Results and discussion

4.4.1 QA/QC

To ensure consistent instrument performance, the RSD% of each targeted phenolic compound in the laboratory QC standard mix, which underwent multiple analyses during sample runs, was monitored. The closest calibration curve was used for quantification. In both the storage and thermal treatment experiments, all targeted phenolic compounds maintained an RSD% below 20%, with p-hydroxybenzoic acid demonstrating an RSD% of 5.8% and 10.7 ± 8.3%, respectively.

Moreover, the mass accuracy and retention time shift for all tested phenolic compounds were evaluated to assess the quality and reliability of the LC-MS data. The mass accuracy across all analyzed phenolic compounds averaged at -0.43 ± 1.14 ppm (ranging from -2.17 to 2.36 ppm) in

the honey storage experiment, and -0.24 ± 1.32 ppm (ranging from -2.25 to 3.10 ppm) in the honey thermal treatment study. In the honey storage experiment, the RSD% of the retention time shift varied from 0.06% to 1.43% across all targeted phenolic compounds and ranged from 0.08% to 3.85% for all targeted phenolic compounds in the honey thermal treatment experiment. Particularly, for p-hydroxybenzoic acid, identified as the marker for buckwheat honey, the mass accuracy and RSD % of the retention time shift were identified as 1.36 ± 0.61 ppm and 0.16%, respectively, for the honey heat treatment experiment and 0.62 ± 0.38 ppm and 0.17%, respectively, for the storage experiment.

Furthermore, for the thermal treatment experiment, statistical analysis revealed no significant difference ($p > 0.05$) between the detected signal of the unheated and heated procedural blanks for all tested phenolic compounds. This suggests that the heating process did not induce notable alterations in the honey matrix that could impact the quantification of phenolic compounds in honey samples. Thus, employing a matrix-matched calibration curve prepared using unheated honey samples is suitable for quantifying phenolic compounds in heat-treated honey samples.

Regarding the limit of detection (LOD) and method detection limit (MDL), values for each phenolic compound in both the storage and thermal treatment experiments align closely with those specified in Chapter 3, as outlined in Table 3.2. Specifically, the LOD for p-hydroxybenzoic acid in the storage and thermal treatment experiments were calculated as 153.49 ng/g honey and 353.59 ng/g honey, respectively, which are comparable to the value reported in Chapter 3. The MDLs for the storage experiment were 662.42 ng/g and 899.18 ng/g of honey for white and dark honey matrices, respectively. For the thermal treatment experiment, the MDLs were 325.19 ng/g of honey and 437 ng/g of honey for white and dark honey matrices, respectively. Although slightly higher than those reported in Chapter 3, these MDLs remain significantly below the buckwheat threshold mark of 5318 ng/g of honey.

4.4.2 Effect of honey storage on its phenolic profile

A Principal Component Analysis (PCA) plot was prepared from the phenolic profiles of all the stored honey samples to assess the impact of honey storage at different time and temperature conditions on the phenolic profile of honey (Figure 4.1). In the PCA plot using the first two PCs, honey samples stored at 65°C for 1, 3, and 6 months exhibited a distinct clustering away from samples stored at -80°C, -20°C, 4°C, and 25°C. This clustering suggests that storage at elevated

temperatures impacts the phenolic composition of honey compared to samples stored at lower temperatures. Furthermore, a closer examination of the PCA plot revealed an interesting trend regarding the phenolic profiles of honey samples stored at 65°C for different durations. Specifically, honey samples stored at 65°C for 1 month appeared to cluster closer to the group of samples stored at -80°C, -20°C, 4°C, and 25°C along PC1. This observation suggests that after one month of storage at 65°C, the phenolic profile of honey retains some similarities to that of honey stored at lower temperatures. However, as the storage duration at 65°C increased to 3 and 6 months, the clustering of samples shifted further away from the cluster associated with lower storage temperatures along PC1. This indicates a progressive alteration in the phenolic profile of honey with prolonged exposure to elevated temperatures.

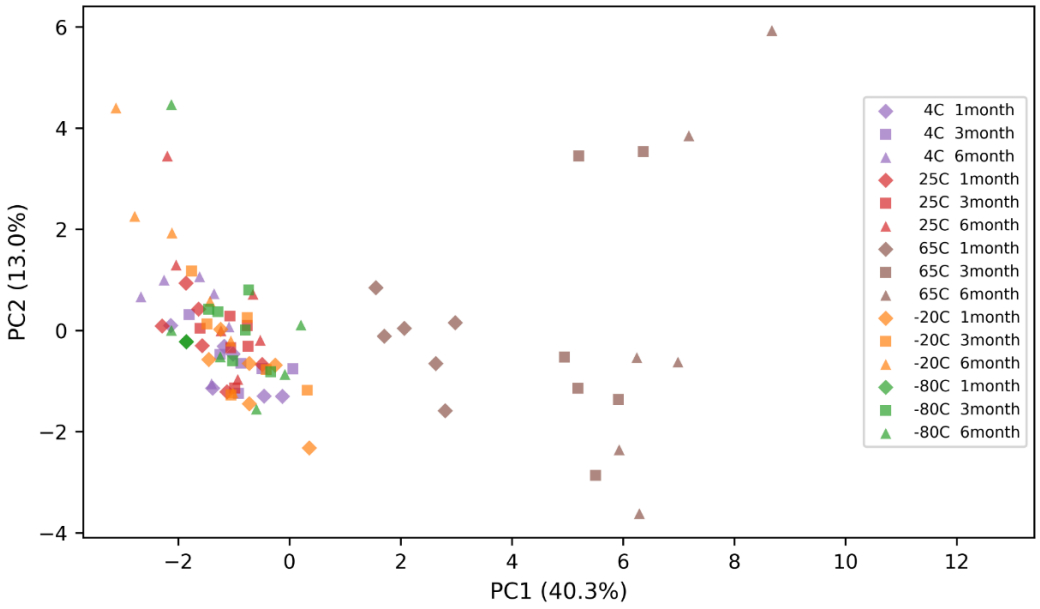

Figure 4.1. Principal Component Analysis (first 2 components) for the impact of storage conditions on the quantified phenolic profile (18 features) in selected honey samples (n=6). PC1 and PC2 explained 40.3% and 13.0 % of the total variance, respectively.

A one-way ANOVA test was conducted to assess the influence of storing honey at 65°C on the tested phenolic compounds compared to lower storage temperatures, regardless of storage duration or honey type. As illustrated in Figure 4.2, epicatechin, 2,5-dihydroxybenzoic acid, p-coumaric acid, syringic acid, chlorogenic acid, and homogentisic acid exhibited significant changes in concentration following storage at 65°C compared to all lower temperatures. A significant increase ($p < 0.05$) in concentration after storage at 65°C was demonstrated for

epicatechin, chlorogenic acid and homogentisic acid, while a significant decrease ($p < 0.05$) was shown for 2,5-dihydroxybenzoic acid, p-coumaric acid, and syringic acid. Specifically, epicatechin showed an increase of 100%, chlorogenic acid increased by 277%, and homogentisic acid increased by 114%. Conversely, 2,5-dihydroxybenzoic acid decreased by 92%, p-coumaric acid decreased by 76%, and syringic acid decreased by 65%. Sinapic acid displayed a significant increase ($p < 0.05$) when stored at 65°C compared to -80°C, 4°C, and 25°C, showing an increase of 206%. Moreover, vanillic acid demonstrated a significant increase ($p < 0.05$) only when stored at 65°C compared to storage at 4°C, showing an increase of 115%. No significant difference ($p > 0.05$) was observed in the concentration of other tested phenolic compounds between storage temperatures when storage time and honey group were not considered as factors.

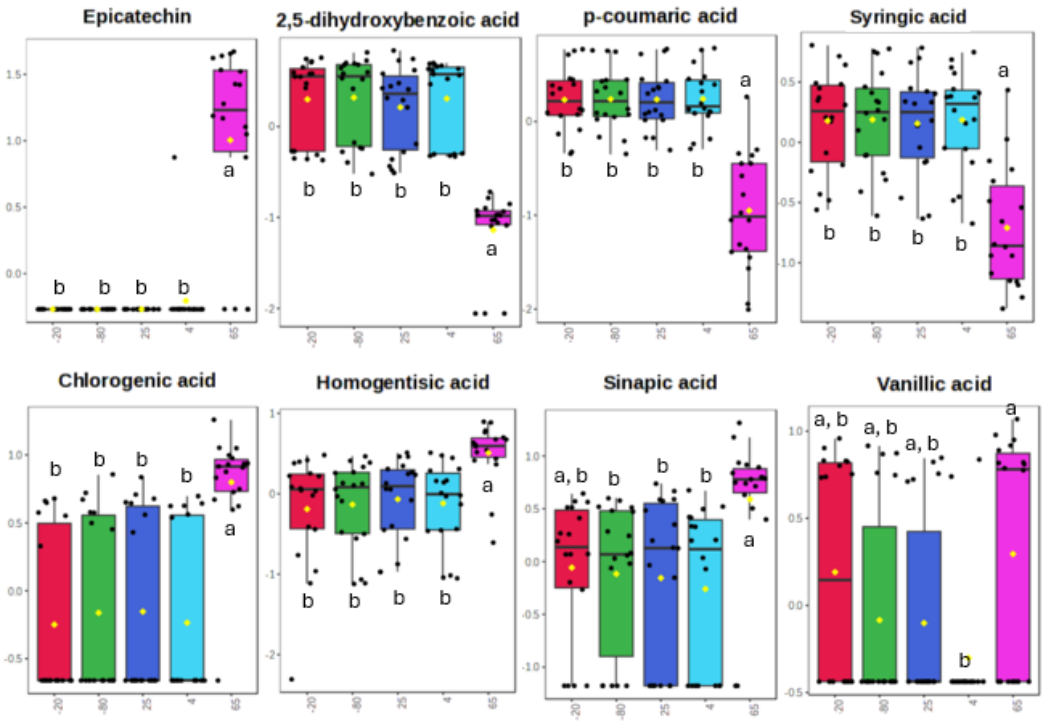

Figure 4.2. Phenolic compounds demonstrating significant differences between different honey storage temperatures. Concentrations were normalized using log transformation (based 10) and pareto scaling. Significant differences (one-way ANOVA) between different storage temperatures are represented by different letters, according to Tukey's test ($p < 0.05$).

A study by Braghini et al. (2020) has found that honey stored at 40°C for 90 days experienced a significant reduction in most tested phenolic compounds, whereas those stored at 4°C for 365

days generally preserved their phenolic content. Similarly, samples stored at 22°C for 90 days maintained their phenolic profile compared to the initial stages of storage. However, six phenolic compounds, including p-hydroxymethylbenzoic acid, gallic acid, protocatechuic acid, quercetin, vanillin, and kaempferol, were detected in honey stored at both 22°C and 40°C, despite their absence in fresh samples. While the emergence of these compounds was not observed in the conducted study, a decrease in the phenolic content was noted for the majority of phenolic compounds following storage at 65°C. Additionally, consistent with the findings by Braghini et al. (2020), lower temperatures, such as refrigeration, appear more conducive to preserving the phenolic profile of honey. Studies have demonstrated that treatments at high temperatures, inflict more severe impacts on phenolic content compared to milder heating temperatures (Braghini et al., 2019; Šarić et al., 2013). Typically, prolonged exposure to high temperatures leads to a reduction in phenolic content due to structural changes such as isomerization, polymerization, and oxidation (Larsen & Ahmed, 2022; Sęczyk et al., 2019).

These findings emphasize the importance of temperature control in honey storage to preserve the integrity of honey's phenolic profile and ensure the maintenance of its quality attributes. The results suggest storage of honey, especially below refrigeration temperatures, is a promising approach for maintaining the phenolic profile in honey. The observed changes in the phenolic composition of honey stored at 65°C highlight the susceptibility of phenolic compounds to thermal degradation or increase over time. Moreover, the identification of distinct clustering patterns among honey samples stored at different temperatures provides valuable insights into the potential impact of storage conditions on the authenticity and quality of honey. Furthermore, this storage experiment justifies the significance of the research being conducted. Had the honey samples been subjected to storage conditions that could potentially modify their phenolic profile, the validity of the investigations into using these honey samples for botanical origin assessment would have been compromised. Altered phenolic composition could lead to inaccurate predictions regarding the botanical authenticity of honey. Thus, all the honey samples tested in this study were stored at -20°C to safeguard their phenolic profile, ensuring more reliable conclusions regarding the botanical authenticity of honey and the identification of phenolic markers.

4.4.2.1 Impact of honey storage on p-hydroxybenzoic acid, a candidate buckwheat honey marker

To gain deeper insights into how storage conditions affect the assessment of honey's botanical authenticity, a focus was set on investigating the change in concentration of p-hydroxybenzoic acid, a compound previously identified as a buckwheat honey marker, under various storage conditions. As depicted in Figure 4.3, the percent change in the mean z-score of honey samples stored at 25°C or below, over 1 to 6 months, varied from 0.7% for honey stored at -20°C for 6 months to -10.6% for honey stored at -20°C for 3 months. In contrast, a more important percent change in the mean z-score of honey samples was exhibited for honey stored at 65°C, ranging from -35.7% for a 1-month storage to -67.7% for a 6-month storage.

A Two-Way ANOVA was conducted on the mean z-score of all storage treatments for a given sample. The results revealed a significant difference in the mean z-score observed between groups based on time and temperature. Moreover, a significant interaction was observed between time and temperature, highlighting the intricate relationship between storage duration and temperature in shaping the phenolic composition of honey.

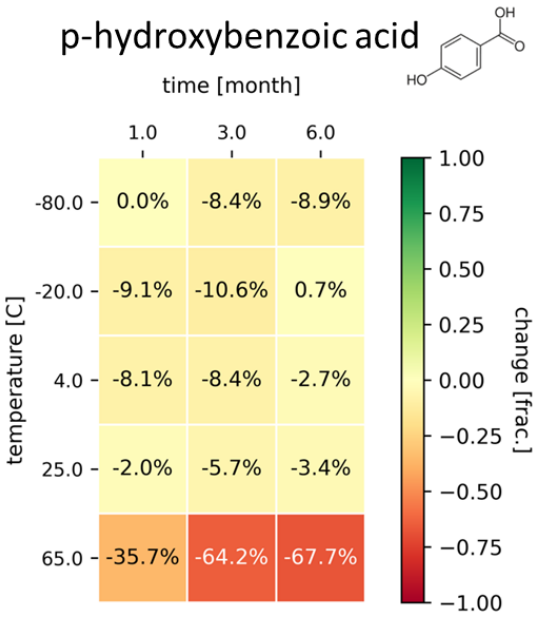

Figure 4.3. Heat map explaining the impact of storage conditions on the percent concentration change of p-hydroxybenzoic acid in selected honey samples (n=6).

In line with these findings, previous studies have reported similar trends in the impact of storage conditions on p-hydroxybenzoic acid in other food commodities. For instance, a study

investigating the impact of freezing raspberry cultivars at -22°C for 6 months has reported no significant differences in the concentration of p-hydroxybenzoic acid before or after the storage period (Türkben et al., 2010). Conversely, another paper studying the impact of soybean flour storage at refrigeration (4°C), room temperature (20°C) and heating (45°C) revealed significant increase ($p < 0.05$) in the concentration of p-hydroxybenzoic acid after 48 weeks of storage at these temperatures compared to the control. When comparing the concentration of p-hydroxybenzoic acid in soybean flour stored for 48 weeks across all three temperature conditions, no significant difference ($p > 0.05$) was reported amongst their concentrations (Mayakrishnan et al., 2018).

The changes in p-hydroxybenzoic acid concentration over the course of storage time may be attributed to the sensitivity of phenolic acids, such as p-hydroxybenzoic acid, to heat. This sensitivity arises from the presence of hydroxyl groups making them prone to various types of thermal reactions including dehydration, decarboxylation, hydrolysis, and oxidation, ultimately resulting in a decrease in their concentration (Castada et al., 2020; Chaaban et al., 2017; Criquet & Leitner, 2015; Ferreyra et al., 2023). When honey is subject to high temperatures, p-hydroxybenzoic acid may undergo these reactions, leading to the breakdown of its molecular structure and the formation of degradation products during prolonged exposure to elevated temperatures. In addition, a possible explanation for why a more important decrease in p-hydroxybenzoic acid concentration was observed for a more extensive storage duration at elevated temperatures could be explained by reaction kinetics. The rate of chemical reactions, such as degradation reactions, typically increases with temperature following the Arrhenius equation (Zapata et al., 2022). Higher temperatures provide more energy to the molecules, increasing their kinetic energy and facilitating reaction pathways that lead to the degradation of p-hydroxybenzoic acid. Therefore, storing honey at 65°C accelerates the degradation kinetics of p-hydroxybenzoic acid molecules, leading to a more significant decrease in concentration over time compared to storage at lower temperatures (Castada et al., 2020).

4.4.2.2 Impact of honey storage on buckwheat honey classification

In light of the observed impact of honey storage at 65°C on p-hydroxybenzoic acid, further investigations were conducted on its influence on honey classification. This section aimed to evaluate whether alterations in compound concentrations due to storage would affect the

accuracy of a classification model in distinguishing buckwheat from non-buckwheat honey, utilizing a previously established threshold from Chapter 3.

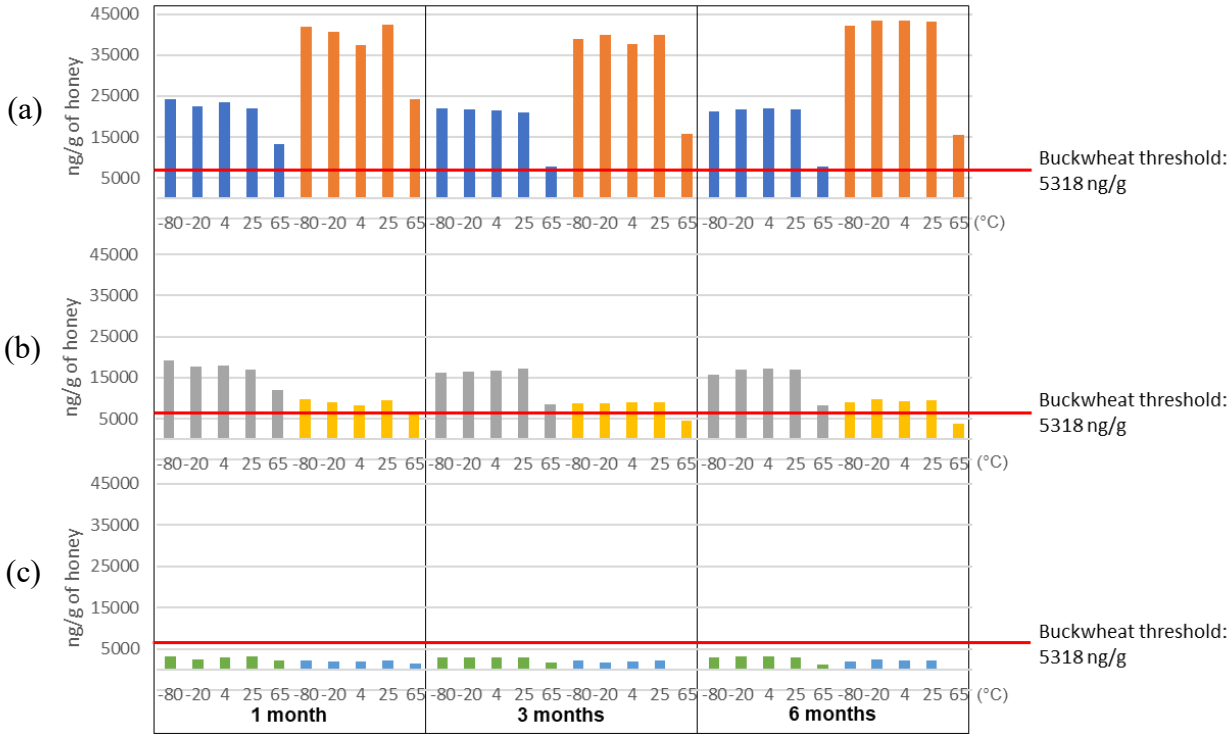

Figure 4.4. Impact of storage conditions on the concentration of p-hydroxybenzoic acid in three classes of honey where each color corresponds to a different honey: (a) honeys labeled and confirmed by pollen analysis as being buckwheat honey (n=2); (b) honeys labeled as buckwheat and confirmed by pollen analysis as polyfloral honey (n=2); (c) other monofloral honeys not containing buckwheat pollen (<0.1%) (n=2).

Figure 4.4 illustrates the variations in p-hydroxybenzoic acid concentrations in honey subjected to different storage durations and temperatures. As a general rule, honey samples stored below 25°C maintained consistent p-hydroxybenzoic acid concentrations regardless of storage duration while those stored at 65°C exhibited a decrease over time. To evaluate how storage affects the classification model's accuracy in predicting buckwheat honey, the concentration of p-hydroxybenzoic acid across three honey classes was compared against a predetermined threshold of 5318 ng/g. Despite a decrease at 65°C, confirmed buckwheat honey samples (Figure 4.4, (a)) stayed above the threshold, ensuring accurate classification when p-hydroxybenzoic acid was the

sole determinant for categorizing honey as either buckwheat or non-buckwheat. However, it's worth noting that depending on the initial concentration of this marker in honey, even confirmed buckwheat honey samples showing slight concentrations above the threshold may fall below it after storage at 65°C, resulting in misclassification as non-buckwheat honey. Furthermore, in samples labeled as buckwheat but confirmed by pollen analysis as polyfloral honey (Figure 4.4, (b)), although they do not meet the monofloral buckwheat honey criteria according to pollen analysis, storage below 25°C leads to their classification as buckwheat honey due to the p-hydroxybenzoic acid concentration surpassing the threshold. This suggests that despite not being strictly monofloral buckwheat honey, the concentration of p-hydroxybenzoic acid remains above the buckwheat honey threshold, as the presence of buckwheat nectar, albeit not dominant, contributes to this compound's levels. Additionally, these honey samples may contain nectar from other botanical species that may also contain some p-hydroxybenzoic acid, potentially contributing to the level of this compound in honey. The gray-colored honey maintained p-hydroxybenzoic acid levels above the buckwheat threshold even after storage at 65°C, retaining its classification as buckwheat honey. In contrast, the yellow-colored honey fell below the threshold after 3-6 months at 65°C, resulting in its classification as non-buckwheat. This demonstrates that initially low levels of p-hydroxybenzoic acid can degrade below the threshold at high temperatures, indicating it should not be the sole determinant for buckwheat honey classification. Since this marker is not temperature stable, relying solely on it could lead to misclassification. For instance, if the marker degrades under high thermal conditions, authentic buckwheat honey might be incorrectly classified as non-buckwheat. Therefore, p-hydroxybenzoic acid should be used as a complementary marker alongside more heat-stable markers to provide a more accurate assessment of the botanical authenticity of buckwheat honey. While effective at room temperature or below, its reliability diminishes at elevated temperatures, highlighting the need for a multi-marker approach. Moreover, in the case of honey samples classified as other monofloral honey not containing buckwheat pollen (Figure 4.4, (c)), regardless of storage conditions, the concentration of p-hydroxybenzoic acid in both samples falls below the threshold for buckwheat honey. Consequently, these honeys are correctly categorized as non-buckwheat honey when utilizing this compound as the indicator of buckwheat authenticity.

These findings underscore the importance of exercising caution during honey authentication, as storage can significantly impact the marker used for honey's botanical origin classification. For instance, certain storage conditions may lead to a decrease in the marker's concentration so important that its level may fall below the threshold value, leading to inaccurate conclusions regarding the honey's botanical origin. Despite 65°C not reflecting real-world scenarios, they offer valuable insights into the changes experienced by p-hydroxybenzoic acid under extreme storage conditions. Therefore, maintaining honey samples at appropriate temperatures is vital for preserving their phenolic profile, particularly the compounds serving as markers for botanical origins, thus ensuring accurate assessment of honey's botanical authenticity. Nonetheless, while this study offers valuable insights into the effects of storage on buckwheat honey classification, a larger sample size would be beneficial to draw more definitive conclusions.

4.4.3 Effect of honey thermal treatment on phenolic profiles

In a second experiment aiming at understanding the impact of thermal treatments on a shorter time scale, honey samples were subject to 55°C, 74°C, 85°C, and 100°C for 5, 10, 30, 60, 90 min. A temperature of 55°C was chosen to emulate the liquefaction operation commonly utilized in the honey industry (Aydoğan Coşkun et al., 2020; Escriche et al., 2008; Eshete & Eshete, 2019). Considering the typical commercial pasteurization range of 70-78°C, a temperature of 74°C was selected as a midpoint (Eshete & Eshete, 2019). To gain insights into how honey's phenolic profile is influenced by its application in commercial food products, a temperature of 85°C was selected as this temperature was utilized in the production of honey candies (Dibyakanta Seth & Mishra). Additionally, a temperature of 100°C was selected to simulate honey's use as a sweetener in hot beverages and baked goods (von Eyken & Bayen, 2020).

To examine the effects of thermal treatment on the phenolic profile of honey, a comprehensive analysis employing Principal Component Analysis (PCA) was conducted where PC1, accounting for 47.7% of the total variance, was plotted against PC3, showcasing 10.2% of the total variance.

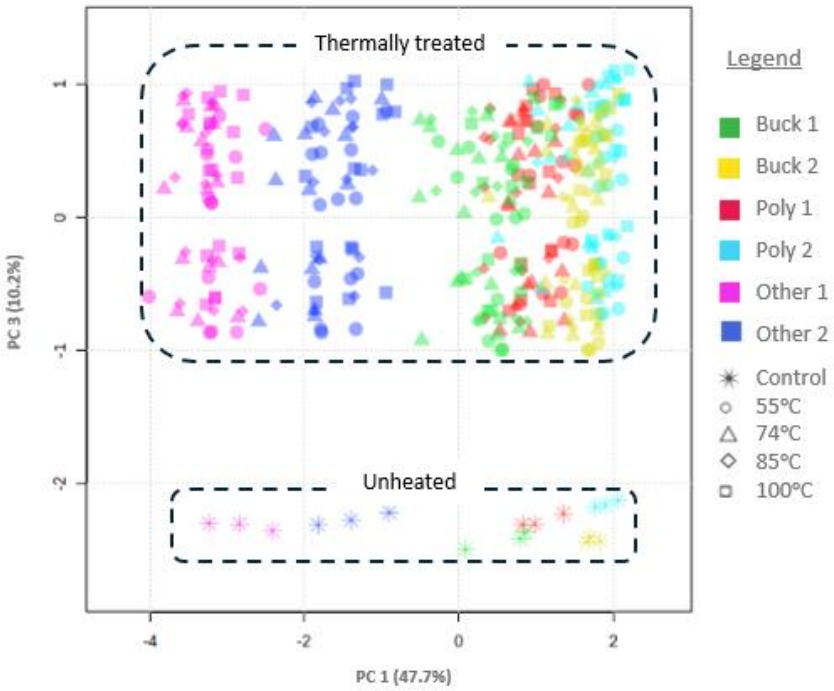

Figure 4.5. Principal Component Analysis (PC1 and PC3) comparing the quantified phenolic profile (13 features) of thermally treated honey to unheated honey (n=6 selected honey samples). Data normalized using log (base 10) transformation and pareto scaling.

Figure 4.5 unveiled a distinct clustering pattern in which unheated honey ("control") clustered away from those subjected to thermal treatment. It is important to note that this clustering pattern is not random and is not due to instrumental variation as honey samples were analyzed on the LC-QTOF-MS in a randomized order. Additionally, to account for the high sample volume, two different matrix match calibration curves were applied, further confirming the non-random nature of the control samples' clustering pattern. To determine if the difference between unheated and thermally treated honey is significant, a one-way ANOVA test was performed on the normalized data (log (base 10) transformation and pareto scaling). It revealed that, across all six honey samples tested, there was no significant difference ($p > 0.05$) in the concentrations of any phenolic compounds between the heat-treated samples and the control. Moreover, the PCA highlights that PC3 is the principal component delineating the impact of honey heat treatment. Samples positioned higher on the PC3 axis corresponded to honey exposed to more intense thermal conditions (85°C and 100°C), whereas those closer to the "control" samples on the PC3 axis underwent treatment at lower temperatures (55°C and 74°C, representing liquefaction and

pasteurization processes, respectively). This pattern along PC3 outlines the range of thermal stress experienced by the honey samples, offering valuable insights into the relationship between temperature exposure and phenolic profile in honey.

According to the literature, the impact of thermal treatment on the phenolic profile in honey appears to be highly dependent on the temperature and duration of thermal exposure. One study suggests that the impact of the liquefaction process is less pronounced than pasteurization in terms of altering phenolic compound concentrations. While liquefaction tends to slightly elevate the total phenolic content (TPC) of honey compared to unheated samples, the difference is not statistically significant ($p > 0.05$). Conversely, pasteurized honey samples exhibit a significant increase in TPC ($p < 0.01$) (Aydoğan Coşkun et al., 2020). The increase in phenolic content may be attributed to protein denaturation which releases phenolic compounds from their association with proteins (Flanjak et al.; Larsen & Ahmed, 2022). Regarding the effect of higher temperatures on TPC in honey, findings vary across studies. For instance, heat treatment at 90°C for 23 minutes has been associated with a significant increase in honey TPC (Rababah et al., 2024). While many studies support the notion that exposure to elevated temperatures increases honey TPC (Antony & Farid, 2022; Trinh et al., 2022), others present contrasting results. For example, a study investigating the impact of phenolic compounds on honey samples heat-treated at 80°C found a decrease in TPC for buckwheat honey and no effect for clover honey (Pimentel-González et al., 2016). These contradictory outcomes suggest that the distinct phenolic profiles of honey from various botanical origins may influence fluctuations in honey TPC, with certain compounds being more vulnerable to thermal degradation than others. Therefore, considering the botanical origin of honey samples is imperative when interpreting the effects of heat treatment on honey's phenolic profile.

Furthermore, to gain a better understanding into the potential impact of industrial processes (such as liquefaction or pasteurization or incorporating honey into food production) on altering the botanical classification of honey, a Principal Component Analysis (PCA) was conducted. This analysis aimed to assess whether the botanical class of the six honey samples chosen for this case study would be influenced by various thermal treatment conditions.

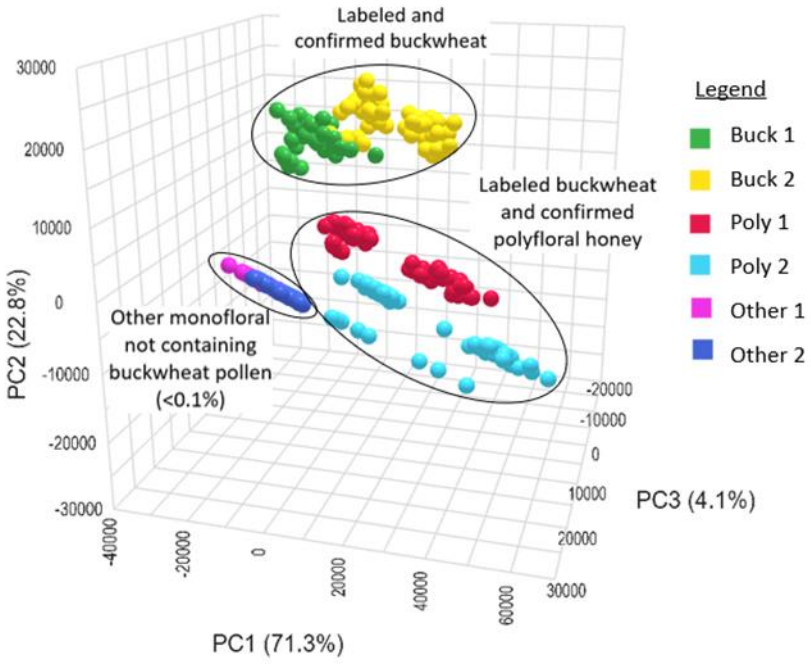

Figure 4.6. Principal Component Analysis (first three components) showing the impact of thermal treatment on the quantified phenolic profile (13 features) of six selected honey samples.

Figure 4.6 depicts a 3D PCA illustrating that regardless of the thermal treatment conditions applied to honey, the phenolic profile remained largely unchanged, allowing for classification based on botanical origin when considering PC1 and PC2, which represent the principal components carrying the majority of the total variance. In essence, the botanical origin of honey seems to exert a greater influence on its classification than the effects of thermal treatment. Similar findings were reported by a study that examined the effects of industrial thermal treatment (liquefaction and pasteurization) on honey's floral authenticity. This study has found that industrial thermal treatment does not significantly alter phenolic compounds to the extent of impacting honey discrimination based on botanical origin (Escriche et al., 2014). This suggests that these processing conditions are not sufficiently aggressive to modify honey's intrinsic properties concerning botanical origin. Additionally, the phenolic profiles of honey within each class exhibit notable similarities, as evidenced by their grouping together in distinct clusters across PC1 and PC2, regardless of thermal treatment conditions.

The findings from this study suggest that while thermal treatment may have some effect on the phenolic profile of honey, its influence appears to be secondary to the inherent botanical origin of

the honey. The observed clustering patterns in the PCAs reinforce the notion that the phenolic profile of honey remains relatively stable despite variations in thermal treatment conditions. These insights contribute to a better understanding of how industrial processes, such as liquefaction, pasteurization, or integration of honey into food production, may impact the botanical classification of honey. However, further research with larger sample sizes and broader experimental parameters is advised to validate these findings and elucidate the underlying mechanisms governing the relationship between thermal treatment and honey's phenolic profile.

4.4.3.1 Impact of honey thermal treatment on buckwheat marker and buckwheat honey classification

To assess the influence of thermal treatment on the buckwheat honey marker (p-hydroxybenzoic acid), a bar chart was generated for all four temperature conditions tested (55°C, 74°C, 85°C, 100°C), focusing on the longest exposure time (90 minutes) to elucidate the impact of these temperatures on the marker in honey (refer to Figure 4.7).

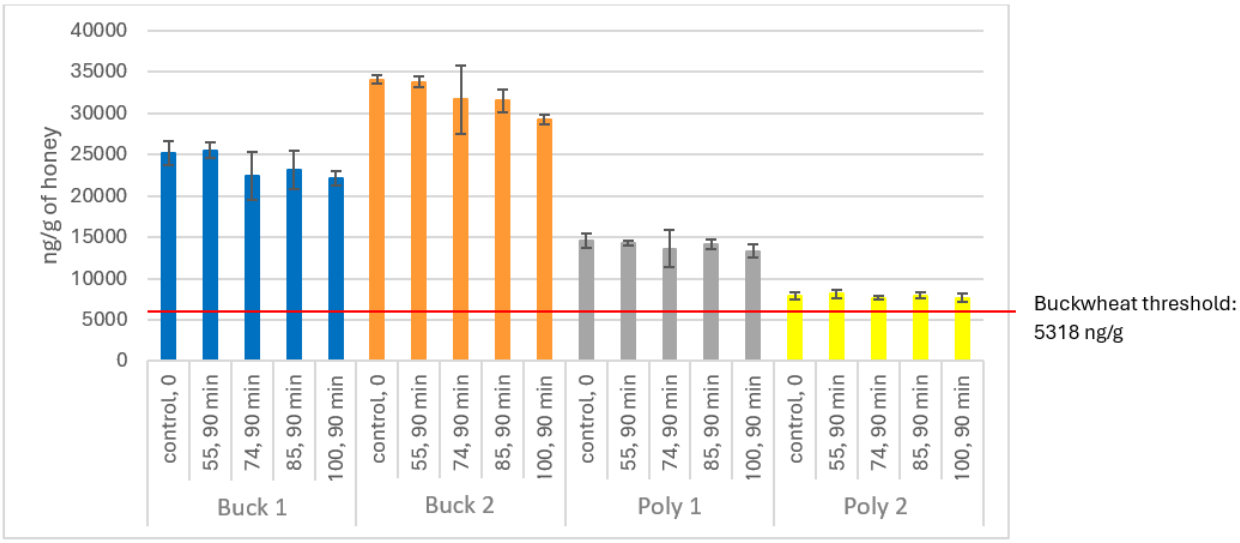

Figure 4.7. Impact of thermal treatment on the concentration of p-hydroxybenzoic acid in honey samples. No significant difference ($p > 0.05$) was observed in p-hydroxybenzoic acid concentration across honey thermal treatment within the same sample ID. Honey samples corresponding to the class “other monofloral honeys not containing buckwheat pollen” are not

shown in this figure as the concentration of this compound in these honeys (n=2) is below MDL (381 ng/g of honey).

In Figure 4.7, initial observation reveals a decrease in p-hydroxybenzoic acid concentration for 'Buck 1' and 'Buck 2' following heating, particularly at temperatures of 74°C or higher. However, upon conducting statistical analysis on these honey samples, no significant difference ($p > 0.05$) was detected in the concentration of p-hydroxybenzoic acid across different thermal treatment conditions within the same sample ID. Similarly, no significant variance ($p > 0.05$) was observed for 'Poly 1' and 'Poly 2' across different thermal treatments. Moreover, when evaluating the impact of thermal treatment on this buckwheat honey marker, it becomes evident that irrespective of the heating conditions to which honey is subjected in this case study, its concentration consistently remains above the threshold level. This ensures accurate classification of honey by the model as buckwheat honey, considering p-hydroxybenzoic acid as the sole determinant used to assess buckwheat honey classification. These findings suggest that, within the parameters of this study, thermal treatment does not significantly ($p > 0.05$) affect the concentration of p-hydroxybenzoic acid in buckwheat honey, reinforcing its reliability as a marker for the classification of buckwheat honey.

4.5 Conclusion

In conclusion, to assess the impact of storage and thermal treatment on the phenolic profile of honey, six honey samples were selected for this case study. The findings from the storage experiment indicate that the p-hydroxybenzoic acid marker threshold, established in untreated honey samples as 5318 ng/g in buckwheat honey, remains applicable to honey samples stored at temperatures up to 25°C, accurately predicting them as buckwheat honey. However, storage at 65°C leads to a decrease in the concentration of this marker, potentially misclassifying true buckwheat honey as non-buckwheat. Moreover, the thermal treatment experiment suggests that non-thermally treated honey samples exhibit a similar phenolic profile compared to heat-treated honey. Nevertheless, regardless of the thermal treatment conditions applied to honey, the phenolic profile exhibited minimal alteration, thereby enabling classification based on botanical origin. Ultimately, the classification of honey seems to be more influenced by its botanical origin than by the impact of thermal treatment. In addition, no significant difference ($p > 0.05$) was

detected in the concentration of p-hydroxybenzoic acid across different thermal treatment conditions within the same honey sample.

These results emphasize the necessity of being vigilant during the authentication process of honey, as storage conditions can greatly influence the marker utilized for classifying honey based on its botanical origin. Hence, it is crucial to store honey samples at suitable temperatures, particularly at or below 25°C as indicated by this study, to safeguard their phenolic composition, especially the compound that serves as a marker of botanical origin, thereby enabling accurate evaluation of the botanical authenticity of honey. Furthermore, although thermal treatment does not appear to significantly impact the phenolic profile or the buckwheat honey marker, it is essential to note that this conclusion is based on a limited sample size of only six selected honey samples. Further research with larger sample sizes and broader experimental parameters is recommended to validate these findings.

CHAPTER 5. DISCUSSION AND CONCLUSION

5.1 Conclusion

This study developed a multi-targeted analysis workflow for detecting phenolic compounds in honey using a simple dilute-and-shoot HPLC-QTOF-MS method. Validated for 29 phenolic compounds in honey, the method demonstrated acceptable linearity, recoveries, precision, and repeatability. The sample preparation and analysis took less than 20 minutes per sample, requiring only 0.2g of honey, making it suitable for routine use. Applied to 465 honey samples from Canada and internationally, the multi-targeted analysis of phenolic compounds in honey is proven to be a successful method in differentiating buckwheat honey from other botanical origins. Notably, p-hydroxybenzoic acid emerged as a reliable single marker for buckwheat honey, with a threshold of 5318 ng/g. This marker's reliability was confirmed with a high true positive rate of 96.7% and a positive predictive rate of 92.6% through a confusion matrix. To further validate the robustness of the dilute-and-shoot method developed for the detection of p-hydroxybenzoic acid in honey and assess the reliability of the buckwheat honey marker, cross-instrumental validation was conducted using LC-QqQ-MS/MS. The method was successfully validated on the LC-QqQ-MS/MS with acceptable linearity, method precision, repeatability, and recovery. The study showed that the marker is highly transferable from one LC-MS instrument to another, achieving 99.14% consistency in honey sample classification compared to LC-QTOF-MS results. In addition, both instruments had the same true positive rate, but the LC-QqQ-MS/MS had a slightly lower positive predictive rate at 91.6%.

Furthermore, following the identification of p-hydroxybenzoic acid as a reliable marker of buckwheat honey and its successful validation across instruments, its effectiveness in distinguishing buckwheat honey was further tested under various storage and thermal treatment conditions. This case study was carried out on six selected honey samples. Firstly, the storage experiment revealed that the p-hydroxybenzoic acid threshold of 5318 ng/g, effective for untreated buckwheat honey, also applies to samples stored up to 25°C, ensuring accurate classification as buckwheat honey. However, at 65°C, the marker concentration decreases, leading to potential misclassification of true buckwheat honey as another type. Moreover, the concentration of p-hydroxybenzoic acid remained consistent across various thermal treatments within the same honey sample, showing no significant variation ($p > 0.05$) reinforcing the

suitability of using p-hydroxybenzoic acid with a threshold of 5318 ng/g as a reliable marker for the thermal treatment conditions selected in this study. Additionally, while the phenolic profile of honey changed with storage at 65°C compared to those stored at room temperature or lower, with some phenolic compounds showing an increase and others decrease, thermal treatment did not significantly impact the overall phenolic profile (14 compounds) compared to non-heat-treated samples ($p > 0.05$). These findings suggest that since the phenolic profile of honey showed minimal changes despite varying thermal treatments, honey may therefore be reliably classified by botanical origin with the classification of honey seeming to be more influenced by its botanical origin than by the impact of thermal treatment.

5.2 Contribution to knowledge

This research represents a significant advancement in the field of honey authentication by analyzing 29 phenolic compounds in a substantial sample size of 465 honey samples. To our knowledge, this is the first study to investigate such a wide array of phenolic compounds in honey from various botanical origins with such a substantial sample size. Specifically, it is the first study to analyze this extensive number of phenolic compounds in Canadian honey. The development of a robust analytical method tailored to Canadian honey lays the groundwork for future research and standardization efforts, aiding in the creation of regulatory guidelines specific to the Canadian honey industry. Furthermore, although p-hydroxybenzoic acid has been reported in various studies as a marker for buckwheat honey, no prior research has identified a specific threshold level for this compound or any other phenolic marker. This study is the first to establish a threshold level for a phenolic marker in honey, thereby providing a more precise tool for authenticating buckwheat honey. In addition, several studies have proposed phenolic compounds as potential markers for determining the botanical origin of honey. However, there has been a notable gap in validating these markers for robust and accurate assessments of honey authenticity. This research stands out as the first investigation to assess the transferability of such a marker across different analytical instruments, demonstrating its efficiency and reliability beyond its discovery on a single instrument. Moreover, despite numerous studies suggesting phenolic compounds as reliable markers of honey's botanical authenticity, there is limited understanding of how thermal processing and storage affect honey's phenolic profile and the phenolic marker and, consequently, its botanical classification. This research marks the first

study to address these gaps by exploring the effects of storage and thermal processing on the phenolic content and specifically the marker, providing insights into the marker's reliability after honey is exposed to these conditions. Overall, this research enhances our understanding of the changes in honey's phenolic profile under various conditions and contributes to our comprehension of the parameters to consider when assessing the classification of honey's botanical origin, particularly for buckwheat honey.

5.3 Recommendations for future research

To further advance the field of honey authenticity verification and build on the findings of the present study, several key recommendations for future research are proposed. Expanding the sample size and geographical diversity of honey samples, beyond the primarily Canadian samples used in this study, will significantly reinforce the reliability of p-hydroxybenzoic acid as a marker for buckwheat honey. By collecting samples from a wider range of locations, researchers can validate the consistency of this marker across different environments and determine if it serves as a universal marker for buckwheat honey or if it varies regionally. This approach will provide a valuable tool for verifying honey authenticity and ensure that findings are generalizable and robust. It will also help ascertain if Canadian buckwheat honey can be distinguished from honey produced in other regions, enhancing the specificity and accuracy of botanical origin markers. While this study focused on buckwheat honey, future research should explore other botanical origins to identify additional markers. Applying the same methodologies to different types of honey will enable scientists to develop a comprehensive set of markers for various botanical origins. This broader approach will facilitate the creation of standardized methods for determining honey authenticity across different types. Beyond p-hydroxybenzoic acid, future studies should explore other potential chemical markers in honey, such as organic acids, amino acids, sugars, and enzymes. Additionally, identifying multiple markers for buckwheat honey will enhance the accuracy of its botanical origin confirmation. A multi-marker approach will provide a more robust framework for honey authentication, reducing the likelihood of false positives or negatives. Moreover, current conclusions regarding the effects of storage and thermal treatment on buckwheat honey classification are based on a limited sample size. Future research should include larger sample sizes and broader experimental parameters, such as time and temperature, to validate these findings and understand the underlying mechanisms. A more

extensive dataset will provide more definitive conclusions and improve the generalizability of the results. Non-targeted analysis methods can also be utilized to identify new phenolic compounds that may emerge after storage or thermal treatment. These compounds could serve as markers for the conditions the honey has been subjected to. In addition, a non-targeted approach should also be employed to identify additional phenolic compounds as markers of buckwheat honey. Tools like SIRIUS could help discover new markers, which can be confirmed by analytical standards, enhancing the specificity and reliability of honey authentication. This approach will broaden the scope of phenolic compounds tested and improve the overall robustness of authenticity verification. Furthermore, to facilitate the adoption and implementation of robust analytical strategies by regulatory bodies, future research should prioritize similar systematic studies. These studies should include cross-instrument validations to ensure consistent marker performance across different analytical tools. By improving the reliability and accuracy of honey authenticity testing, researchers can support regulatory efforts, protect consumer interests, and maintain market integrity. Implementing these recommendations will significantly advance the scientific community's ability to authenticate honey based on its botanical origin. By increasing sample diversity, expanding geographical scope, exploring additional markers, and standardizing methods, researchers can provide more reliable tools for honey authentication.

5.4 Final conclusion

Overall, this study highlighted that p-hydroxybenzoic acid is a promising marker for buckwheat honey. Despite numerous methods proposed in the literature for authenticating honey's botanical origin, they have proven inadequate, underscoring the need for robust, reliable, and standardized techniques. This study has developed and validated a dilute-and-shoot HPLC-QTOF-MS method for detecting phenolic compounds in honey, focusing on p-hydroxybenzoic acid, revealed to be a promising marker that has been demonstrated to be reliable across different LC-MS instruments. This study has also assessed the marker's stability under different storage and thermal processing conditions which has not been tested in previous studies. This research represents a significant step toward standardizing methods for honey authentication. By providing a dependable approach to verify the botanical origin of honey, this study contributes to ensuring the quality and authenticity of honey. These advancements pave the way for more reliable honey authentication, enhancing consumer trust and industry standards.

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APPENDIX

Table 1. LC-QqQ-MS/MS MRM parameters.

Chemicals	Formula	Ion Mode	RT[a] (min)	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)	RF[b] Lens (V)	Min Dwell Time (ms)
(±)-2-cis,4-trans-Abscisic acid	$C_{15}H_{20}O_4$	[M-H]-	4.68	263.129	153	10	44	6.348
					219	14	44	6.348
2,3,4-Trihydroxybenzoic acid	$C_7H_6O_5$	[M-H]-	2.26	169.016	151	14	33	7.794
					125	15	33	7.794
2,5-Dihydroxybenzoic acid	$C_7H_6O_4$	[M-H]-	2.85	153.021	108	22	38	7.05
					109	14	38	7.05
Apigenin	$C_{15}H_{10}O_5$	[M-H]-	5.42	269.046	117	36	92	7.07
					149	25	92	7.07
Benzoic acid	$C_7H_6O_2$	[M-H]-	4.38	121.029	77	12	30	6.348
					93	13	30	6.348
Caffeic acid	$C_9H_8O_4$	[M-H]-	3	179.035	135	15	55	7.034
					107	22	55	7.034
Chlorogenic acid	$C_{16}H_{18}O_9$	[M-H]-	2.34	353.088	191	17	50	7.794
					85	46	50	7.794
Chrysin	$C_{15}H_{10}O_4$	[M-H]-	6.13	253.054	143	28	50	13.187
					209	14	50	13.187
Ellagic acid	$C_{14}H_6O_8$	[M-H]-	4.26	300.999	284	30	109	6.348
					229	27	109	6.348
Epicatechin	$C_{15}H_{14}O_6$	[M-H]-	2.76	289.072	245	15	62	7.076
					203	19	62	7.076
Ferulic acid	$C_{10}H_{10}O_4$	[M-H]-	3.72	193.051	134	16	43	6.388
					178	13	43	6.388
Galangin	$C_{15}H_{10}O_5$	[M-H]-	6.24	269.046	213	25	92	13.244

					223	27	92	13.244
Gallic acid	$C_7H_6O_5$	[M-H]-	1.48	169.014	125	15	50	15.674
					79	24	50	15.674
Genistein	$C_{15}H_{10}O_5$	[M-H]-	5.13	269.048	133	31	103	6.348
					159	29	103	6.348
Hesperetin	$C_{16}H_{14}O_6$	[M-H]-	5.08	301.071	164	24	90	6.348
					286	18	90	6.348
Homogentisic acid	$C_8H_8O_4$	[M-H]-	1.63	167.035	123	11	30	15.655
					122	23	30	15.655
Kaempferol	$C_{15}H_{10}O_6$	[M-H]-	5.33	285.04	185	27	103	7.07
					239	28	103	7.07
Luteolin	$C_{15}H_{10}O_6$	[M-H]-	5.06	285.042	133	32	103	6.348
					151	26	103	6.348
Morin	$C_{15}H_{10}O_7$	[M-H]-	4.59	301.035	125	20	109	6.348
					107	29	109	6.348
Myricetin	$C_{15}H_{10}O_8$	[M-H]-	4.34	317.031	151	24	88	6.348
					179	20	88	6.348
p-Coumaric acid	$C_9H_8O_3$	[M-H]-	3.65	163.04	119	14	34	6.388
					93	32	34	6.388
p-Hydroxybenzoic acid	$C_7H_6O_3$	[M-H]-	2.87	137.024	93	16	38	7.05
					65	29	38	7.05
Protocatechualdehyde	$C_7H_6O_3$	[M-H]-	2.49	137.024	108	23	38	7.794
					93	16	38	7.794
Protocatechuic acid	$C_7H_6O_4$	[M-H]-	2.07	153.019	109	14	43	7.794
					108	22	43	7.794
Quercetin	$C_{15}H_{10}O_7$	[M-H]-	4.59	301.035	151	21	88	6.348
					179	18	88	6.348
Rosmarinic acid	$C_{18}H_{16}O_8$	[M-H]-	4.12	359.078	161	17	51	6.348
					197	17	51	6.348
Rutin	$C_{27}H_{30}O_{16}$	[M-H]-	3.98	609.146	301	31	165	6.348
					300	40	165	6.348
Salicylic acid	$C_7H_6O_3$	[M-H]-	4.61	137.024	93	16	38	6.348

					65	29	38	6.348
Sinapic acid	$C_{11}H_{12}O_5$	[M-H]-	3.67	223.061	208	13	53	6.388
					164	15	53	6.388
Syringic acid	$C_9H_{10}O_5$	[M-H]-	3.08	197.035	182	14	37	7.034
					123	24	37	7.034
trans-Cinnamic acid	$C_9H_8O_2$	[M-H]-	5.04	147.045	103	11	30	6.348
					77	24	30	6.348
Vanillic acid	$C_8H_8O_4$	[M-H]-	3.03	167.035	152	13	30	7.034
					108	20	30	7.034
Vanillin	$C_8H_8O_3$	[M-H]-	3.33	151.04	136	13	45	7.034
					92	20	45	7.034

[a] Retention time, [b] Radio Frequency